Crystal Structure of the Homing Endonuclease I-CvuI Provides a New Template for Genome Modification*

Received for publication, July 17, 2015, and in revised form, September 8, 2015  Published, JBC Papers in Press, September 11, 2015, DOI 10.1074/jbc.M115.678342

Rafael Molina‡, Pilar Redondo†, Blanca López-Méndez‡, Maider Villate§, Nekane Merino‡, Francisco J. Blanco**‡, Julien Valton†, Silvestre Grizot‡, Phillipe Duchateau§, Jesús Prieto†, and Guillermo Montoya‡,§,‡

From the ‡Structural Biology and Biocomputing Programme, Spanish National Cancer Research Centre (CNIO), Macromolecular Crystallography Group, C/ Melchor Fernández Almagro 3, 28029 Madrid, Spain, the §Protein Structure & Function Programme, Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen, Denmark, the *Structural Biology Unit, CIC bioGUNE, Parque Tecnológico de Bizkaia 800, 48160 Derio, Spain, **IKERBASQUE, Basque Foundation for Science, María Díaz de Haro 3, 48013 Bilbao, Spain, and †CELLECTIS S. A., 8 rue de la croix Jarry, 75013 Paris, France

Background: Homing endonucleases are one of the templates used in genome engineering.

Results: Structural and biochemical analysis shows that I-CvuI can be a new scaffold to engineer novel DNA specificities.

Conclusion: The increase in catalytic ion concentration may strengthen I-CvuI selectivity.

Significance: This finding expands the homing endonuclease repertoire for redesigning new protein-DNA interactions.

Homing endonucleases recognize and generate a DNA double-strand break, which has been used to promote gene targeting. These enzymes recognize long DNA stretches; they are highly sequence-specific enzymes and display a very low frequency of cleavage even in complete genomes. Although a large number of homing endonucleases have been identified, the landscape of possible target sequences is still very limited to cover the complexity of the whole eukaryotic genome. Therefore, the finding and molecular analysis of homing endonucleases identified but not yet characterized may widen the landscape of possible target sequences. The previous characterization of protein-DNA interaction before the engineering of new homing endonucleases is essential for further enzyme modification. Here we report the crystal structure of I-CvuI in complex with its target DNA and with the target DNA of I-CreI, a homologue enzyme widely used in genome engineering. To characterize the enzyme cleavage mechanism, we have solved the I-CvuI DNA structures in the presence of non-catalytic (Ca²⁺) and catalytic ions (Mg²⁺). We have also analyzed the metal dependence of DNA cleavage using Mg²⁺ ions at different concentrations ranging from non-cleavable to cleavable concentrations obtained from in vitro cleavage experiments. The structure of I-CvuI homing endonuclease expands the current repertoire for engineering custom specificities, both by itself as a new scaffold alone and in hybrid constructs with other related homing endonucleases or other DNA-binding protein templates.

Homing endonucleases (HEs), also known as meganucleases, generate an accurate double-strand break that can be used to promote gene targeting through homologous recombination. These enzymes recognize long DNA sequences; their recognition sites vary between 12–45 bp in length. HEs are highly sequence-specific enzymes and display a very low frequency of off-target cleavage, even in whole genomes. Due to this rare-cutting property, they have been used for the manipulation of the genomes of mammalian and plant cells (1–3). However, the use of meganuclease-induced recombination has long been limited by the repertoire of natural meganucleases amenable of customization.

HEs are grouped in several families; among them the LAGLIDADG family is the most abundant. The family name derives from the conserved amino acid sequence motif containing the catalytic aspartic residue. The nuclease of this family cleave their DNA target along the minor groove to generate cohesive 4-bp-long 3’-OH overhangs. Two classes of LAGLIDADG nucleases can be found. One of them contains a single copy of the motif acting as homodimers, which bind palindromic or near-palindromic DNA target sequences. The other class contains two copies of the LAGLIDADG motif acting as monomers, which recognize and cleave non-palindromic DNA sequences (4). Catalysis requires the conserved acidic residue at the C termini of the two LAGLIDADG sequences in the active site and the coordination of divergent metal cations for phosphodiester hydrolysis (5). Members of the LAGLIDADG family have been engineered to specifically target new DNA sequences emerging as powerful tools for gene targeting. The most used template is I-CreI, whose scaffold has been redesigned using a combinatorial approach to target new specific DNA sequences (6).

