Published online 26 May 2018

Nucleic Acids Research, 2018, Vol. 46, No. 12 ***6229–6237***
doi: 10.1093/nar/gky344

Single-site DNA cleavage by Type III restriction endonuclease requires a site-bound enzyme and a trans-acting enzyme that are ATPase-activated

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Received January 30, 2018; Revised April 16, 2018; Editorial Decision April 17, 2018; Accepted April 26, 2018

ABSTRACT

Endonucleolytic cleavage of DNA by Type III restriction-modification (RM) enzymes requires long-range communication between at least two recognition sites in inverted orientation. This results in convergence of two nuclease domains, one each from the enzymes loaded at the recognition sites with one still bound to the site. The nucleases catalyze scission of the single-strands leading to double-strand DNA break. An obscure feature of the Type III RM enzymes EcoP1I and EcoP15I is their ability to cleave DNA having a single recognition site under certain conditions. Here we demonstrate that single-site cleavage is the result of cooperation between an enzyme bound to the recognition site in *cis* and one in *trans*. DNA cleavage is catalyzed by converging nucleases that are activated by hydrolysis-competent ATPase in presence of their respective DNA substrates. Furthermore, a single activated nuclease cannot nick a strand on its own, and requires the partner. Based on the commonalities in the features of single-site and two-site cleavage derived from this study, we propose that their mechanism is similar. Furthermore, the products of two-site cleavage can act as substrates and activators of single-site cleavage. The difference in the two modes lies in how the two cooperating enzymes converge, which in case of single-site cleavage appears to be via 3D diffusion.

INTRODUCTION

Long-range communication between two distal DNA sites, in *cis*, regulates fundamental cellular functions of transcription regulation and DNA metabolism. The communication is mediated by active and directional movement (translocation) or 1D diffusion along DNA, or looping of DNA. ATPase motors power translocation of proteins along DNA, hydrolyzing one nucleotide per base pair. These motors can be categorized as helicases that unwind double-stranded DNA and actively translocate on single-stranded DNA, and translocases that translocate on double-stranded DNA. In contrast to the motors, an emerging theme is that of ATPases that function as switches and use far fewer nucleotides to promote their one dimensional (1D) diffusion along DNA (1–4).

The phenomenon of switch mediated 1D diffusion has been proposed through single-molecule studies on the MutS-MutL family of mismatch repair proteins (3,4). A mechanism involving 1D diffusion has also been proposed for the endonucleolytic activity of Type III restriction-modification (RM) enzymes EcoP1I and EcoP15I (2). Both these enzyme systems use diffusion along DNA to allow communication between distant sites of action. MutS bound to ATP diffuses along the DNA between the site of mismatch and the site of nicking of the unmethylated strand (5). ATP-dependent enzyme diffusion facilitates DNA cleavage by Type III RM enzymes between distant recognition sites. In both these systems, though the presence of ATP is essential, its role is not fully understood. Here we report a study that unravels the role of ATP at various stages of the nucleolytic activity of Type III RM enzymes.

Type III RM enzymes are bacterial defense systems that protect the host from invading foreign DNA by nucleolytically cleaving them at specific recognition sites. The RM enzyme is composed of two subunits of methyltransferase (Mod) and a restriction subunit (Res) having an ATPase and an endonuclease domain. The dimeric Mod or the trimeric Res-Mod complex catalyzes methylation of the recognition site. Endonucleolytic cleavage of DNA by Type III RM enzyme requires the presence of at least two unmethylated recognition sites in inverted orientation (6), which will henceforth be referred to as two-site cleavage. The recognition site is asymmetric and as a consequence only one strand is methylated. Methylation of genomic DNA protects it from the restriction activity of the

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enzyme. A battery of biophysical, biochemical and single-molecule studies carried out over the last decade on the Type III RM enzymes EcoP1I and EcoP15I has led to different models of DNA cleavage (2,7). According to a recent model derived from magnetic tweezers combined with TIRF microscopy, a Type III RM enzyme, on binding to its recognition site and in presence of ATP, undergoes a change in its conformational state, which promotes its 1D diffusion along the DNA (2). An alternate model based on atomic force microscopy, proposes the convergence of the two enzymes through DNA looping and limited ATP hydrolysis for translocation (7,8). DNA cleavage without looping has been observed in magnetic tweezers assay (9). Consequently, the requirement and role of DNA looping for cleavage needs further studies.

According to the 1D diffusion model, encounter of the diffusing enzyme with another enzyme bound to a recognition site brings together a nuclease domain each from the respective enzymes. The two nucleases catalyze the nicking of alternate single-strands resulting in a double-strand break at a fixed distance downstream of the recognition site of the stationary enzyme. Independent of the spacing between the recognition sites, which can be as large as 3000 bp, the nucleolytic reaction requires the hydrolysis of about ten ATP molecules (10,11). The hydrolysis of ATP by Type III RM enzyme alters its conformational state to diffusion-competent (2). However, the requirement of the nucleotide for DNA cleavage *per se* is not fully clear. For example, it is not known if the ATPase of the stationary enzyme, like that of the diffusing enzyme, has to hydrolyse ATP for DNA cleavage.

Although the canonical substrates for Type III RM enzymes have at least two recognition sites in inverted orientation, there have been reports of Type III RM enzymes cleaving linear and circular DNA having only a single recognition site in presence of ATP (12–18). Earlier reports indicated a circular plasmid having a single recognition site was cleaved better than the linearized plasmid (17). Cleavage of the linearized plasmid could be improved on addition of sinefungin (17). The site of this cleavage has been mapped to 25–27 bp downstream of the recognition site of EcoP15I and 26–28 bp downstream of EcoP1I recognition site (19). This is identical to that mapped for cleaved canonical substrates (20). However, the mechanism of the non-canonical ATP-dependent single-site cleavage is unknown. An endonucleolytic reaction entails hydrolysis of a phosphodiester bond on both strands. A Type III RM enzyme, which has only one nuclease domain, can catalyse single-site cleavage by binding to the recognition site and (i) nicking the two strands, akin to the monomeric restriction endonuclease BfiI (21) or (ii) nicking one strand while recruiting another enzyme to nick the other strand, like the endonuclease FokI (22) (Figure 1A).

