Divalent Metal Ion Differentially Regulates the Sequential Nicking Reactions of the GIY-YIG Homing Endonuclease I-BmoI

Benjamin P. Kleinstiver¹, Wesley Bérubé-Janzen¹, Andrew D. Fernandes¹,², David R. Edgell¹*¹

¹ Department of Biochemistry, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, Ontario, Canada; ² Department of Applied Mathematics, The University of Western Ontario, London, Ontario, Canada

Abstract

Homing endonucleases are site-specific DNA endonucleases that function as mobile genetic elements by introducing double-strand breaks or nicks at specific locations. Of the major families of homing endonucleases, the modular GIY-YIG endonucleases are least understood in terms of mechanism. The GIY-YIG homing endonuclease I-BmoI generates a double-strand break by sequential nicking reactions during which the single active site of the GIY-YIG nuclease domain must undergo a substantial reorganization. Here, we show that divalent metal ion plays a significant role in regulating the two independent nicking reactions by I-BmoI. Rate constant determination for each nicking reaction revealed that limiting divalent metal ion has a greater impact on the second strand than the first strand nicking reaction. We also show that substrate mutations within the I-Bmol cleavage site can modulate the first strand nicking reaction over a 314-fold range. Additionally, in-gel DNA footprinting with mutant substrates and modeling of an I-BmoI-substrate complex suggest that amino acid contacts to a critical GC-2 base pair are required to induce a bottom-strand distortion that likely directs conformational changes for reaction progress. Collectively, our data implies mechanistic roles for divalent metal ion and substrate bases, suggesting that divalent metal ion facilitates the re-positioning of the GIY-YIG nuclease domain between sequential nicking reactions.

Introduction

Homing endonucleases are DNA endonucleases that primarily function as mobile genetic elements by introducing double-strand breaks or nicks at specific homing sites in naive genomes [1,2]. DNA repair, recombination and replication pathways repair the double-strand break (or nick) using the endonuclease-containing genome as a template, resulting in the mobilization of the homing endonuclease gene and surrounding DNA to the recipient genome [1]. In model laboratory systems, the efficiency of endonuclease-mediated homing is extraordinary, reaching 80–100% in some cases [3,4,5], implying that homing endonucleases can quickly spread through populations of naive genomes. The rapid accumulation of genome sequence data has revealed an abundance of homing endonuclease genes in bacterial, archaeal, viral, and organellar genomes, including the mitochondrial and nuclear genomes of eukaryotes [6,7]. Five distinct homing endonuclease families, defined by conserved amino acids, have been characterized to date. They are the LAGLIDADG, HNH, His-Cys box, GIY-YIG, and PD-(D/E)xK families [8,9].

Homing endonucleases have attracted much interest as potential reagents for manipulating complex genomes, and substantial effort has been devoted to reprogramming the specificity of a select few LAGLIDADG endonucleases to cleave clinically relevant targets [10,11,12,13,14]. Understanding the mechanism of DNA hydrolysis by homing endonucleases is critical for their utilization as reagents for targeted manipulation of complex genomes. From a mechanistic perspective, the least understood of the homing endonuclease families are the GIY-YIG enzymes. GIY-YIG homing endonucleases are modular in nature and contain an N-terminal cleavage domain of ~100 amino acids that includes the class-defining GIY-YIG motif, and a C-terminal DNA-binding domain that is composed of distinct modules (Figure 1) [15,16]. The N- and C-terminal domains are connected by a flexible linker that functions as a molecular ruler to position the catalytic domain at the correct distance on substrate from the DNA-binding domain [17,18,19,20]. Two well-studied GIY-YIG endonucleases are the isocaudomers I-TevI and I-BmoI, encoded within group I introns interrupting the thymidylate synthase genes of bacteriophage T4 and Bacillus mojavensis, respectively [21,22,23]. I-TevI and I-BmoI introduce a staggered double-strand break by two independent and sequential nicking reactions at the same positions of their substrates, with the bottom (non-coding) strand of substrate nicked before the top (coding) strand [24,25]. DNA bending assays and in-gel footprinting demonstrated that significant DNA distortions on the bottom strand occur independently of the first nicking reaction [24,25]. Interestingly, studies with mutant DNA substrates revealed distinct sequence
requirements for efficient double-strand break formation by each endonuclease. In particular, I-BmoI absolutely requires a GC base pair at position -2 immediately 3' to the top-strand nicking site [26], yet displays no sequence preference for bases flanking the bottom-strand nicking site, while I-TevI requires an additional CG base pair 5' to the bottom-strand nicking site for cleavage activity [27].