I-CvuI is a homologue of I-CreI, and therefore a homodimeric LAGLIDADG homing endonuclease. This enzyme is

---

* This work was supported by the European Community’s Seventh Framework Programme (FP7/2007-2013) under BioStruct-X (Grant 283570). This work was also supported by the Ministerio de Economía y Competitividad (BFU2011-23815/BMC (to G. M.) and CTQ2014-56966-R (to F. J. B.)), the Ministerio de Educación y Ciencia (to P. S. G. and J. V.) and the European Social Fund under the 2007-2013 Spanish National Research Council (MICINN). This work was also supported by the Ministerio de Economía y Competitividad through the Infrastructures National Network ( несколькие статьи с различными титулами и текстами).
coded by the genome of the chloroplast large subunit ribosomal RNA gene of the green algae *Chlorella vulgaris*. I-CvuI recognizes and cleaves a 24-bp-long DNA palindromic sequence (see Fig. 1). Here we report the crystal structure of I-CvuI in complex with its target DNA, hereafter termed Sro1.3, and with the target DNA of I-CreI, hereafter termed C1221 (7). We have solved the structures in the presence of Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ to characterize I-CvuI cleavage and its DNA recognition pattern, and we have performed a comparison with I-CreI, the best characterized meganuclease template. The structure of this novel HE expands the current repertoire for engineering custom specificities used as a new scaffold or in hybrid constructs with other related HEs (8). Furthermore, after evaluating the metal dependence of I-CvuI catalysis, we analyzed *in vitro* the metal concentration dependence of the phosphodiester hydrolysis reaction using catalytic ions ranging from non-cleavable to cleavable concentrations.

**Experimental Procedures**

**Protein Expression, Purification, and Crystallization—I-CvuI expression, purification, protein-DNA complex formation in the presence of Sro1.3 (5'-TCAGAAGCTGCTACGACGGTCTGTA-3') and crystallization have been described previously (9). Here we have incubated the complex in the presence of both non-catalytic ions (Ca$^{2+}$ at 2 mM) and catalytic ions (Mg$^{2+}$ and Mn$^{2+}$ at 2−20 and 2 mM, respectively). For comparison reasons, we also obtained the I-CvuI-DNA complex using the analogue DNA target from I-CreI, C1221 (5'-TCAAAAACGTCGTACGCCGGTCTGTA-3'), in the presence of 2 mM Mg$^{2+}$. The C1221, a palindromic 24-bp DNA target, derived from the pseudo-palindromic I-CreI wild-type target (10), was used here as a template for the *in vitro* cleavage experiments because this palindromic scaffold is cleaved *in vivo* by I-CreI with the same efficiency as the wild-type pseudo-palindromic target (11).

**I-CvuI DNA Binding Studied by Isothermal Titration Calorimetry—Isothermal titration calorimetry experiments were performed in a MicroCal Auto-iTC200 (Malvern). Both protein and DNA samples were extensively dialyzed in 25 mM Heps, 150 mM NaCl, 10 mM CaCl$_2$, pH 7.4. I-CvuI (6 μM) and Sro1.3 or C1221 (both at 65 μM) were loaded into the calorimeter cell and titration syringe, respectively. Titrations were carried out using 25 injections of 1.5 μl each injected at 180−240-s intervals. Data analysis was performed on the concentration-normalized heats by nonlinear regression using the MicroCal PEAQ-ITC analysis software (Malvern).

**I-CvuI DNA Binding Studied by Microscale Thermophoresis—**Microscale thermophoresis experiments were performed on a NanoTemper Monolith NT.115 instrument using 40% LED and 20−40% IR-laser power. Laser on and off times were set at 30 and 5 s, respectively. Cy5-labeled oligonucleotides were purchased from TAG Copenahgen A/S (Copenhagen, Denmark). The concentration of the labeled oligonucleotides was kept constant at 25 nM, and the corresponding endonuclease binding patterns were titrated in 1:1 dilutions with the highest concentrations at 500 nM or 8 μM. Samples were prepared in buffer containing 25 mM Heps, 50 mM NaCl, 0.2% Pluronic F-127, 0.5 mg/ml BSA, pH 7.4, supplemented with either 10 mM CaCl$_2$ or 10 mM MgCl$_2$, and loaded into standard treated capillaries (NanoTemper Technologies) for measurements. The $K_D$ was determined by fitting the change in thermophoretic depletion to the quadratic solution of the mass action law. (The fluorescence during thermodiffusion was averaged for 1 s after the 10 s of IR-laser heating due to the higher signal to noise in this region. Overall, the IR-laser was on for 30 s.) Error bars are the standard deviation between three independent repeats ($n = 3$).