Using a heterologous cooperation assay involving two Type III RM enzymes, EcoP1I and EcoP15I, we demonstrate here which of the two modes lead to single-site cleavage. Furthermore, we reveal the role of ATP in single-site cleavage. We also address if ATP has any other role in cleavage of canonical substrates, in addition to facilitating 1D diffusion. Finally, this study allows us to present a common model for single-site and two-site cleavage. Together, this study reveals how an ATPase switch regulates various stages of a multistep enzymatic process.

MATERIALS AND METHODS

DNA substrates

DNA substrates were purchased from Integrated DNA Technologies and Sigma Aldrich. The two complementary DNA strands were annealed using temperature gradient from 95 to 25°C. The annealed DNA substrates were further purified using an 8 ml Mono Q 10/100 GL column. The resulting duplex was washed thoroughly with Milli-Q water, and concentrated using Vivaspin concentrator (MWCO 3 kDa; GE Healthcare). The concentrated DNA was stored at -30°C until further use. The various oligonucelotides used in biochemical studies are mentioned in Supplementary Table S1.

Protein purification of EcoP1I and EcoP15I

Escherichia coli BL21(DE3) cells were transformed with wild type EcoP1I operon in recombinant vector pRSF with a 6xHis tag at the N-terminus of the Mod subunit. An 8 L culture was grown at 37°C till the OD_{600} reached 0.4 after which the culture was shifted to a lower temperature of 18°C. The culture was induced at OD_{600} of 0.6 with 1 mM IPTG and grown further for 12–14 h with constant shaking. The culture was pelleted and harvested by dissolving in lysis buffer [50 mM Tris–HCl (pH 8), 500 mM NaCl, 10% glycerol, 10 mM $MgCl_2$]. The re-suspended cell pellet were lysed by sonication. A sonication cycle was carried out for 3 min with 1 s on and 3 s off. For complete lysis of cells the sonication cycle was repeated two times. To prevent generation of heat during sonication, the lysate was kept on ice during the entire process. The lysate was spun at 37 000 rpm for 1 h at 4°C using Optima XE ultracentrifuge (Beckman Coulter). As the first purification step, EcoP1I was purified by affinity chromatography using a 5 ml Ni-NTA column (GE Healthcare). Fractions containing EcoP1I were pooled and dialyzed against B-50 buffer [50 mM Tris–Cl (pH 8), 50 mM NaCl, 1 mM EDTA, 1 mM DTT] for 6 h at 4°C. The dialyzed protein was further purified using an 8 ml Mono Q 10/100 GL column (GE Healthcare). The EcoP1I obtained after Mono Q contained impurities, which were removed by hydrophobic interaction chromatography using a 5 ml Hi Trap Phenyl HP Low substitution column (GE Healthcare). EcoP1I thus obtained was highly pure. To check the homogeneity of EcoP1I, size exclusion chromatography (SEC) using Superdex 200 10/300 (GE Healthcare) was carried out as a last step. EcoP1I obtained after SEC was pooled concentrated and stored in buffer 10 mM Tris–HCl pH 8, 100 mM KCl and 1 mM DTT.

Escherichia coli BL21(AI) cells were transformed with wild type EcoP15I operon in recombinant vector pHIS (23) with a 6xHis tag at the C-terminus of the Res subunit. A 6 L culture was grown at 37°C till OD_{600} reached 0.4, after which culture was shifted to 18°C. The culture was induced with 2 g/l L-arabinose and kept further for 10 h with constant shaking. EcoP15I was purified using a protocol similar to that used for purification of EcoP1I, except that the HIC purification step was not included.

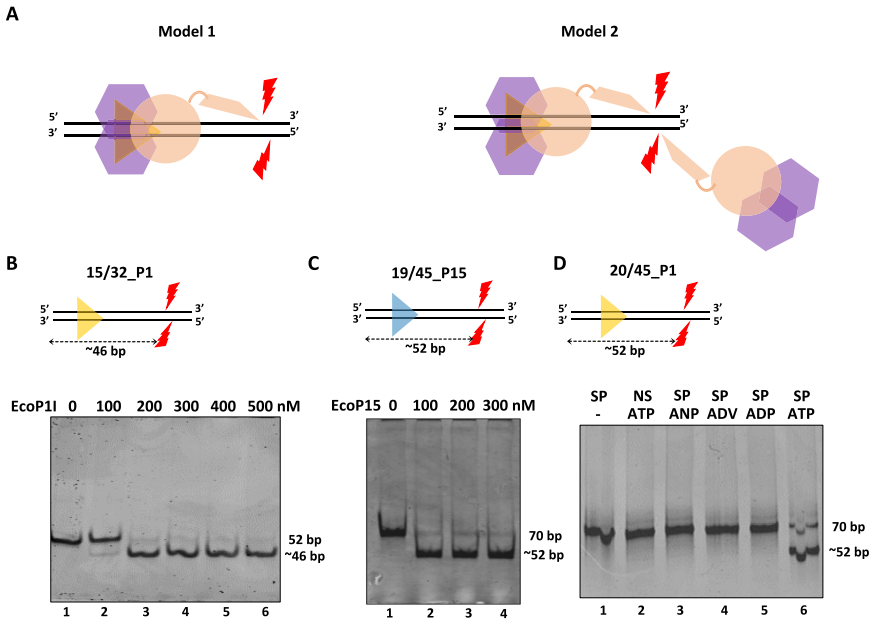

Figure 1. Single-site cleavage by EcoP1I and EcoP15I. (**A**) Cartoons illustrating the possible models for single-site cleavage. Model 1: a single Type III RM enzyme (Mod colored purple and Res colored beige) bound to its recognition site (yellow arrowhead) performs a double-strand DNA break by nicking both the strands. Model 2: an enzyme bound to its recognition site cooperates with a free enzyme from solution to cleave the DNA. (**B**) 300 nM of DNA, 15/32_P1, containing single recognition site of EcoP1I (yellow arrowhead) and (**C**) 300 nM 19/45_P15 having a single recognition site of EcoP15I (blue arrowhead) was incubated with increasing concentration of their respective enzymes (the reaction was performed twice and similar results obtained). The reaction was started by the addition of ATP. Red arrows mark the site of nicks, and the position of cut of the top strand from the upstream end is mentioned. Length of DNA substrate and cleaved product are given on the right hand side of the gels respectively. (**D**) Single-site cleavage was catalyzed by 200 nM of EcoP1I only in presence of ATP and 300 nM of its specific DNA (SP, 20/45_P1) having the corresponding recognition site but not in the presence of a non-specific DNA (NS). Cleavage failed to happen in presence of ADP or non-hydrolysable ATP analogues AMP-PNP (ANP), ADP-vanadate (ADV).