The GIY-YIG nuclease motif is not unique to homing endonucleases, as the domain is found in a variety of protein scaffolds [28], including DNA repair proteins [29], retrotransposon elements [30], and restriction endonucleases [31,32,33]. Structural studies of diverse GIY-YIG domains have revealed a compact α/β/γ-fold composed of a central three-stranded antiparallel β-sheet flanked by three α-helices [29,34,35,36,37,38]. A set of conserved residues comprise the active site that uses a one-metal ion mechanism to catalyze DNA hydrolysis [36,37]. The function of the GIY-YIG nuclease domain varies in a scaffold-dependent manner, as the domain has acquired additional structural units that are interspersed within the GIY-YIG domain to direct oligomerization or DNA binding. For instance, the GIY-YIG restriction enzyme Eco29kI utilizes additional folds to function as a dimer [39], with each monomer nicking one strand of substrate. In the case of the UvrC nucleotide excision repair endonuclease, the single GIY-YIG domain nicks 3' to a lesion site to initiate a repair event [40]. The homing endonucleases are distinct from other GIY-YIG family members, as they have an extended structure and bind DNA as monomers [25]. They are believed to successively use the single active to generate a double-strand break [30], but how the active size is reorganized to accommodate different DNA strands for the sequential nicking reactions is unknown.

Here, we investigate the contributions of divalent metal ion and DNA sequence to the sequential nicking reactions of I-BmoI. We show that cleavage by I-BmoI is sensitive to the identity and concentration of divalent metal ion, and that the second strand nicking rate is affected to a greater extent by low metal ion concentration of divalent metal ion, and that the second strand shows that cleavage by I-BmoI is sensitive to the identity and DNA sequence to the sequential nicking reactions of I-BmoI. We selected a range of MgCl2 concentrations to dissect reaction progress as previous data indicated that the sequential reactions have distinct metal requirements [24]. We performed time-course cleavage assays with a supercoiled plasmid (pBmoHS) containing the I-BmoI intronless thyA target site (Figure 2). I-BmoI reaction progress can be visualized using supercoiled substrate, as the first nicking reaction generates a nicked plasmid intermediate and the second nicking reaction converts the nicked intermediate to linear product [41]. Cleavage assays were performed under single turnover conditions (protein excess) with a range of MgCl2, CaCl2, CuCl2, MnCl2, NiCl2, and ZnCl2 concentrations (Figure 2). Reactions with CoCl2 did not yield any products. The overall divalent metal ion preference of I-BmoI was Mn2+ > Mg2+ > Ni2+ > Ca2+ > Zn2+ > Cu2+. In particular, cleavage was extremely efficient in the presence of MnCl2 for all concentrations tested, while reactions in MgCl2 displayed a second strand defect at 0.25 mM MgCl2, near complete conversion of nicked intermediate to linear product at 2 mM MgCl2, and complete conversion to product at 10 mM MgCl2 (Figure 2A). NiCl2 appeared to be inhibitory at 10 mM, yet similar levels of cleavage were observed at 0.5 mM NiCl2 and MgCl2. Cleavage was observed in the presence of CaCl2 (albeit greatly reduced versus MgCl2), whereas ZnCl2 and CuCl2 were inhibitory at most concentrations tested, with the exception that 0.05 mM ZnCl2 supported nicking only. These findings correlate well with the typical roles ofF divalent metal observed for the majority of site-specific DNA endonucleases, including the tetrameric GIY-YIG restriction enzyme Cfr42I, apart from the observation that cleavage by Cfr42I was most efficient in CoCl2-containing buffers [31,42,43,44].

Site-specific endonucleases have been shown to exhibit increased activity in the presence of manganese, but at the cost of fidelity [45,46]. To determine if I-BmoI displayed a similar loss of fidelity in the presence of MnCl2 relative to MgCl2, we performed cleavage assays on a plasmid substrate with a G-2T mutation, against which I-BmoI is known to retain only limited activity [Figure 2B] [26]. We observed minimal nicking of the pBmoHS G-2T substrate by I-BmoI in the presence of 10 mM MgCl2, while 2 mM and 10 mM MnCl2 were conducive for cleavage (but reduced versus intronless thyA substrate). Collectively, these data suggested that the observed increase in efficiency in the presence of MnCl2 is partly due to decreased fidelity, allowing I-BmoI to cleave non-cognate sites. Furthermore, based on these results we determined that of the divalent metal ions tested, a selection of manganese concentrations would provide the optimal level of efficiency and specificity.