**I-CvuI in Vitro DNA Cleavage—**To analyze the effect of cations, the I-CvuI enzymatic activity was assayed against Sro1.3 and C1221 included either in a BamHI-linearized yeast plasmid or in an XmnI-linearized pGEM-T plasmid, respectively, as described previously (12). The cleavage of linearized plasmid was performed at variable cation concentrations (between 1 and 10 μM for MgCl$_2$ or MnCl$_2$) against 100 ng of substrate and with 150 ng of protein. In the assays, the wild-type I-CvuI was incubated with the DNA at 37 °C in 10 mM Tris pH 8, 50 mM NaCl and the corresponding cation using a final volume of 25 μl. The reactions were stopped by the addition of 6× Buffer Stop (45% glycerol, 95 mM EDTA (pH 8), 1.5% (w/v) SDS, 1.5 mg/ml proteinase K, and 0.048% (w/v) bromophenol blue) and incubated at 37 °C for 15 min. Samples were then electrophoresed in 0.6% agarose gels, and the intensity of the bands was observed under UV light.

**Structure Determination, Model Building, and Refinement—**All data were collected at 100 K, using a PILATUS 6M detector both at the PX beamline at the Swiss Light Source (SLS, Villigen) and at XLALC (ALBA, Barcelona, Spain). Data processing and scaling were accomplished using XDS (13) and SCALA from the CCP4 package (14) (see Tables 1 and 2). The structures were solved by molecular replacement as implemented in the program PHASER (15). The search model was based on the Protein Data Bank (PDB) entries 1G9Z (I-CreI-DNA-Mg$^{2+}$) or 1G9Y (I-CreI-DNA-Ca$^{2+}$) according to the *a priori* knowledge of catalytic or non-catalytic conditions. The structures were then subjected to iterative cycles of model building and refinement with Coot (16) and PHENIX (17). The identification and analysis of the protein-DNA hydrogen bonds and van der Waals contacts were achieved with the Protein Interfaces, Surfaces and Assemblies service (PISA) at the European Bioinformatics Institute. DNA structures were analyzed using 3DNA (18).

**Results**

**Overall Structure of the I-CvuI-Sro1.3 Complex—I-CvuI in complex with a 24-bp DNA duplex, corresponding to the palindromic sequence of its wild-type target, was crystallized as an enzyme-substrate complex with Ca$^{2+}$ and as an enzyme-product complex with Mn$^{2+}$. The overall fold of I-CvuI in complex with its DNA target is similar to I-CreI (Fig. 1). The structure shows a homodimer (with monomer A and monomer A') with a clear two-fold symmetry axis between the LAGLIDADG helix of each monomer (α1 and α1'). Each monomer contains the typical αββαββα topology of the LAGLIDADG family having three extra helices at the C terminus. Two antiparallel β-sheets, β1-β4 and β1'-β4, form a concave surface with an inner cylindrical shape where the DNA molecule is accommodated (Fig. 1A). The non-cleaved DNA and the cleaved I-CvuI
DNA structures differ in the status of the DNA molecule (Fig. 2), but the protein moieties are similar with a Cα helix/H9251 rmsd of 0.27 Å.

**I-CvuI versus I-CreI DNA Binding**—I-CreI is the member of the LAGLIDADG family that has been extensively used in protein-DNA interaction redesign (7). Therefore, due to our final aim to provide a new scaffold for meganuclease design, we have compared the I-CvuI structure with this member of the family. I-CvuI shows respectively 37% identity and 55% sequence similarity with I-CreI. The crystal structures of I-CvuI and I-CreI (19) share a common overall folding; nevertheless some secondary structure differences are observed (Fig. 1B). As a consequence of their primary sequence differences, the main topology differences are located at α1, α3-α4, and α6. Particularly interesting are the higher length of α6 in I-CvuI than in I-CreI and the presence of η2 at the end of α4 in I-CvuI, whereas in I-CreI, η2 is located at the end of α3.

The DNA interaction patterns in I-CvuI/Sro1.3 and I-CreI/C1221 complexes are very different (Fig. 3A), although the DNA sequences of the Sro1.3 and the C1221 are almost identical, differing only at positions ±9 (Fig. 3A). However, the targets conserve only the interactions between the residues Gln-29 (Gln-26 in I-CreI) with strand B and Arg-73 (Arg-70 in I-CreI) with η symbol refers to a 310-helix) are displayed as purple cylinders. β-Strands are rendered as blue arrows, and strict η turns are rendered as T7 letters.