Mutagenesis of EcoP1I and EcoP15I

Nuclease dead mutants of EcoP1I^{E916A} EcoP15I^{D898A} and Walker A and Walker B mutants EcoP1I^{K90R} EcoP1I^{E227A} EcoP15I^{E227A} were generated using quick-change site directed mutagenesis. Positive clones were confirmed by sequencing the entire gene. The mutants of EcoP1I and EcoP15I were purified using the same protocol as wild type enzymes.

Labelling of DNA substrate with ^{32}P at 5′-end

dsDNA substrate 100 nM was labeled at 5′-end with T4 polynucleotide kinase (New England Biolabs) in presence of (gamma-^{32}P ATP) at 37°C for 30 min. Polynucleotide kinase was heat inactivated at 65°C for 20 min. The DNA was purified using MicroSpin column (GE Healthcare) and stored at –30°C until further use.

DNA cleavage assays

DNA cleavage assays were carried out in Buffer R [50 mM Tris–HCl pH 8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) at 25°C. Protein and DNA were incubated in Buffer R supplemented with 20 μM sinefungin for 45 minutes after which the reaction was started by the addition of 1 mM ATP. The reaction was carried out for 45 min after which the reaction was stopped by the addition of 0.5× stop buffer (10 mM Tris–HCl pH 8, 60 mM EDTA, 60% glycerol, 0.025% SDS, 0.03% xylene cyanol).The samples were kept at 25°C for 20 min and then loaded on 18% NATIVE PAGE [18% acrylamide:bisacrylamide (19:1), 1× TBE]. In case of denaturing urea-formamide gel runs, the reaction was stopped with formamide stop dye (95% formamide, 0.025% SDS, 0.5 mM EDTA and 0.03% bromophenol blue). For complete denaturation of DNA, the samples were heated at 99°C for 10 min before loading on 18% urea-formamide PAGE gel (18% acrylamide: bis-acrylamide, 7 M urea and 20% formamide, 1× TBE). The gels were stained with a water solution containing ethidium bromide and scanned using Typhoon TRIO+ variable mode imager.

Heterologous cooperation assays

EcoP1I and EcoP15I were incubated with respective DNAs separately in presence of 20 μM sinefungin at 25°C for 45 min. After which the sample was mixed and reaction was started by addition 1 mM of ATP. The reaction was further carried out for 45 min at 25°C after which the reaction was stopped with 1 volume of formamide stop dye. The samples were heated for 10 min at 99°C and loaded on 18% urea-formamide PAGE gel. The gel was run at 220 V for 90 min stained with ethidium bromide and scanned using Typhoon TRIO+ variable mode imager (GE Healthcare)

For reactions visualized using radioactive labels, 5 nM of ^{32}P DNA was incubated with EcoP1I in presence of 20 μM sinefungin for 45 min at 25°C. Similarly 300 nM of unlabeled 5/24_P15 DNA was incubated with 250 nM of Ecop15I in presence of 20 μM sinefungin. After 45 min the two protein–DNA complexes were mixed and a reaction was started with 1 mM of ATP. The reaction was in-

cubated for 45 min at 25°C after which the reaction was stopped with equal volume of formamide gel loading dye. The samples were heated at 99°C for 10 min, and immediately loaded on 18% urea–formamide PAGE gel. The gel was run at 10 W for 90 min. The gel was imaged by placing on phosphor imager plate and kept at –80°C for 3 h. The phosphor imager plate was scanned using Typhoon TRIO+ variable mode imager.

RESULTS

Prerequisites for single-site cleavage

Both EcoP1I and EcoP15I cleaved DNA having just one recognition site (Figure 1B). As expected from the previous reports of location of cleavage of a single-site substrate by EcoP1I (19) and our analysis (Supplementary Figure S1), EcoP1I cleaved the DNA 15/32_P1 (a 52 bp long DNA with the 5 bp EcoP1I recognition site, AGACC, flanked by 15 bp upstream and 32 bp downstream, see Supplementary Figure S2) into ∼46 and ∼6 bp fragments (Figure 1B). The ∼6 bp fragment could not be visualized either because staining with ethidium bromide was not sensitive enough or because the fragment had run out of the gel (Figure 1B). Similarly, as expected based on previous mapping studies (19), EcoP15I cleaved the DNA 19/45_P15 (a 70 bp long DNA with the 6 bp long EcoP15I recognition site, CAGCAG, flanked by 19 bp upstream and 45 bp downstream, see Supplementary Figure S2) into ∼52 and ∼18 bp fragments (Figure 1C). Based on previous reports (16–18), we included sinefungin and potassium chloride to enhance efficiency of single-site cleavage by EcoP1I and EcoP15I. The shorter fragment could not be visualized on the gel. The enzymes did not cleave a non-specific (NS) DNA lacking recognition sites (Figure 1D, Lane 2). Cleavage was noticed only in presence of ATP, and was not observed in absence of ATP or with ATP analogue AMP-PNP, or with transition state ATP mimic ADP-vanadate or with ADP (Figure 1D, lanes 3–6).

Single-site cleavage is a trans activity

EcoP1I and EcoP15I have been shown to cooperate to cleave DNA having one each of their respective recognition sites oriented head-to-head (23). We used this knowledge to design a 'heterologous cooperation assay' to find if the single-site cleavage is a *cis* or a *trans* activity. The heterologous cooperation assay involved use of two separate short pieces of DNA each with a single recognition site for EcoP1I or EcoP15I, respectively. Reaction was initiated by addition of ATP to a mix containing EcoP15I and nuclease-dead EcoP1I^{E916A} bound to their respective single-site DNA. The products of the reaction were analyzed on a denaturing urea-formamide PAGE gel (Figure 2A and B). The 35 bp long 5/24_P15 was chosen as specific DNA for EcoP15I, as this DNA was one base pair short of getting cleaved, as is clear from Figure 2C, lanes 3–5. This minimized the number of cleaved DNA products on the denaturing gel, simplifying their analysis.