Limiting divalent metal ion has a more pronounced regulation of second strand nicking

To gain insight into the substrate conversion process by I-BmoI, we selected a range of MgCl2 concentrations to dissect reaction progress as previous data indicated that the sequential reactions have distinct metal requirements [24]. We performed time-course cleavage assays with the thyA supercoiled substrate and determined rate constants based on the following reaction scheme:

\[
C \xrightarrow{k_1} N \xrightarrow{k_2} L
\]
where $k_1$ is the rate constant for conversion of supercoiled plasmid (C) to nicked intermediate (N), and $k_2$ is the rate constant for conversion of nicked intermediate to linear product (L). Representative time course experiments in 0.5 mM and 10 mM MgCl$_2$ are shown in Figure 3, with $k_1$ and $k_2$ rate constants for all MgCl$_2$ concentrations tested summarized in Table 1. We observed a 5.2-fold reduction in $k_1$ when MgCl$_2$ was reduced from 10 mM to 0.5 mM MgCl$_2$, while a 12.2-fold decrease was observed for $k_2$. The distinct metal requirements for each nicking reaction is represented in the log10($k_1$) - log10($k_2$) versus MgCl$_2$ concentration plot, which shows a more pronounced decrease in $k_2$ as the concentration of MgCl$_2$ is reduced (Figure 3C). These data suggest that divalent metal ion regulates the rate of both nicking reactions, but that the second strand nicking reaction has a more stringent requirement for divalent metal than the first nicking reaction.

**Assays with mutant substrates reveal three distinct cleavage phenotypes**

To further probe the contribution of divalent metal ion and DNA sequence to the sequential nicking reactions by I-BmoI, we performed time-course cleavage assays with various supercoiled mutant substrates at 2 mM and 10 mM MgCl$_2$. We took advantage of a series of previously constructed substrates [26], whereby positions -6 through -1 of intronless *thyA* substrate were individually and in combination changed to the corresponding intron-containing sequence (Figure 1 and Table 2), which I-BmoI does not cleave. End-point assays with these substrates highlighted the importance of the GC-2 base pair in the generation of a DSB by I-BmoI [26]. The previous assays, however, were incapable of distinguishing defects in each independent nicking reaction because they were performed on linearized plasmid substrates in 10 nM MgCl$_2$.

**Time-course assays using mutant cleavage site plasmids revealed that the substrates segregated into three distinct classes (Figure 4 and Table 2).** Class I mutants were defined as substrates that behaved essentially as intronless *thyA* substrate, showing slightly reduced nicking and slower conversion of nicked intermediate to linear product at 2 mM versus 10 mM MgCl$_2$ (Figure 4A). The substrates were those with substitutions C-4A or G-6T, and the rate constants for the first and second strand nicking reactions in low and high MgCl$_2$ were very similar to those obtained for intronless *thyA* substrate (Table 2 and Table S3).

Class II mutants were defined as substrates that exhibited a greater impairment of first and second strand nicking in low versus high MgCl$_2$ conditions. This class included the singly mutated substrates T-1G, C-3G, and C-5A, as well as the multiply substituted C-3G/C-4A, C-4A/C-5A, C-5A/G-6T and C-4A/C-5A/G-6T substrates (Table 2). The general characteristics of substrates in this class were the accumulation and persistence of the nicked intermediate in 2 mM MgCl$_2$ (which resulted in impaired double-strand break formation), and the rescue of cleavage in 10 mM MgCl$_2$ resulting in the near complete conversion to linear product by 180 seconds (Figure 4B and Table 2). Rate constant analysis indicated a further segregation of substrates within class II by differential base-specific effects on first and second strand nicking reactions (Table 2). The T-1G substitution decreased the first ($k_1$) and second ($k_2$) strand rates by roughly 2- and 3-fold versus intronless *thyA* substrate in the presence of 2 mM and 10 mM MgCl$_2$, respectively, suggesting a more profound modulation of second-strand nicking. The C-3G substitution, which displayed similar effects in the presence or absence of a C-4A substitution, generated approximately 5- and 3-fold reductions in $k_1$ and $k_2$ versus intronless substrate, regardless of MgCl$_2$ concentration. Interestingly, the C-5A substitution, alone or in the context of a G-6T substitution, decreased $k_1$ by roughly 6- and 3-fold in the presence of 2 mM and 10 mM MgCl$_2$, respectively. The addition of a C-4A substitution to the C-5A template exacerbated the $k_1$ defect to 12- and 9-fold in the presence of 2 mM and 10 mM MgCl$_2$. Surprisingly, the C-5A substitution showed less of an influence on the second-strand nicking rate ($k_2$), evidenced by a 1.5-fold rate reduction in both MgCl$_2$ concentrations. This result was observed with the C-5A substitution alone or in the context of the double mutation with C-4A.