**FIGURE 1. Structural characterization of I-CvuI-DNA complex.** A, crystal structure of I-CvuI in complex with its target Sro1.3. The DNA sequence is shown at the bottom of the figure. Red triangles indicate the phosphodiester cleavage site. The box indicates the catalytic region. The two calcium ions are shown as cyan spheres. B, structure-based sequence alignment of I-CvuI versus I-CreI using ESPRIT (28). α-Helices and 310-helices (η symbol refers to a 310-helix) are displayed as purple cylinders. β-Strands are rendered as blue arrows, and strict η turns are rendered as T7 letters.
used the 3DNA program (18), revealing clear changes in the twist parameter at those regions. Thus, the local base step parameter twists in Sro1.3 at the transition A/G, G/A and T/C, C/T are higher (30.15°, 50.64° and 44.06°, and 32.02°) than at the transition A/A, A/A and T/T, T/T in the C1221 (31.46°, 33.52° and 31.25°, and 37.14°), and this punctual difference is responsible for the higher twisting of the overall folding. To further understand the molecular basis of the higher overall twisting in Sro1.3 as compared with C1221, we analyzed in detail the interactions at positions ±9. As indicated in Fig. 3C, Arg-33 of I-CvuI interacts with −9GstrandA in the Sro1.3 target, whereas in the C1221, DNAQ38 from I-CreI shows an analogue interaction with −9AstrandA. In addition, C1221 shows an extra interaction with the complementary base to −9AstrandA, −9TstrandB through Asn-30 from I-CreI. The longer side chain of Arg-33 in I-CvuI as compared with Gln-38 in I-CreI along with the extra interaction through Asn-30 from I-CreI explained the over-twisting of the Sro1.3 target. Isothermal titration calorimetry data indeed show an unfavorable entropic contribution (−TΔS = 18.2 kcal/mol at 25 °C) for the I-CvuI-I-Sro1.3 complex that is nevertheless offset by a most favorable enthalpy contribution (ΔH = −28.1 kcal/mol at 25 °C).

I-CvuI Target Recognition Pattern and Site Recognition Flexibility—Previous work reveals that I-CvuI is a highly specific enzyme. It displays no or low activity when it binds the DNA targets of homologous enzymes such as for example the one of I-CreI (8). We demonstrate here that the binding affinity of I-CvuI for the C1221 palindromic target of I-CreI is lower as compared with the affinity for its own target in the presence of both non-catalytic and catalytic cations (Figs. 4 and 5). I-CvuI shows a 3-fold higher affinity (53.3 versus 131.9 nM) for its own target versus the C1221 as measured by isothermal titration calorimetry (Figs. 4B and 5B). However, I-CreI cleaves the Sro1.3 in a similar degree to C1221 (8). Thus, to address specificity issues and how they could affect the site recognition flexibility for redesign purposes, we also solved the crystal structure of the complex formed by I-CvuI and C1221 (Fig. 6A, bottom), thus deciphering the protein-DNA contacts with this sequence. The Sro1.3 and C1221 are palindromic. They differ only in the two positions ±9 of the whole 22-bp target (Fig. 6A). Exchanging the G/C base pairs at ±9 in the Sro1.3 by A/T base pairs in the C1221 promoted rearrangements in the protein-DNA interaction interface. Seven out of eight amino acids are involved in direct interactions to bases with Sro1.3 (Fig. 6A, top). These interactions were conserved in the I-CvuI-C1221 complex structure. A new extra residue, Gln-41, appeared involved in the I-CvuI-C1221 interface (Fig. 6A, bottom). This new interaction does not alter the conformation of the DNA target (Fig. 6B). However, although −9GstrandA in Sro1.3 interacts specifically with Arg-33, the corresponding base in the palindromic C1221 (−9AstrandA) interacts with Gln-41 and Arg-43 through water-mediated contacts (Fig. 6C). Furthermore, −9CstrandB in Sro1.3 does not display protein contacts, whereas at 10 Å. The calcium ions are shown as cyan spheres. The manganese ions are shown as black spheres. Black arrows indicate the phosphodiester bond cleavage on each strand.

**FIGURE 2.** Detailed view of the I-CvuI-Sro1.3 active site in the presence of non-catalytic and catalytic ions. A, schematic representation of LAQLI-\(\text{DADGK}\text{H}\) active site, B, detailed view of the active site in the presence of 2 mM Ca\(^{2+}\) with the anomalous map superimposed showing electron density contoured at 3.5 sigma. C, detailed view at the active site in the presence of 2 mM Mn\(^{2+}\) with the anomalous map superimposed showing electron density contoured at 10 sigma.
the base in the same position in C1221 (~9T_strandB) interacts specifically with Arg-33. An additional difference is that ~8T_strandB in Sro1.3 interacts with Arg-43 through a water-mediated contact, but its counterpart in C1221 shows a direct interaction with Arg-33.

