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*Fok*I dimerization is required for DNA cleavage

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ABSTRACT ***Fok*I is a type IIs restriction endonuclease comprised of a DNA recognition domain and a catalytic domain. The structural similarity of the *Fok*I catalytic domain to the type II restriction endonuclease *Bam*HI monomer suggested that the *Fok*I catalytic domains may dimerize. In addition, the *Fok*I structure, presented in an accompanying paper in this issue of *Proceedings*, reveals a dimerization interface between catalytic domains. We provide evidence here that *Fok*I catalytic domain must dimerize for DNA cleavage to occur. First, we show that the rate of DNA cleavage catalyzed by various concentrations of *Fok*I are not directly proportional to the protein concentration, suggesting a cooperative effect for DNA cleavage. Second, we constructed a *Fok*I variant, *Fok*N13Y, which is unable to bind the *Fok*I recognition sequence but when mixed with wild-type *Fok*I increases the rate of DNA cleavage. Additionally, the *Fok*I catalytic domain that lacks the DNA binding domain was shown to increase the rate of wild-type *Fok*I cleavage of DNA. We also constructed an *Fok*I variant, *Fok*D483A, R487A, which should be defective for dimerization because the altered residues reside at the putative dimerization interface. Consistent with the *Fok*I dimerization model, the variant *Fok*D483A, R487A revealed greatly impaired DNA cleavage. Based on our work and previous reports, we discuss a pathway of DNA binding, dimerization, and cleavage by *Fok*I endonuclease.**

The type IIs restriction endonuclease *Fok*I, isolated from *Flavobacterium okeanokoites*, recognizes an asymmetric nucleotide sequence and cleaves both DNA strands outside of the recognition site: 5′-GGATG$(N)_{9/13}$ (1). The *fokIRM* genes have been cloned and sequenced (2, 3). The endonuclease consists of 587 aa with a molecular mass of 65.4 kDa (2, 4). *Fok*I has been shown to exist as a monomer in solution, based on gel filtration and sedimentation experiments (4). It has been concluded that, when bound to DNA and analyzed by gel-mobility shift experiments, only one monomer of *Fok*I was bound to its DNA recognition sequence (5). The gel-mobility shift experiments, where a 1:1 complex was observed (5), were performed with a *Fok*I precleaved DNA. Therefore this complex represents *Fok*I bound to the DNA product not to the DNA substrate.

Unlike *Fok*I, the typical type II restriction endonucleases, such as *Eco*RI or *Eco*RV, form a tight homodimer in solution and bind to DNA as a homodimer. Each monomeric subunit of homodimer contains one catalytic center. Mutational analysis by Waugh and Sauer (6) suggests that there is a single catalytic center per *Fok*I monomer. This led them to conclude that either *Fok*I must rearrange its catalytic center for sequential cleavage of each DNA strand or it must form a higher order complex to cleave both strands of DNA (6).

Based on proteolytic studies, it was shown that *Fok*I endonuclease contains two separate structural domains, one for DNA recognition and one for DNA cleavage (7). A purified 41-kDa N-terminal proteolytic fragment bound the recognition sequence specifically but did not cleave DNA, whereas the 25-kDa C-terminal proteolytic fragment in the presence of Mg^{2+} cleaved DNA nonspecifically (7). Mutational analysis further supported a modular structure of *Fok*I. A C-terminal deletion variant of *Fok*I showed the same DNA binding properties as the wild-type enzyme (8). On the other hand, a single amino acid substitution in the C-terminal half of the *Fok*I protein disabled endonuclease activity but did not affect the ability of the enzyme to bind specifically to DNA (6). Other studies have demonstrated that *Fok*I catalytic domain can be fused to DNA binding proteins to yield catalytically active chimeric proteins with novel recognition specificities (9–12).

Yonezawa and Sugiura (13), by using four different footprinting techniques, showed that *Fok*I interacts with the target recognition sequence from the major groove side of the DNA helix and that DNA protection at the site of cleavage in the absence of divalent metal ion is very weak. Waugh and Sauer (6) used DNase footprinting and methylation protection with cleavage-deficient variants of *Fok*I in the presence of divalent metal ion to show also a weak protection pattern around the site of cleavage (6).