Class III mutants were defined as substrates with drastically reduced reaction rates that displayed little or no cleavage in either low- or high-metal ion conditions (Figure 4C and Table 2). All substrates with mutations at the -2 position fell into this category, in addition to substrates with mutations at both the -5 and -3 positions. Interestingly, we observed that the G-2T substitution alone, and in combination with substitutions at positions -1 or -4 and -6, demonstrated limited accumulation of nicked intermediate at 180s in high metal. First strand nicking rates ($k_1$) for class III substrates were 84- to 314-fold reduced as compared to intronless *thyA* substrate, indicating a substantial first strand defect. Additionally, we observed that the substrates containing a combination of C-3G and C-5A mutations retained low levels of...
double-strand break formation after 180 s in 10 mM MgCl₂, and that the addition of a T-1G or C-4A mutation did not abolish the low amount of cleavage. Importantly, the intron-containing substrate showed no evidence of nicking or cleavage after 180 seconds under either condition tested (Table 2).

Collectively, the cleavage assays indicated that substrates with individual mutations at positions -4 or -6 had little effect on first- or second-strand nicking in either low or high metal ion conditions. Additionally, substrates with single mutations at positions -1, -3 or -5 exhibited defects in both first- and second-strand nicking in low metal conditions and an observable rescue of cleavage by high divalent metal ion. Substrates with mutations at both the -3 and -5 positions were not significantly rescued by high metal ion, and those with mutations at position -2 were defective for cleavage under all conditions tested.

In-gel footprinting reveals multiple minor groove distortions dependent on GC-2

The cleavage data suggested that divalent metal ion and the GC-2 base pair are required for efficient double-strand break formation. To gain further insight into the role of the GC-2 base pair in the cleavage pathway, we performed in-gel footprinting with the minor groove-specific reagent 1,10-copper phenanthroline (OP-Cu). We previously used in-gel footprinting to show that significant OP-Cu hypersensitive sites were localized to positions -2 and -1 of the bottom strand of the I-BmoI-thyA complex [24], consistent with protein-induced DNA distortions that make the minor groove more accessible to OP-Cu. The distortions were also present in I-BmoI-substrate complexes formed with the catalytic mutants R27A and E74A, implying that the distortions precede first-strand nicking and do not require the presence of a metal ion.

Table 1. Rate constants for first- and second-strand nicking reactions in different MgCl₂ concentrations.

| [MgCl₂] (mM) | \(k_1\) (s⁻¹) | \(k_2\) (s⁻¹) |
|--------------|-------------|-------------|
| 10           | 0.10±0.007  | 0.078±0.008 |
| 7            | 0.10±0.005  | 0.060±0.003 |
| 5            | 0.079±0.004 | 0.050±0.002 |
| 2            | 0.056±0.001 | 0.036±0.001 |
| 1            | 0.037±0.0006| 0.013±0.0004|
| 0.5          | 0.019±0.0002| 0.0064±0.0002|

\(k_1\) and \(k_2\) rate constants are reported as the best fit value of three independent experiments with standard error.

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Figure 3. Limiting divalent metal ion has a greater effect on second strand than first strand nicking. Shown are representative images of time-course cleavage assays with supercoiled substrate containing the intronless thyA target site and I-BmoI in A. 0.5 mM MgCl₂ and B. 10 mM MgCl₂, as well as progress curves for each condition. Circular substrate (C), nicked intermediate (N) and linear product (L) are indicated on the gel images. Individual data points from three independent replicates are shown in the progress curves, and the solid continuous lines are the best fit of the data to equations 1 and 2. C. Plot of the log₁₀\(k_1\) - log₁₀\(k_2\) value for MgCl₂ concentrations tested.
Previous footprinting studies, however, did not explore the requirement of the GC-2 base pair in formation of OP-Cu hypersensitive sites. We performed in-gel footprinting with a 74-mer duplex oligonucleotide substrate corresponding to the intron-containing substrate (In+), which contains an alternative sequence upstream of the insertion site relative to the intronless substrate and notably has a G-2T substitution (Figure 5). As shown in Figures 5b and 5c, the OP-Cu hypersensitive sites at positions -2 and -1 on the bottom strand were significantly reduced in the UC