**Active Site and Cleavage Mechanism**—Divalent metal ions play an essential role in the phosphodiester catalysis in meganucleases (20). The conserved acidic residues at the active site coordinate the divalent metal ions. The general mechanism of cleavage of the phosphodiester bonds of DNA requires a nucleophile to attack the electron-deficient phosphorus atom, a general base to activate the nucleophile, and a general acid to protonate the leaving group. Positively charged groups are also needed to stabilize the phosphoanion transition state. The role of divalent metal ions during cleavage in LAGLIDADG meganucleases has been thoroughly studied (21, 20). Three metal sites have been observed in the active center called A, B, and C. Sites A and B are located in opposite locations, whereas site C occupies the center of the active site (Fig. 2A). The metal at site C is shared between the two catalytic sites and participates in a two-metal ion cleavage mechanism to hydrolyze the phosphodiesters of both strands (20).

The positions of the metal ions in the crystal structures of the I-CvuI were analyzed by collecting anomalous diffraction data, using crystals grown in the presence of CaCl₂ and MnCl₂ (Fig. 2B and C). The structure of the enzyme-substrate complex included the non-catalytic Ca²⁺ ions, which were visualized by examining the anomalous difference Fourier maps. In contrast with I-CreI, which shows three Ca²⁺ atoms in the active site (5), only two anomalous peaks could be detected in sites A and B of the I-CvuI active center (Fig. 2B). The Ca²⁺ atoms are hexacoordinated with phosphates from both DNA strands (+3G_strandA, −1A_strandB, and +1A_strandA, −3G_strandB, respec-
tively), three water molecules, and the side chains of Asp-23-Asp-23 in each LAGLIDADG motif.

We followed a similar strategy for the enzyme-product complex including the presence of Mn$^{2+}$ ions (Tables 1 and 2). Three anomalous peaks in sites A, B, and C were detected due to the presence of this metal in the I-CvuI active center (Fig. 2C). The central Mn$^{2+}$ ion is shared between the two acidic catalytic residues. The two external Mn$^{2+}$ ions are located in positions equivalent to the Ca$^{2+}$ cations. They also display similar interactions with neighboring atoms. The Mn$^{2+}$ ion in site A is coordinated with the side chain of Asp-23, the carbonyl of Ala-22, the 5’-phosphate of +3G$_{strandA}$, the phosphate of −1A$_{strandB}$, and a water molecule outside the active site. The Mn$^{2+}$ in site B interacts with the 5’-phosphate of +1A$_{strandA}$, the phosphate of −3G$_{strandB}$, the main chain carbonyl of Ala-22, the side chain of Asp-23 in the LAGLIDADG motif of the other monomer, and a water molecule outside the active site. The Mn$^{2+}$ in site C interacts with both catalytic residues Asp-23-Asp-23, the carbonyls of the 3’-phosphates +3G$_{strandA}$, −3G$_{strandB}$, and the 5’-hydroxyls of +2C$_{strandA}$, −2C$_{strandB}$. Thus, our data show that the cleaved complex contains the expected three metal sites and follows a mechanism similar to other members of the LAGLIDAG family (20, 21). The comparison of the I-CvuI Ca$^{2+}$ and Mn$^{2+}$ anomalous maps (Fig. 2) suggests that I-CvuI may follow a mechanism similar to the monomeric I-DmoI (22). However, the structural organization of the homodimeric I-CvuI active site is symmetric, suggesting that there is no cleavage preference for one strand over the other during catalysis, such as in the case of I-DmoI (20).

Besides the residues involved in the metal binding, other critical residues in HE catalysis are responsible for the generation of the basic pocket needed for favoring the transition state (23). In this sense, Lys-102 is located in the enzyme active site and is a candidate to act as a Lewis acid (stabilizing the pentacoordinate transition state) or to activate a proton donor in the cleavage reaction. A sequence alignment with other homodimeric HEs suggests that the other residue involved in the basic pocket in I-CvuI should be Arg-54. Interestingly, in I-CvuI, a glutamine is found in this position instead of the conserved arginine/lysine (8). By analogy with I-CreI, additional residues such as Arg-73 in I-CvuI (Arg-70 in I-CreI) were located at the active site in the same positions. The mutation of these amino acids abolishes I-Crel endonuclease activity (24).