EcoP1I cleaved 15/32_P1 resulting in two single strands of 46 and 48 bases (Figure 2C, lane 2). As above, the shorter fragments could not be visualized on the denaturing gel using ethidium bromide. Despite the use of the nuclease-dead EcoP1I^{E916A}, the DNA containing its recognition site was nicked, but not cleaved, in presence of EcoP15I bound to its 35 bp long specific DNA 5/24_P15 (Figure 2C, lane 3). However, EcoP15I did not nick EcoP1I specific DNA in the absence of the specific DNA of EcoP15I (Figure 2C, lanes 4). Also, EcoP15I in presence of 5/24_P15 and EcoP1I nicked only the specific DNA of EcoP1I and not a non-specific one (Figure 2C, lanes 3 and 5). Consequently, we concluded that the nicking of EcoP1I specific oligomer was a result of cooperation between EcoP1I^{E916A} and EcoP15I bound to their specific DNA. It should be noted that the experiment does not rule out the possibility that the DNA bound state is only required for activation of the enzyme to a reaction competent conformational state, and not essential during the reaction. However, the fixed distance of cleavage from the recognition site of the *cis*-bound enzyme, i.e. EcoP1I^{E916A} in this case, indicates that this enzyme is site bound during catalysis. In comparison to the efficiency of single-site cleavage by a single enzyme, EcoP1I or EcoP15I (Figure 1B and C, 2C, lane 2), the efficiency of nicking noticed in case of heterologous cooperation involving both EcoP1I and EcoP15I was much less (Figure 2C, lane 3), suggesting that the heterologous cooperation is not as optimized as homologous cooperation.

Although the heterologous cooperation assay revealed that single-site cleavage happened by the cooperation of two enzymes in *trans*, it did not rule out the possibility of a single enzyme bound to its recognition site also performing double-strand break on its own in *cis*. Due to the high concentration of enzyme–DNA complex used in the above assay, it was hard to delineate cleavage due to a single enzyme and cleavage due to cooperation between two enzymes encountering each other in solution. To overcome the ambiguity, we studied single-site cleavage reaction at low concentrations of enzyme–DNA complex. Our reasoning was that if single-site cleavage required the enzymes to cooperate in *trans*, then the reaction would not occur at very low concentrations of enzyme-substrate complex due to a reduced frequency of two of the complexes coming together.

In this set of reactions, as the amount of DNA used was much less than what could be visualized using ethidium bromide stain, we used radiolabeled EcoP1I DNA substrates instead (Figure 2B). Reaction mixes with varying concentrations of EcoP1I (5–40 nM) was incubated with 5 nM substrate DNA. Under such low concentrations of enzyme-DNA complex, amount of either single-strand nick or double-strand break observed was negligible (Figure 2D, lanes 1, 3, 5, 7 and 9). This suggested that an enzyme bound to its recognition site could not catalyze a nick or double-strand break on its own. However, a significant increase in DNA cleavage was noticed on addition of 250 nM of EcoP15I and 300 nM of its substrate DNA (Figure 2D, lanes 8 and 10). This led us to conclude that the single-site cleavage of a DNA required cooperation between an enzyme bound to the DNA in *cis* and an enzyme with its specific DNA in *trans*.

We next asked the question if the DNA product of a single-site cleavage reaction could in complex with the enzyme participate in cleaving other DNA substrate