Figure 4. Magnesium concentrations reveal three distinct classes of cleavage site substitutions. Cleavage assays with I-BmoI were conducted on supercoiled plasmid substrates containing substitutions at positions -6 to -1 in the presence of 2 mM or 10 mM MgCl2 (see also Table 2). Mutant substrates were arranged into three classes. (A) Substrates that showed a phenotype similar to wild-type intronless thyA substrate (class I); (B) substrates that demonstrated poor cleavage in 2 mM MgCl2 and rescued cleavage in 10 mM MgCl2 (class II); (C) substrates with significantly reduced or no cleavage (class III). Shown are representative gel images of time-course cleavage assays, where the second lane from the left contains unreacted plasmid substrate (-). Nicked (N), linear (L), and circular (C) plasmid forms indicated to the right. Beneath each gel image is a graphical representation of reaction progress over time in 2 mM and 10 mM MgCl2 using dashed and solid lines, respectively. Data points representing three independent experiments for class I and II, and two experiments for class III are shown.

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bound by E74 [24].
consisting of I-BmoI-In+ substrate as compared to UC formed with intronless thyA substrate. We next mutated the GC-2 base pair within the context of the intronless substrate to TA-2, AT-2 or CG-2, and measured the extent of the OP-Cu hypersensitive sites at positions -2 and -1 on the bottom strand. These three mutations reduced the OP-Cu hypersensitive sites to the same extent as was observed with intron-containing substrate (Figure 5b and 5c), indicating the GC-2 base pair is critical for inducing DNA distortions that result in enhanced OP-Cu sensitivity. To provide further evidence for this model, we tested a T-2G substitution within the context of the intron-containing substrate (Figure 5a), reasoning that this mutation should restore the OP-Cu hypersensitivity if contacts to the GC-2 base pair are required for minor-groove distortions. Indeed, footprinting reactions on the T-2G intron-containing substrate revealed a restoration of OP-Cu hypersensitivity at positions -2 and -1 to approximately half of those observed on thyA intronless substrate. Products seen at the -4 position are indicative of bottom-strand nicks observed when I-BmoI-thyA complexes were formed in-gel in the absence of exogenously added metal [24], as Cu²⁺ is not a productive divalent metal ion for cleavage (Figure 2A).

Modeling of an I-Bmol-substrate complex
To gain insight into the role of divergent metal ion and substrate contacts in the I-Bmol reaction pathway, we used the recently solved co-crystals of the GIY-GIY restriction enzymes Eco29kI and Hpy188I with their respective substrates to model an I-Bmol-substrate complex [36,37]. Eco29kI and Hpy188I function as dimers to cleave palindromic sites, with each GIY-YIG monomer nicking one strand of the substrate [37,39]. We first generated a homology model of residues 1-92 of I-BmoI based on the closely related I-TevI GIY-YIG domain structure [38]. Next, we superimposed the I-BmoI model on a monomer of the dimeric Eco29kI and Hpy188I structures by aligning residues 6-10 of I-BmoI with the structurally analogous amino acids of Eco29kI (residues 47-51) and Hyp188I (residues 61-65) (which included key structural resides of the β-sheet 1). We observed that the position of the I-BmoI active site residues (Y6, Y17, R27, H31, E74 and N87) aligned well with the homologous residues in both restriction enzymes (Figure 6A and data not shown), as was observed in two other structure superposition studies [36,37]. To model the position of the substrate in the I-Bmol active site, we included the substrate DNA from the Eco29kI structure, and similar results were obtained using the DNA from the Hpy188I structure. As

| Table 2. Summary of cleavage data for intronless and mutant substrates. |
|------------------------|-----------------|-----------------|-----------------|
| Substrate | 2mM MgCl₂ | 10mM MgCl₂ |
|            | k₁⁺ | k₁⁻ | k₂⁺ | k₂⁻ |
| (intronless) | G C C C G T | 0.057 | 0.026 | 0.11 | 0.074 |
| Class I | -4A | 0.050 | 0.028 | 0.10 | 0.057 |
| (like wild-type) | -6T | 0.057 | 0.024 | 0.087 | 0.058 |
| Class II | -1G | 0.031 | 0.0095 | 0.065 | 0.025 |
| (rescue) | -3G | 0.010 | 0.0084 | 0.025 | 0.025 |
| | -4A | 0.0095 | 0.016 | 0.031 | 0.042 |
| | -4A | 0.0082 | 0.0091 | 0.024 | 0.022 |
| | -5A | 0.0044 | 0.015 | 0.014 | 0.043 |
| | -6T | 0.011 | 0.017 | 0.028 | 0.048 |
| | -5A | 0.0045 | 0.015 | 0.014 | 0.047 |
| Class III | -2T | 0.00027 | n.d. | 0.00061 | n.d. |
| (no rescue) | -3G | n.d. | n.d. | n.d. | n.d. |
| | -2T | n.d. | n.d. | n.d. | n.d. |
| | -4A | n.d. | n.d. | n.d. | n.d. |
| | -5A | n.d. | n.d. | n.d. | n.d. |
| | -3C | 0.0037 | 0.00053 | 0.0013 | 0.014 |
| | -2T | n.d. | n.d. | n.d. | n.d. |
| | -5A | 0.00016 | n.d. | 0.00035 | 0.0026 |
| | -3G | n.d. | n.d. | n.d. | n.d. |
| | -2T | n.d. | n.d. | n.d. | n.d. |
| | -5A | n.d. | n.d. | n.d. | n.d. |
| | -4A | 0.00020 | 0.0017 | 0.00052 | 0.0091 |
| | -3G | n.d. | n.d. | n.d. | n.d. |
| | -2T | n.d. | n.d. | n.d. | n.d. |
| | -5A | n.d. | n.d. | n.d. | n.d. |
| (intron-containing) | T A A G T G | n.d. | n.d. | n.d. | n.d. |