### Figure 4

In vitro cleavage and binding experiments, with I-CvuI using the Sro1.3 target. A, I-CvuI in vitro plasmid cleavage experiments in the presence of Mg$^{2+}$ at concentrations ranging from 1 to 10 mM. The plasmid was linearized with BamHI before the assay with the enzyme. The top band represents the linearized plasmid, and the two lower bands are the products of I-CvuI cleavage. Mw, molecular weight markers. B, Isothermal titration calorimetry binding curve in the presence of 10 mM Ca$^{2+}$. C, binding curves by microscale thermophoresis in the presence of 10 mM Ca$^{2+}$. D, binding curves by microscale thermophoresis in the presence of 10 mM Mg$^{2+}$. The binding curves by microscale thermophoresis represent the data points from three measurements. Error bars indicate means ± S.E.
Catalysis Is Metal Type- and Concentration-dependent—As described previously (8), I-CvuI catalysis is dependent on the type of metal. To fully understand the cleavage characteristics of I-CvuI, we analyzed the activity of the enzyme in the presence of Ca\(^{2+}\)/H\(^{+}\) and Mg\(^{2+}\)/H\(^{+}\), combining structural, binding, and in vitro cleavage information using its target Sro1.3. As has been observed in other meganucleases, the presence of 2 mM Ca\(^{2+}\)/H\(^{+}\) did not promote cleavage of the DNA target, in agreement with our enzyme-substrate structure (Fig. 2B). On the other hand, in the presence of the same concentration of Mn\(^{2+}\)/H\(^{+}\), the crystal structure revealed that both DNA strands were cleaved and that three metal ions were located at the active site (positions A, B, and C, Fig. 2C).

To investigate the metal ion type and concentration dependence, we performed in vitro cleavage experiments (Fig. 4A), showing a metal concentration dependence of phosphodiester hydrolysis when using Mg\(^{2+}\). Cleavage at 2 mM Mg\(^{2+}\) is not complete (Fig. 4A), whereas at 10 mM, the enzyme cleaves the target completely (Fig. 4A).

In addition, by in vitro cleavage experiments, we checked the I-CvuI activity of the C1221 target in the presence of different concentrations of Mg\(^{2+}\). In contrast with the I-CvuI:Sro1.3 complex, high concentrations of Mg\(^{2+}\) inhibited the cleavage of the C1221 target, whereas low concentrations allowed partial cleavage (Fig. 5A). Moreover, the crystal structure of I-CvuI:C1221 in presence of 2 mM Mg\(^{2+}\) showed a mixture between the non-cut and cut target, in agreement with the in vitro cleavage experiments. We could not obtain crystals of this protein-DNA complex at higher Mg\(^{2+}\) concentrations, hampering the collection of structural information. Therefore, to address whether this behavior is related to a catalytic cation-dependent affinity, we performed microscale thermophoresis experiments for the I-CvuI:C1221 complex at different magnesium concentrations (Fig. 5D). Interestingly, I-CvuI binding decreases for its non-specific target DNA at higher cation concentrations (Fig. 5D). In fact, the presence of 10 mM Mg\(^{2+}\) completely abolishes the binding, in agreement with the in vitro cleavage experiments that showed no cleavage at 10 mM Mg\(^{2+}\) (Fig. 5, A and D).

Discussion

LAGLIDAG homing endonucleases are one of the scaffolds widely used to create highly precise protein “cutters” capable of generating a double-strand break in a desired genome region.
Structure and DNA Binding Properties of Endonuclease I-CvuI

A

Hydrogen bound to DNA (<3.5Å)

- Direct contact
- Water-mediated contact
- H-bond acceptor
- H-bond donor

B

C

I-CvuI:Sro1.3

I-CvuI:C1221
once their DNA specificity has been redesigned. However, their use is limited to the repertoire of known HEs not covering the whole genome sequence. To overcome this problem, we could redesign already known scaffolds or characterize novel HEs whose DNA pattern recognitions fulfill our DNA target requirements. Usually, the re-engineering of well-known scaffolds, such as I-Crel, to recognize a given DNA sequence is expensive and time-consuming. Therefore, it could be cheaper to find other HEs that may offer the same or similar effects. Indeed, the current massive genome sequencing helps us find new scaffolds for these biotechnological purposes that greatly expand the number of DNA sequences that can be targeted. I-CvuI is an example of a novel HE with another DNA target and its quasi-analogue palindromic I-CreI target. Following some of our previous studies with members of the LAGLIDADG homing endonuclease family, we suggest that the concentration influences I-CvuI catalysis using its DNA target according to their sequence differences imposes important conformational differences. This effect has been observed previously in I-Crel variants targeting the human XPC (25) and RAG1 (26) genes. Furthermore, we have shown how the metal concentration influences I-CvuI catalysis using its DNA target and its quasi-analogue palindromic I-Crel target. Following some of our previous studies with members of the LAGLIDADG homing endonuclease family, we suggest that the increase of the cation concentration may induce subtle conformational changes in the target that decrease binding (27). Thus, an increase of Mg$^{2+}$ ions could make more stringent the differences between C1221 and the Sro1.3, leaving the enzyme in a less favorable scenario and avoiding cleavage of the non-target DNA. Therefore, our data suggest that the DNA pattern recognition of I-CvuI can be altered with few changes in the DNA sequence, suggesting the possibility to use this new scaffold for HE redesign.