The three dimensional structure of *Fok*I complexed to DNA confirmed that the protein is comprised of two functional domains, a N-terminal DNA recognition domain and a C-terminal catalytic domain (14). Together, the *Fok*I structure in the presence of DNA (14) and in the absence of DNA (15) provides a basis for the biochemical and mutational studies reported here. The structure in the presence of DNA showed a single *Fok*I molecule approaching DNA from the major groove side, with the recognition domain making all of the base-specific contacts at the recognition site. The catalytic domain of *Fok*I revealed a structure remarkably similar to a monomer of the type II restriction endonuclease *Bam*HI (14–16). Three catalytically important residues of *Fok*I (Asp-450, Asp-467, and Lys-469) superimpose with the catalytic residues Asp-94, Glu-111, and Glu-113 of *Bam*HI. Therefore, whether examined by mutational analysis (6) or by structural comparison (14), there appears to be only one catalytic site per *Fok*I molecule. This raises the question, "How does monomeric *Fok*I cleave both DNA strands by using a single catalytic center?" The *Fok*I structure showed that the catalytic domain is sequestered alongside of the recognition domain and does not contact DNA at the cleavage site, in accordance with the earlier footprinting studies (6, 13). From the structure, the *Fok*I catalytic domain can be relocated at the site of cleavage by a simple rotation around the linker segment so that the catalytic residues are positioned in the vicinity of the scissile phosphodiester bond located 13 nt away from the recognition site (14). However, for the same catalytic domain to cleave the other DNA strand, it would require the enzyme to adopt a second configuration, which is difficult to model without extensive refolding (14).

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Abbreviations: *Fok*N13Y, variant of *Fok*I endonuclease containing Asn13Tyr substitution; *Fok*CD, *Fok*I catalytic domain representing C-terminal 196 aa of the endonuclease; *Fok*CD/D450A, catalytically inactive catalytic domain containing Asp-450 → Ala substitution; *Fok*D483A, R487A, variant of *Fok*I endonuclease containing Asp-483 →Ala and Arg-487 → Ala substitutions.

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In this paper, we provide evidence that *Fok*I dimerization is required for DNA cleavage.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. *Escherichia coli* strain ER2566 [*fhuA2, lacZ::T7 gene1, Δ(mcrC-mrr) 114::IS10, R(mcr-73::miniTn10– TetS)2, endA1*] containing a chromosomal copy of the T7 RNA polymerase gene was constructed by E. Raleigh (New England Biolabs) and used for expression of *Fok*I variants. Plasmid pAFP1 containing the cloned *Fok*I endonuclease gene (2) and plasmid pAFD2 containing the *Fok*I methyltransferase gene (2) were constructed by W. E. Jack (New England Biolabs). The pAFP1 derivative coding for the catalytically inactive D450A variant of *Fok*I; the plasmid pSKFok1, a derivative of pBluescriptSK(−), containing a single *Fok*I site; and the plasmid pSKFok0, the derivative of pSKFok1 in which deletion of the 445-bp *Pvu*II fragment removed the single *Fok*I recognition sequence were constructed by C. Noren (New England Biolabs; unpublished work). pTYB4 and pTYB2 are products of New England Biolabs.

Subcloning of the *Fok*I Gene into the Impact One-Step Protein Purification System. The full length *Fok*I endonuclease gene and the truncated gene coding only for the catalytic domain of *Fok*I endonuclease were amplified by PCR by using plasmid pAFP1 as a template. The 5′ primer, 5′- AGCCCATGGTTTCTAAAATACGTACGTTCGGTTGGG-3′, for amplication of the full length *Fok*I endonuclease, was complementary to the *fokIR* gene except for silent mutations in the 6th and 7th codons to create a *Bsi*WI site. The primer, 5′-GTGCATATGCAACTAGTCAAAAGTGAACTGG-3′, for amplification of the catalytic domain of *Fok*I was flanked by the *Nde*I site. The rest of the primer sequence matched the *fokIR* gene sequence starting from the 384th codon. The 3′ reverse primer, 5′-CATGGCGCCAAAGTTTATCTCGCCGTTATTAA-3′, is complementary to the end of *fokIR* gene and is flanked by the *Ehe*I site. The resulting PCR products were agarose-gel purified and cleaved with restriction endonucleases: *Nco*I and *Ehe*I in the case of the full length *Fok*I gene; or *Nde*I and *Ehe*I in the case of the gene coding for the *Fok*I catalytic domain. The gel-purified restriction fragment containing the full length *fokIR* was ligated into the *Nco*I and *Sma*I digested vector pTYB4. This created the construct pTYB4/FokR (Fig. 1) where the C terminus of the *fokIR* gene was fused directly to the intein of the Impact Purification System (17, 18). The gel-purified restriction fragment containing the *fokIR* catalytic domain coding sequence was ligated into the *Nde*I and *Sma*I digested vector pTYB2. The resulting construct was designated pTYB2/FokCD. No sequence alterations other than desired mutations were detected by DNA sequence analysis.