*Rate constants were determined from three independent experimental trials for all substrates.

k₁, the rate constant for the first nicking reaction that generates nicked intermediate from circular substrate (expressed as the median of a 95% confidence interval, see Table S3).
k₂, the rate constant for the second nicking reaction that generates the linear product (expressed as the median of a 95% confidence interval, see Table S3).
n.d. not determined.
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shown in Figure 6, the path of the DNA follows a previously hypothesized catalytic cleft that is lined by the active site residues of I-BmoI [41], with the metal ion coordinated by E74 positioned in close proximity to the bottom-strand scissile phosphate at position -4. Of particular interest are the OP-Cu hypersensitive sites at positions -2 and -1 of the bottom strand that result from a widening of the minor groove. These distortions could place the base edge of the bottom strand C of the critical GC-2 base pair within hydrogen bonding distance of a number of side chains, highlighting the importance of this base pair in the cleavage pathway. The model also reinforces the notion that the role of divalent metal ion in promoting second-strand nicking must be in repositioning of the substrate-DNA complex rather than by directly participating in DNA hydrolysis.

Discussion

Enzymes that function as DNA endonucleases almost always require the presence of active site divalent metal ions [8,42,44]. In general, the preferred ion is magnesium, which acts to catalyze reaction progress by stabilizing negative phosphoanion transition states, or by acting as Lewis acids to modulate the pKa of coordinated water molecules [42,44]. Metal-activated water molecules can function as catalytic agents by adopting the roles of nucleophiles and general bases, or by protonating leaving groups [44,47]. Families of site-specific DNA endonucleases have distinct metal ion requirements, and generally function by either a one- or two-metal ion mechanism [44,47]. The metal ion requirements for homing endonucleases are best understood for the LAGLIDADG family that have an active-site preference for magnesium, and can uniquely function by a two-metal mechanism where a third divalent metal ion is shared between two active sites [8,48,49]. The H-N-H homing endonuclease I-HmuI, which generates single-stranded nicks in DNA substrates, functions optimally at 1 mM MgCl₂ or MnCl₂ and nicks via a one-metal ion mechanism [43]. Studies of the related H-N-H colicin E9 revealed different divalent metal ion requirements for cleavage of distinct nucleic acid substrates, with magnesium promoting cleavage of dsDNA and zinc being more effective for nicking of ssDNA [50]. I-PpoI, a His-Cys box homing endonuclease, functions by a one-metal ion mechanism and requires zinc and magnesium for folding and cleavage, respectively [8,51]. The focus of this paper, the GIY-YIG homing endonucleases, have traditionally been studied in the presence of magnesium that structural studies indicate is coordinated by a conserved active site glutamate (E74 in I-BmoI) [38]. GIY-YIG enzymes likely use a single-metal ion to promote DNA hydrolysis [36,37], and our data agrees with studies of the tetrameric GIY-YIG restriction enzyme Cfr42I that revealed a broad tolerance to divalent-metal ion [31]. Among the GIY-YIG family of enzymes, there is tremendous diversity in how the GIY-YIG nuclease domain is utilized to hydrolyze DNA. For instance, the restriction enzymes Eco29kI and Hpy188I function as dimers [37,39], with each GIY-YIG monomer nicking one strand, whereas the UvrC nucleotide excision repair protein uses a single GIY-YIG domain to nick 3' to a mutagenic lesion [40]. In this respect, GIY-YIG homing endonucleases differ from characterized GIY-YIG enzymes that make a DSB in that a single catalytic domain is used in two ordered and sequential nicking reactions to generate a DSB [24,25]. How the single active site of the GIY-YIG nuclease...
domain is reorganized on substrate between nicking reactions is unknown, but previous studies suggested an involvement of divalent metal ion in this process [24]. Our current study more accurately defines the roles of divalent metal ion and DNA substrate bases in this process as evidenced by reaction conditions that differentially affect $k_1$ or $k_2$. Not surprisingly, substrates that contained a mutation in the critical GC-2 base pair displayed drastically impaired rate constants, indicating a severe defect in first-strand nicking and an inability to form a DSB. Interestingly, bases surrounding the bottom-strand nicking site at positions -4 and -5 also resulted in reduced nicking rates when mutated. This observation was somewhat surprising as previous studies had not revealed a critical role of these positions for cleavage by I-Bmol [26], likely because past studies examined only linear product formation under high metal conditions rather than employing reactions conditions that could temporally dissect base-specific nicking effects. The I-Bmol-substrate model predicts that multiple amino acid side chains within the catalytic cleft project into the major groove near positions -4 and -5, implying that base pair substitutions result in altered contacts that consequently reduce $k_1$. The observation that $k_1$ defects associated with these substrates were significantly rescued in the presence of 10 mM MgCl$_2$ suggests that magnesium can compensate for lost I-Bmol-substrate interactions by promoting alternative conformations of protein or substrate within the catalytic cleft that facilitate cleavage. Additionally, substitutions near the bottom strand-nicking site