Thus, I-CvuI offers an alternative scaffold to well-established HEs already used in the development of redesigned enzymes for therapeutic and biotechnological applications. The characterization of the I-CvuI-DNA complex should help in the production of intelligent molecular scalpels that recognize and substi-

FIGURE 6. DNA recognition pattern of I-CvuI with Sro1.3 and C1221. A, summary of the DNA interfaces for I-CvuI in the presence of Sro1.3 (top) or C1221 (bottom). Water molecules and water-mediated H-bonds are red. The scissile phosphate groups are black. Protein-DNA base contacts different between each target are shown in bold. Hydrogen bond donors and acceptors on nucleotide bases are denoted by protruding and recessed ovals, respectively. Yellow boxes indicate differences in target sequences. B, overall folding of both DNAs bound to I-CvuI. C, detailed view of the interaction around ± 9 target position with each target.
tute certain DNA sequences, may avoid the high cost of engineering some other traditional HE scaffolds, and may be useful for therapeutic and biotechnological purposes.

**Author Contributions**—G. M. conceived the study and together with J. P. coordinated the work and wrote the paper. R. M., G. M., and J. P. designed, performed, and analyzed the experiments. P. R., M. V. F. J. B., P. D., J. V., S. G., and N. M. provided technical assistance, commented the experiments and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—We thank the Swiss Light Source and XALOC beamline staff from the Spanish Synchrotron Radiation Facility for their support. The Novo Nordisk Foundation Center for Protein Research is supported financially by the Novo Nordisk Foundation (Grant NNF14CC0001).

**References**

1. Choulika, A., Perrin, A., Dujon, B., and Nicolas, J. F. (1994) The yeast I-SceI meganuclease induces site-directed chromosomal recombination in mammalian cells. *C. R. Acad. Sci. III* 317, 1013–1019

2. Choulika, A., Perrin, A., Dujon, B., and Nicolas, J. F. (1995) Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 15, 1968–1973

3. Rouet, P., Smith, F., and Jasim, M. (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 91, 6064–6068

4. Marcaida, M. J., Muñoz, I. G., Blanco, F. J., Prieto, J., and Montoya, G. (2010) Homing endonucleases: from basics to therapeutic applications. *Cell. Mol. Life Sci.* 67, 727–748

5. Chevalier, B. S., Monnat, R. J., and Stoddard, B. L. (2001) The homing endonuclease I-CreI uses three metals, one of which is shared between the two active sites. *Nat. Struct. Biol.* 8, 312–316

6. Prieto, J., Molina, R., and Montoya, G. (2012) Molecular scissors for in situ cellular repair. *Crit. Rev. Biochem. Mol. Biol.* 47, 207–221

7. Arnaoud, S., Chames, P., Perez, C., Lacroix, E., Duclet, A., Epinat, J. C., Stricher, F., Petit, A. S., Patin, A., Guiller, S., Rolland, S., Prieto, J., Blanco, F. J., Bravo, J., Montoya, G., Serrano, L., Duchateau, P., and Pâques, F. (2006) Engineering of large numbers of highly specific homing endonucleases that induce recombination on novel DNA targets. *J. Mol. Biol.* 355, 443–458

8. Lucas, P., Otis, C., Mercier, J. P., Turmel, M., and Lemieux, C. (2001) Rapid evolution of the DNA-binding site in LAGLIDADG homing endonucleases. *Nucleic Acids Res.* 29, 960–969

9. Redondo, P., Merino, N., Villate, M., Blanco, F. J., Montoya, G., and Molina, R. (2014) Crystallization and preliminary X-ray diffraction analysis of the homing endonuclease I-CreI from Chlorella vulgaris in complex with its target DNA. *Acta Crystallogr. F Struct Biol Commun.* 70, 256–259