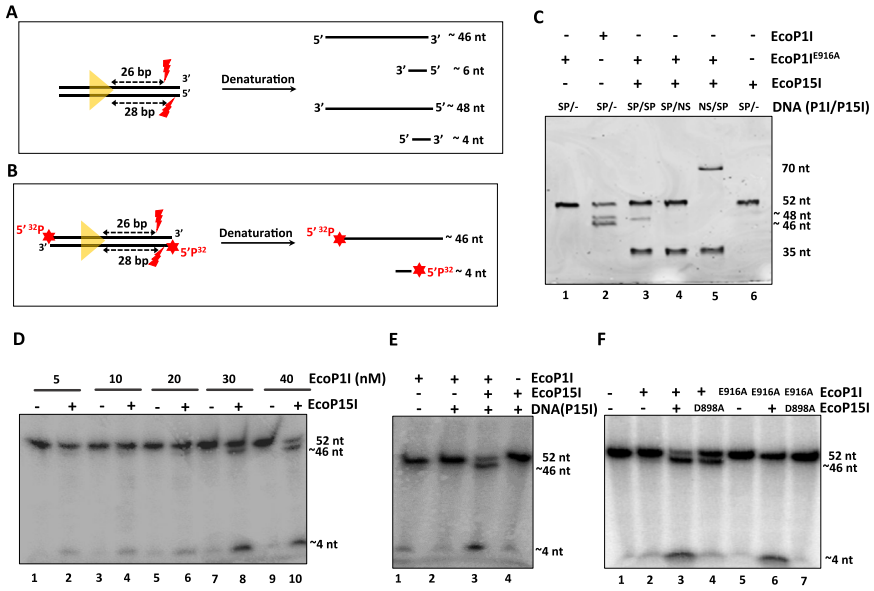

Figure 2. Single-site cleavage is the result of cooperation between a *cis*-bound and a *trans*-acting enzyme. (**A**) A schematic illustrating the products of a hypothetical single-site cleavage as single-strands that are observed when analyzed on a denaturing urea-formamide PAGE using ethidium bromide stain. The position of the cleavage sites from the upstream end is mentioned. These positions are based on previous mapping of single-site cleavage. (**B**) The cleavage products observed when a DNA with its 5′-ends labeled with ^{32}P is analyzed on a denaturing urea-formamide PAGE gel. (**C**) An heterologous cooperation assay with of EcoP1I and EcoP15I. The DNA in the gel were unlabeled and stained using ethidium bromide for visualization. SP/SP represents presence of specific DNA of EcoP1I and EcoP15I, SP/– represents presence of EcoP1I specific DNA only, SP/NS represents the presence of EcoP1I specific DNA and non-specific DNA of EcoP15I, and so on. Lanes 1) 15/32_P1 + EcoP1I^{E916A}, 2) 15/32_P1 + EcoP1I, 3) 15/32_P1 + EcoP1I^{E916A} and 5/24_P15 + EcoP15I, 4) 15/32_P1 + EcoP1I^{E916A} and NS_P15 + EcoP15I, 5) NS_P1 + EcoP1I^{E916A} and 5/24_P15 + EcoP15I, 6) 15/32_P1 + EcoP15I. 300 nM of DNA and 250 nM of enzymes were used. (**D**) Cleavage assay with 5 nM 15/32_P1 DNA labeled at 5′-end with increasing concentration of EcoP1I. dsDNA break happened only when a higher concentration of EcoP15I/DNA complex was added indicating that single-site cleavage happened as a result of cooperation between *cis*-bound and *trans*-acting enzymes (the experiment was repeated thrice and similar results obtained). (**E**) Heterologous cooperation assay demonstrating that the DNA product of a single-site cleavage can participate in further rounds of single site cleavage. Lane 1: 5 nM 15/32_P1 DNA labeled at the 5′-end and 40 nM EcoP1I. Lane 2: 5 nM 15/32_P1 DNA labeled at the 5′-end, 40 nM EcoP1I and 300 nM of purified product of the single-site cleaved 19/27_P15 DNA. Lane 3: 5 nM 15/32_P1 DNA labeled at the 5′-end, 40 nM EcoP1I, 250 nM of EcoP15I and 300 nM of the purified cleaved product of 19/27_P15 DNA. Lane 4: 5 nM 15/32_P1 DNA labeled at the 5′-end, 250 nM of EcoP15I and 300 nM of the purified cleaved product of 19/27_P15 DNA. The experiment was repeated thrice and similar results obtained. (F) Cleavage assay demonstrating that the *cis*-bound enzyme cleaved the top strand and the *trans*-acting enzyme cleaved the bottom strand. 5 nM 15/32_P1 and 300 nM 5/24_P15 was used for the assay (the experiment was repeated thrice and similar results obtained). See text for details.

molecules? Toward this, we purified cleaved DNA product of single-site cleavage by EcoP15I using a native PAGE (Supplementary Figure S3). The purified DNA was then used as the EcoP15I specific DNA in a heterologous cooperation assay. The EcoP1I specific substrate DNA (radiolabelled15/32_P1) was cleaved in presence of the above-mentioned purified product DNA, EcoP1I, EcoP15I and ATP (Figure 2E, lane 3). To verify that the *trans* enzyme activity occurs in single enzyme system too, we carried out two experiments: (i) 5 nM of radiolabeled 15/32_P1 was incubated with 250 nM of EcoP1I for different time periods (Supplementary Figure S4A); (ii) the same experiment as in (i) performed in presence of 250 nM of unlabeled 5/26_P1 (Supplementary Figure S4B). We found cleavage under condition (ii), where unlabeled DNA was supplemented, to be much more efficient than under condition (i) (Supplementary Figure S4A and B). This observation is consistent with the model of the requirement of a *trans* acting enzyme to catalyze single-site cleavage. The DNA cleavage observed at longer periods of incubation under condition (i) could be the result of an activated enzyme that has dissociated from the DNA but still proficient to cooperate with the *cis*-bound enzyme to catalyze cleavage.

The cis-bound enzyme nicks the top strand and the trans-acting enzyme nicks the bottom strand

In the previous section, we had shown that inactivating the nuclease of one of the enzymes resulted in only a nick, indicating that the double-strand break was the result of two nicks each catalyzed by one of the two cooperating enzymes. To identify which of the two enzymes nick the top and the bottom strands, we carried out another heterologous assay. In this assay, we used a combination of wild type and nuclease-dead enzymes, and radiolabelled the EcoP1I DNA substrate. As the 5′-ends of both strands of the DNA were labeled, due to the difference in length of the DNA fragments generated (Figure 2B), we could identify which of the two strands were cleaved by the *cis*-bound and *trans*-acting enzymes, by keeping one of the enzymes active and the other nuclease-dead.

As in the previous experiment, we maintained the EcoP1I or EcoP1I^{E916A} concentration at 40 nM and its substrate DNA at 5 nM to reduce the frequency of two EcoP1I–DNA complexes coming together and causing DNA cleavage. To this reaction mix, we added 250 nM of EcoP15I or its nuclease-dead mutant EcoP15I^{D898A} and 300 nM of its substrate DNA. Cleavage reaction was initiated by ad-