![Figure 6. Model of I-Bmol GIY-YIG domain interactions with substrate.](image-url)
Bacterial strains and plasmids

E. coli strains DH5α and ER2566 (New England Biolabs) were used for plasmid manipulations and protein expression, respectively. pTYBmoI was used to over-express a wild-type, codon-optimized version of I-BmoI for purification as previously described [41]. pBmoHS is a pBS derivative that contains an insert corresponding to 49 bp of the intronless thyA substrate [26]. A complete description of all plasmids used in this study is found in Table S1.

Oligonucleotides

Oligonucleotides used in this study can be found in Table S2.

Cleavage assays on plasmid substrates

Single time-point cleavage assays to examine metal dependence were performed in 10-μl volumes containing 20 nM Tris-HCl pH 8.0, 250 mM NaCl, 2.5% glycerol, 10 nM wild-type or G-2T pBmoHS, and various concentrations of MgCl2, CaCl2, CuCl2, MnCl2, NiCl2, and ZnCl2. Reactions were started by the addition of I-BmoI to final a concentration of 175 nM, allowed to proceed for 90 seconds at 37°C, and stopped by the addition of 4 μl stop dye (100 mM EDTA, 25% glycerol, 0.2% bromophenol blue). Stopped reactions were heated for 5 minutes at 95°C, cooled on ice for 5 minutes, electrophoresed on a 1% agarose gel, and stained in a 1×TAE solution containing ethidium bromide (Caledon) prior to analysis on an AlphaImagerTM3400 (Alpha Innotech). Time course cleavage reactions to determine Mg2+ dependence were performed on supercoiled pBmoHS in 115-μl volumes with conditions similar to those listed above, with either 0.5 mM, 1 mM, 2 mM, 5 mM, 7 mM, or 10 mM MgCl2. A 10-μl aliquot was removed prior to the addition of wild-type I-BmoI to final a concentration of 175 nM, and subsequent 10-μl aliquots were removed at ten time points into 4 μl stop dye. Stopped reactions were visualized as indicated above. Reaction progress was determined by the relative amount of circular substrate, nicked intermediate, and linear product. At least 3 independent trials were conducted for each MgCl2 concentration. Time course cleavage reactions with mutant pBmoHS substrates were performed in 65-μl volumes with conditions similar to those listed above, with either 2 mM or 10 mM MgCl2. A 10-μl aliquot was removed prior to the addition of wild-type I-BmoI to final a concentration of 175 nM, and subsequent 10-μl aliquots were removed at 15, 30, 60, 120, and 180 seconds into 4 μl stop dye. Mutant substrates were classified as follows: class I substrates displayed a reaction progress similar to wild-type intronless substrate, class II substrates displayed defects for both bottom and top-strand nicking reactions at 2 mM MgCl2 and ‘rescue’ of cleavage to near WT levels after 180 seconds in 10 mM MgCl2, and class III substrates showed severe to complete defects in 2 mM MgCl2 and no appreciable rescue of activity in 10 mM MgCl2 after 180 seconds. Cleavage assays for class I, II, and a selection of class III substrates (G-2T, C-3G/C-5A, T-1G/C-3G/C-5A, C-3G/C-4A/C-5A, and In+) were conducted as described above. For the remainder of class III substrates, only the accumulation of nicked intermediate or linear product above background levels was measured after a 180 second incubation with I-BmoI in 2 mM or 10 mM MgCl2. Three independent reactions were performed for substrates that gave measurable rate constants, and two independent trials were performed for class III substrates that displayed minimal or no cleavage. Rate constants for the reaction