10. Wang, J., Kim, H. H., Yuan, X., and Herrin, D. L. (1997) Purification, biochemical characterization and protein-DNA interactions of the I-CreI endonuclease produced in *Escherichia coli*. *Nucleic Acids Res.* 25, 3767–3776

11. Arnaoud, S., Perez, C., Cabaniols, J. P., Smith, J., Gouble, A., Grizot, S., Epinat, J. C., Duclet, A., Duchateau, P., and Pâques, F. (2007) Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *J. Mol. Biol.* 371, 49–65

12. Prieto, J., Epinat, J. C., Redondo, P., Ramos, E., Padró, D., Cédrone, F., Montoya, G., Pâques, F., and Blanco, F. J. (2008) Generation and analysis of mesophilic variants of the thermostable archaeal I-Dmol homing endonuclease. *J. Biol. Chem.* 283, 4364–4374

13. Kabsch, W. (2010) XDS. *Acta Crystallogr. D Biol. Crystallogr.* 66, 125–132

14. Evans, P. (2006) Scoring and assessment of data quality. *Acta Crystallogr. D Biol. Crystallogr.* 62, 72–82

15. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. *J. Appl. Crystallogr.* 40, 658–674

16. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* 66, 486–501

17. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* 66, 213–221

18. Lu, X. J., and Olson, W. K. (2008) 3DNA: a versatile, integrated software system for the analysis, rebuilding and visualization of three-dimensional nucleic-acid structures. *Nat. Protoc.* 3, 1213–1227

19. Jurica, M. S., Monnat, R. J., and Stoddard, B. L. (1998) DNA recognition and cleavage by the LAGLIDADG homing endonuclease I-CreI. *Mol. Cell* 2, 469–476

20. Molina, R., Stella, S., Redondo, P., Gomez, H., Marcaida, M. I., Orozco, M., Prieto, J., and Montoya, G. (2015) Visualizing phosphodiester-bond hydrolysis by an endonuclease. *Nat. Struct. Mol. Biol.* 22, 65–72

21. Stoddard, B. L. (2005) Homing endonuclease structure and function. *Q. Rev. Biophys.* 38, 49–95

22. Marcaida, M. J., Prieto, J., Redondo, P., Nadra, A. D., Albés, A., Serrano, L., Grizot, S., Duchateau, P., Pâques, F., Blanco, F. J., and Montoya, G. (2008) Crystal structure of I-Dmol in complex with its target DNA provides new insights into meganuclease engineering. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16888–16893

23. Chevalier, B., Sussman, D., Otis, C., Noël, A. J., Turmel, M., Lemieux, C., Stephens, K., Monnat, R. J., and Stoddard, B. L. (2004) Metal-dependent DNA cleavage mechanism of the I-CreI LAGLIDADG homing endonuclease. *Biochemistry* 43, 14015–14026

24. Seligman, I. M., Stephens, K. M., Savage, J. H., and Monnat, R. J. (1997) Genetic analysis of the Chlamydomonas reinhardtii I-CreI mobile intron homing system in *Escherichia coli*. *Genetics* 147, 1653–1664

25. Redondo, P., Prieto, J., Muñoz, I. G., Albés, A., Serrano, L., Cabaniols, J. P., Daboussi, F., Arnould, S., Perez, C., Duchateau, P., Pâques, F., Blanco, F. J., and Montoya, G. (2008) Molecular basis of xeroderma pigmentosum group C DNA recognition by engineered meganucleases. *Nature* 456, 107–111

26. Muñoz, I. G., Prieto, J., Subramanian, S., Coloma, J., Redondo, P., Villate, M., Merino, N., Marenchino, M., D’Abramo, M., Gervasio, F. L., Grizot, S., Daboussi, F., Smith, J., Chion-Sotinel, I., Pâques, F., Duchateau, P., Albés, A., Stricher, F., Serrano, L., Blanco, F. J., and Montoya, G. (2011) Molecular basis of engineered meganuclease targeting of the endogenous human RAG1 locus. *Nucleic Acids Res.* 39, 729–743

27. Molina, R., Redondo, P., Stella, S., Marenchino, M., D’Abramo, M., Gervasio, F. L., Epinat, J. C., Valton, J., Grizot, S., Duchateau, P., Prieto, J., and Montoya, G. (2012) Non-specific protein-DNA interactions control I-CreI target binding and cleavage. *Nucleic Acids Res.* 40, 6936–6945

28. Robert, X., and Gouet, P. (2014) Deciphering key features in protein structures with the new ENDScript server. *Nucleic Acids Res.* 42, W320–W324