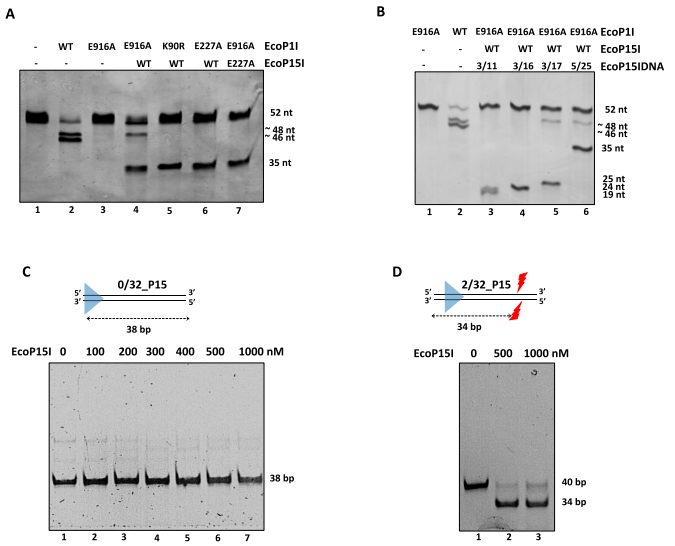

Figure 3. Role of ATP and effect of DNA lengths on single-site cleavage. (**A**) A denaturing urea-formamide PAGE gel stained with ethidium bromide to visualize the cleaved product of a heterologous assay involving the Walker mutants. Lanes 1) 15/32_P1, 2) 15/32_P1 + EcoP1I, 3) 15/32_P1 + EcoP1I^{E916A}, 4) 15/32_P1 + EcoP1I^{E916A} and 5/24_P15 + EcoP15I, 5) 15/32_P1 + EcoP1I^{K90R} and 5/24_P15 + EcoP15I, 6) 15/32_P1 + EcoP1I^{E227A} and 5/24_P15 + EcoP15I, 7) 15/32_P1 + EcoP1I and 5/24_P15 + EcoP15I^{E227A}. 300 nM of DNA and 250 nM of enzymes were used (the experiment was repeated twice and similar results were obtained). The DNA length mentioned on the left hand side of the gel image is that of EcoP1I specific DNA and its cleavage products, and the 35 bp long DNA is 5/24_P15. (**B**) A denaturing urea-formamide PAGE gel stained with ethidium bromide to identify the minimal length of DNA required downstream of the recognition site for activation of single-site cleavage. Lanes 1) 15/32_P1 + EcoP1I^{E916A}, 2) 15/32_P1+ EcoP1I, 3) 15/32_P1 + EcoP1I^{E916A} and 3/11_P15 + EcoP15I, 4)15/32_P1 + EcoP1I^{E916A} and 3/16_P15 + EcoP15I, 5) 15/32_P1 + EcoP1I^{E916A} and 3/17_P15 + EcoP15I, 6) 15/32_P1+ EcoP1I^{E916A} and 5/25_P15 + EcoP15I. 300 nM of DNA and 250 nM of enzymes were used. (**C**) Cleavage assay of 300 nM 0/32_P15 DNA with increasing concentration of EcoP15I resolved on a native PAGE gel. (**D**) DNA cleavage assay of 300 nM 2/32_P15 DNA with increasing concentration of EcoP15I resolved on a native PAGE gel.

dition of ATP. A reaction mix with EcoP1I and EcoP15I yielded two fragments corresponding to nicks on both the top and bottom strands (Figure 2F, lane 3). A reaction involving wild type EcoP1I and EcoP15I^{D898A} resulted in the nick of the top strand (Figure 2F, lane 4), while a reaction involving EcoP1I^{E916A} and EcoP15I resulted in the nick of the bottom strand (Figure 2F, lane 6). These results revealed that the *cis*-bound enzyme cleaved the top strand, while the *trans*-acting enzyme cleaved the bottom strand.

Role of ATP in single-site cleavage

In the preceding sections we demonstrated that single-site cleavage requires two enzymes to cooperate in *trans* and hydrolyze ATP. This led us to the question if ATP binding and/or hydrolysis are required by one or both of the cooperating enzymes. To address this question, we carried out heterologous cooperation assays with one of the enzymes deficient in either ATP binding or hydrolysis. Mutation of Walker A and Walker B motif of the ATPase of EcoP15I has been shown to affect ATP binding and hydrolysis, respectively (25,26). Accordingly, Walker A mutant EcoP1I^{K90R} deficient in ATP binding, and the Walker B mutants EcoP1I^{E227A} and EcoP15I^{E227A} deficient in ATP hydrolysis were used for this experiment. As shown earlier, heterologous cooperation assay with nuclease-dead EcoP1I^{E916A} and wild type EcoP15I resulted in a DNA nick (Figure 2C, lane 3; Figure 3A, lane 4). However, replacement of the nuclease-dead enzyme by the Walker mutants EcoP1I^{K90R} or EcoP1I^{E227A} inhibited any form of nucleolytic activity from either of the enzymes (Figure 3A, lanes 5 and 6). A heterologous assay with EcoP1I^{E916A} and ATP hydrolysis mutant EcoP15I^{E227A} also showed a complete lack of either nick or double-strand break (Figure 3A, lane 7). These observations suggested that both the cooperating enzymes have to be proficient in not only ATP binding but also ATP hydrolysis for single-site cleavage to happen.

Significance of DNA length flanking recognition site for single-site cleavage

In most of the reactions described above, we used 5/24_P15 as substrate for EcoP15I, which itself does not get cleaved but still facilitates the *trans* cleavage of EcoP1I single-site substrates. This prompted us to find if the DNA downstream of the recognition site could be shortened further and, if so, what was the minimal length required for the *trans* nucleolytic activity. Towards this, we used EcoP15I specific DNA of varying lengths downstream (Figure 3B). We saw nicking of the EcoP1I specific DNA even in presence of 3/17_P15, which had only 17 bp downstream of the recognition site (Figure 3B, lane 5). However, on shortening this length to 16 bp or less, the nicking of EcoP1I specific DNA was completely abolished (Figure 3B, lanes 3 and 4).

Next we studied the effect of DNA length upstream of the recognition sequence. For this, instead of using the heterologous cooperation assay, we simply incubated EcoP15I with DNA having 0 bp (0/32_P15) and 2 bp (2/32_P15) upstream of the recognition sequence and monitored their cleavage in presence of ATP. We found that lack of DNA upstream of the recognition site stopped the cleavage (Figure 3C). Cleavage occurred on addition of two or more base pairs upstream (Figure 3D). These analyses revealed that, in addition to ATP hydrolysis, DNA length is an important criterion for the in *trans* nicking activity.

Effect of Walker mutants on cleavage of two-site substrates

We showed above that single-site cleavage requires ATP binding and hydrolysis by both the *trans*-acting enzymes. We, thence, proceeded to see if this conclusion extends to cleavage of the canonical two-site substrate. A previous study using a DNA substrate having an EcoP1I and an EcoP15I recognition site showed that ATP binding mutant EcoP1I^{R394A} in cooperation with active EcoP15I could not cleave DNA while the wild type enzyme could (20). In similar lines, we designed an assay in which a 350 bp long DNA substrate with two recognition sites, one of EcoP1I and the other of EcoP15I, in head-to-head orientation was used. The expected products of cleavage are shown in Figure 4A. The radiolabelled DNA incubated with 20 nM of EcoP1I or EcoP15I showed the occurrence of very faint single-site