\[
C \xrightarrow{k_1} N \xrightarrow{k_2} L
\]

were estimated for the two-constant irreversible kinetic model using Prism3 (GraphPad Software) or via the Bayesian bootstrap [61]. The time-course data were fit to the three following equations.
where \( [C_0] \) is the initial concentration of circular substrate \((\text{nM})\), \([X]\) is the concentration of nicked DNA \((\text{nM})\), \([L]\) is the concentration of linear product \((\text{nM})\), \(k_1\) is the first nicking rate constant \((\text{s}^{-1})\), \(k_2\) is the second nicking constant \((\text{s}^{-1})\), and \(t\) is time \((\text{seconds})\). For each bootstrap replicate, parameters were optimized for minimal discrepancy with the data under the half-taxi metric \([62]\). This procedure was found to be more robust than a standard least-squares estimation due to the constraint that the total amount of circular, nicked, and linear DNA is constant. Posterior parameter medians and 95% confidence intervals are reported in Table S3. The ratio of \(k_1/k_2\) at various MgCl2 concentrations was reported as the value of the \(\log(10)k_1/\log(10)k_2\) to minimize over-weighting the end points if plotted on a linear scale.

**OP-Cu in-gel footprinting**

1,10-phenanthroline copper (OP-Cu) footprinting experiments were conducted as previously described \([24]\). Substrates used included a 74-mer duplex oligonucleotide substrate corresponding to the intronless thyA target site \([\text{DE-116/DE-117}]\), the intronless 74-mer with G-2T \([\text{DE-446/DE-447}]\), G-2A \([\text{DE-459/DE-460}]\), or G-2C \([\text{DE-461/DE-462}]\) substitutions, the intron-containing 74-mer \([\text{DE-444/DE-445}]\), and the intron-containing 74-mer with A-2G substitution \([\text{DE-463/DE-464}]\) (see Table S2). Gel images were analyzed using ImageQuant 5.2 (GE Healthcare Life Sciences). To quantify hypersensitive sites observed in the OP-Cu footprint, two bands outside of the I-Bmol protection region were selected to normalize phosphorimager units \((\text{positions } +9 \text{ and } +10 \text{ for intronless substrates, and } +11 \text{ and } +12 \text{ for intron-containing substrates, relative to the intron insertion site})\). Hypersensitivity to OP-Cu at positions -1 and -2 was calculated by expressing the ratio of normalized phosphorimager units at sites in the shifted footprint (UC, upper complex) to the units in the unbound substrate reaction (UNB, unbound) \([24]\).

**Molecular modeling**

The I-Bmol GIY-YIG domain homology model was built as previously described \([41]\), and alignments to annotated structures were performed using MacPyMOL v1.2r. Residues 6-10 of the homology model were aligned to residues 47-51 of the solution structure of E142Q Eco29kI \((3MX1)\) (Figure 6A) \([36]\). A subsequent alignment was performed with the I-Bmol homology model and a single subunit of the Y49F/L69K Eco29kI structure in complex with its 15 base pair substrate \((3NIc)\) \([36]\). For illustration purposes, only nucleotides \((\text{AGCGCGGC})-(\text{+5})\) of the Eco29kI substrate were shown. Additional alignments were performed using structures of the I-TevI GIY-YIG domain \((1KM0\) and \(1LN0)\) in place of the I-Bmol homology model \([30]\), and of Hpy180I in complex with substrate \((3OQG)\) in place of the Eco29kI structure \([37]\), yielding nearly identical results.

**Supporting Information**

Table S1 Strains and plasmids used in this study. (DOC)

Table S2 Oligonucleotides used in this study. (DOC)

Table S3 Confidence intervals for rate constants determined on mutant substrates in low and high MgCl2 concentrations. (DOC)

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**Author Contributions**

Conceived and designed the experiments: BPK DRE. Performed the experiments: BPK WBJ ADF. Analyzed the data: BPK ADF DRE. Contributed reagents/materials/analysis tools: BPK DRE. Wrote the paper: BPK DRE.
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