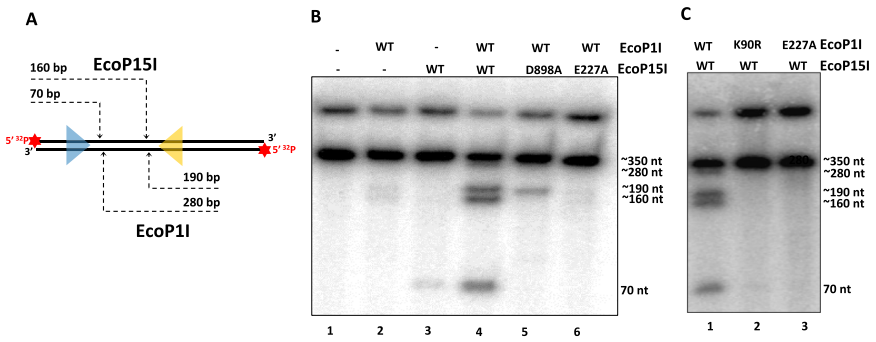

Figure 4. ATP hydrolysis by both enzymes is essential for two-site cleavage. (**A**) A schematic illustrating that the cleavage product of a 350 bp DNA labeled at 5′-end with ^{32}P having a single recognition site of both EcoP1I and EcoP15I in head-to-head orientation will result in four single-strands of length 70, 160, 190 and 280 nt when resolved on a denaturing urea-formamide PAGE gel. (**B**) Denaturing 18% urea-formamide PAGE showing that ATP hydrolysis by EcoP15I is essential for nicking or cleavage. Lanes 1) 350 bp DNA, 2) 350 bp DNA + EcoP1I, 3) 350 bp DNA + EcoP15I, 4) 350 bp DNA + EcoP1I + EcoP15I, 5) 350 bp DNA + EcoP1I + EcoP15I^{D898A}, 6) 350 bp DNA + EcoP1I + EcoP15I^{E227A}. 3 nM of DNA and 20 nM of enzymes were used. Normalized intensities of lanes 4 and 5 are shown in Supplementary Figure S4. (**C**) Denaturing 18% urea-formamide PAGE showing that ATP hydrolysis by EcoP1I is essential for nicking or cleavage. Lanes 1) 350 bp DNA + EcoP1I + EcoP15I, 2) 350 bp DNA + EcoP1I^{K90R} + EcoP15I, 3) 350 bp DNA + EcoP1I^{E227A} + EcoP15I. 3 nM of DNA and 15 nM of enzymes were used (the experiment was repeated twice and similar results obtained). Normalized intensities of Lane 1 are shown in Supplementary Figure S4. The gels in panel B and C demonstrate conclusively the ATP hydrolysis by both the stationary and diffusing enzymes are required for DNA nicking/cleavage. The top most bands in the gels B and C possibly are the result of incomplete denaturation of the 350 bp DNA.

cleavage (Figure 4B, lanes 2 and 3). Incubation with an enzyme mix of 20 nM each of EcoP1I and EcoP15I resulted in DNA cleavage pattern that would be expected from cleavage of a canonical two-site substrate (Figure 4B, Lane 4 and 4C Lane 1). We observed four fragments corresponding to ∼190 nt and ∼160 nt resulting from cleavage next to the EcoP1I recognition site, and ∼70 nt and ∼280 nt resulting from cleavage next to the EcoP15I site (Figure 4A and B Lane 4 and 4C Lane 1).

A similar experiment with the nuclease-dead EcoP15I^{D898A} resulted in only the ∼280 nt and ∼190 nt fragments (Figure 4B, lane 5). We interpreted the appearance of the faint ∼280 nt fragment to be the result of a nick of the bottom strand catalyzed by a 1D diffusing EcoP1I in cooperation with a site bound EcoP15I^{D898A}. The ∼190 nt fragment to be the result of a nick of the top strand catalyzed by site-bound EcoP1I in cooperation with a 1D diffusing EcoP15I^{D898A}, in *cis*. Replacement of nuclease-dead EcoP15I^{D898A} by Walker B mutant EcoP15I^{E227A} abolished nicking of the DNA (Figure 4B, lane 6). A similar experiment in which Walker mutants of EcoP1I were used in combination with active EcoP15I did not result either in nick or double-strand break (Figure 4C, lanes 2 and 3). These results indicated that ATP binding and hydrolysis by the stationary as well as the diffusing enzymes were required for cleavage of a two-site substrate.

DISCUSSION

The canonical substrates of EcoP1I and EcoP15I have at least two sites in inverted orientation. Although observation of ATP-dependent endonucleolytic cleavage of DNA substrates with single-site has previously been mentioned in a few reports, its mechanism, hitherto, remained unknown (12–18). This study demonstrates that single-site cleavage is solely a result of the interaction between two independent enzymes - a *cis*-acting enzyme which is bound to its recognition site in the DNA and a *trans*-acting enzyme either

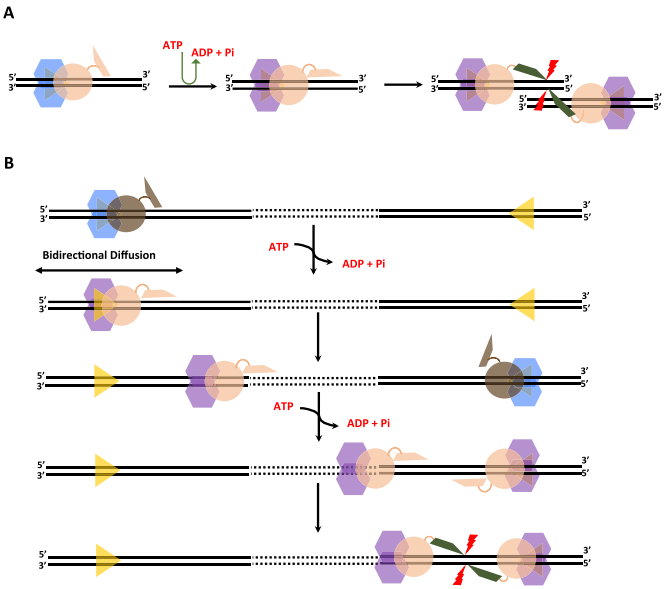

Figure 5. Model for DNA cleavage by Type III RM enzymes EcoP1I and EcoP15I. (**A**) A cartoon illustrating a model for single-site cleavage emphasizing that a target bound enzyme upon ATP hydrolysis changes to a nuclease activated state (represented as a change in color of the enzyme). However, nucleolytic activity manifests only when the enzyme encounters another activated nuclease (represented as change in color of the nuclease to green). (**B**) The model for two-site cleavage is similar to that of single-site cleavage except that the encounter between two activated nuclease is facilitated by 1D diffusion along the DNA and/or looping. For the sake of simplicity and clarity only 1D diffusion is shown.

bound to or activated by its substrate in ATP-dependent manner. The two cooperating enzymes in solution can come together via diffusion.

For cleavage to happen, the interaction between the two nucleases should occur within a time period shorter than the time required by the enzyme in *cis* to leave its recognition site. In case of EcoP15I, this time is measured to be about 6–

17 seconds (2). Furthermore, based on stopped-flow tryptophan fluorescence measurements, it has been proposed that the ATP-activated conformation of the enzyme retains for ∼25 seconds even after it has dissociated from a short linear DNA substrate (2). It is possible that the activated *trans*-acting enzyme dissociates from its specific DNA but can still cooperate and catalyze DNA cleavage for a short period. As mentioned earlier, our experiments do not address this possibility. However, it has been shown previously that supercoiled plasmids that lack free ends are substrates for single-site cleavage by EcoP15I (17). This suggests that the activated enzyme in *trans* does not have to dissociate from the DNA to catalyse the cleavage. This also rules out the potential requirement of the *trans* enzyme to thread on to the DNA, as supercoiled plasmids do not have free ends.

Single-site cleavage shares two striking similarities with two-site cleavage – 1) in both the modes, the nucleolytic cut maps to identical positions with respect to the recognition site (19,20); 2) the site-bound enzyme nicks the top strand and the cooperating enzyme nicks the bottom strand (this study for single-site cleavage and Janscak *et al.* (20) for two-site cleavage). Based on these similarities, we conclude that the configurations of two nucleases required to catalyze double-strand break are very similar in the two modes. The difference lies in how this configuration is achieved. In two-site cleavage, the nucleases converge by 1D diffusion and/or looping, while in single-site cleavage they come in proximity via 3D diffusion. Therefore, the two-site cleavage is a special form of single-site cleavage, where the motion of the enzyme is constrained to the length of the DNA. To achieve similar configuration in both the modes of cleavage, one would expect the two nucleases to be structurally flexible. This requirement is consistent with the reported crystal structure of EcoP15I in which the electron density for the C-terminal nuclease domain is poorly defined, possibly because of its mobility within the crystal lattice, preventing determination of the structure of the domain (27). A long linker domain connects the nuclease to the rest of the enzyme, which could contribute to the flexibility of the nuclease (27).

Previous studies have characterized in great detail the importance of ATP hydrolysis promoting 1D diffusion. It has been shown that about ten ATPs are hydrolyzed to initiate sliding of the enzyme along DNA (12). However, it remains, as yet, unaddressed if ATP hydrolysis is required only to initiate 1D diffusion, or if it also regulates the subsequent nucleolytic activity. In this study, we used Walker mutants to demonstrate that in both single-site and two-site cleavage ATP hydrolysis is required to activate the nucleases. Both the enzymes that cooperate to catalyze the cleavage have to be ATP hydrolysis-competent (Figure 4). Therefore, we conclude that ATP hydrolysis not only changes the conformational state of the enzyme to initiate sliding along DNA, but also activates the nuclease. This commonality in the two modes of cleavage is consistent with our above conclusion that the two have the same mechanism, and that they only differ in the manner in which the nucleases come together.

Using single-site heterologous cooperation assay, we also identified the requirement of a critical length of 17 bp DNA downstream of the recognition site, below which single-site cleavage ceases. To understand the significance of the critical length, we modeled the structure of EcoP15I bound to a DNA having 17 bp downstream of the recognition site using the available crystal structure. In its crystal structure EcoP15I is bound to a DNA that has 11 bp downstream of the recognition site and is not long enough to fully engage the ATPase domain. Increasing this length to 17 bp by structural modeling completely engaged the ATPase domain with the DNA. Hence, we conclude that for cleavage to fructify, the ATPase domain of the two cooperating enzymes have to be fully engaged. A fully engaged ATPase may either be required for ATP hydrolysis and/or to achieve the nuclease-activated state.

Although ATP hydrolysis is essential for nuclease activation, an activated nuclease bound to its recognition site on its own is not proficient for strand nicking. We came to this conclusion from the results of single-site cleavage carried out at low concentrations of the enzyme, which, despite satisfying all the prerequisites, showed that the enzyme bound to its recognition site did not nick the DNA in presence of ATP. Instead, the nick catalyzed only in presence of another hydrolysis competent enzyme (Figure 2C). Based on these results we conclude that strand nicking and the subsequent endonucleolytic cleavage requires interaction between an ATPase-activated nuclease bound to its recognition site with another activated nuclease. The same holds true in case of two-site cleavage (Figure 4).

In conclusion, the study reported here leads to a model (Figure 5) of DNA cleavage in which a recognition site bound Type III RM enzyme would undergo a conformational change induced by the hydrolysis of ATP making it both diffusion-competent and nucleolytically active. However, the activated nuclease can catalyze single-strand scission only in cooperation with another ATP-activated nuclease. We propose that this model is valid for both single-site and two-site cleavage, except that in single-site cleavage the cooperating nucleases come together by 3D diffusion, and in two-site cleavage they converge by 1D diffusion and/or looping (Figure 5). Our data suggests that single-site cleavage requires higher amount of enzyme than two-site cleavage. Due to decrease in dimension of the search space, convergence of enzymes by 1D diffusion and/or looping is expected to be more efficient than 3D diffusion due to higher local concentration of the enzymes at the site of action. A similar suggestion has been made by Butterer *et al.* (19). Hence, the role of single-site cleavage is likely to be less pronounced, and thus alleviating its toxic affects, in particular in a cell with newly replicated genomic DNA having unmodified daughter strand. However, under right enzyme, DNA and cofactor concentrations, the single-site cleavage could very well supplement two-site cleavage *in vitro* and perhaps *in vivo* (18). In particular, the study highlights that the products of cleavage by EcoP1I or EcoP15I can be substrates for and activators of single-site cleavage.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful to Profs. Desirazu N. Rao and Mark D. Szczelkun for their feedback on the work.

FUNDING

Science and Engineering Research Board, Department of Science and Technology (DST), Government of India [EMR/2016/000872 to K.S.]; I.A. thanks University Grants Commission, India, for a research fellowship; A.G. thanks DST India for an INSPIRE Scholarship. Funding for open access charge: Science and Engineering Research Board, Department of Science and Technology (DST), Government of India [EMR/2016/000872].
Conflict of interest statement. None declared.

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