Construction of Catalytically Inactive *Fok*I Variants. The 1.13-kb *Cla*I–*Msc*I fragment of pTYB4/FokR was replaced by the same fragment from the pAFP1/D450A construct (see above) to create the plasmid derivative pTYB4/FokDa450A coding for a catalytically inactive *Fok*I variant. Similarly, the 0.5-kb *Spe*I–*Sap*I fragment of pTYB2/FokCD was replaced by the *Spe*I–*Sap*I fragment from pAFP1/D450A to create pTYB2/FokCD/D450A.

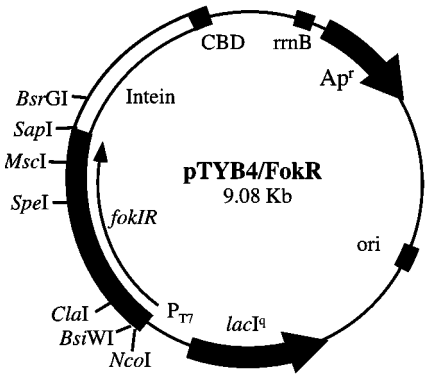

FIG. 1. Plasmid map of the pTYB4/FokR. The *fokIR* gene is placed downstream of the T7 promoter, and *fokIR* is in-frame with the 5′ end of the intein coding sequence. CBD, chitin binding domain.

Construction of a Binding-Deficient *Fok*I Variant. To create a binding-deficient *Fok*I variant, Asn13 was replaced by a bulky amino acid, tyrosine. The following mutagenic primers were used to amplify the 0.29-kb *Bsi*WI–*Cla*I fragment of the *Fok*I gene: 5′-ATACGTACGTTCGGTTGGGTTCAATATCCAGGTAATTTGAG-3′ (introduces N13Y) and 5′-GCCCAACGCAAAAAACCGTCAGATGA-3′.

The PCR product was cleaved with *Bsi*WI and *Cla*I restriction endonucleases, and the gel-purified restriction fragment was ligated into the *Bsi*WI and *Cla*I digested pTYB4/FokR vector. This created a new derivative, pTYB4/FokN13Y, coding for the *Fok*I variant with altered base-specific contacts. The sequence of the replaced DNA fragment was verified by DNA sequence analysis.

Construction of Dimerization-Deficient *Fok*I Variant. The *Fok*I gene segment flanked by *Msc*I and *Bsr*GI restriction sites was amplified by PCR by using pTYB4/FokR as a template and a mutagenic primer that introduced substitutions D483A and R487A, 5′-CAATTGGCCAAGCAGCTGAAATGCAAGCATATGTCGAAGAAAATCAAACACG-3′. The second primer matched the DNA sequence downstream the *fokIR* gene and was flanked by *Bsr*GI restriction site 5′- ACGCTGTACATAGTTTCTCTTCC-3′. The *Msc*I–*Bsr*GI fragment of pTYB4/FokR was replaced with *Msc*I and *Bsr*GI digested mutagenic PCR product. This created a new derivative, pTYB4/Fok483A,487A, which coded for a dimerization-deficient *Fok*I variant.

Target Protein Expression and Purification. Wild-type *Fok*I restriction endonuclease was purified to homogeneity as described earlier (19). The concentration of wild-type *Fok*I protein was determined spectrophotometrically at 280 nm by using the extinction coefficient of 72,520 M^{-1} cm^{-1} (20).

The One-Step Protein Purification System (Impact) (17) was used to facilitate purification of *Fok*N13Y, *Fok*CD, *Fok*CD/D450A, and *Fok*D483A, R487A variants. This technique is based on the self-catalyzed cleavage of the intein that can be controlled *in vitro* by DTT (18). The chitin binding domain is fused genetically to the C terminus of the intein and serves as an affinity tag to bind *Fok*I–intein fusion onto a chitin column. The *Fok*I is released from the fusion after the induction of the intein cleavage reaction by adding 50 mM DTT, while intein–chitin binding domain fusion remains bound to the column (17).

E. coli ER2566 cells containing mutant pTYB-Fok constructs were grown at 37°C in Luria–Bertani medium supplemented with 0.1 mg/ml ampicillin. Expression of the target protein was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 0.1 mM at mid-log phase of growth (Klett = 90). After induction, the cultures were incubated overnight at 15°C. The cells were harvested, resuspended in column buffer (20 mM potassium phosphate, pH 7.4/0.1 mM EDTA/0.5 M NaCl) and disrupted by sonication. The crude extract containing *Fok*I–intein fusion protein was loaded onto a chitin column (New England Biolabs) and washed with two column volumes, and the column was flushed quickly with 2 column volumes of 20 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, and 50 mM NaCl containing 50 mM DTT. The column was incubated at 4°C overnight to induce self-cleavage of the protein fusion (17). Next, the target protein was eluted from the column with column buffer and dialyzed to remove excess DTT. The purified proteins were concentrated by dialysis against 20 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, and 50% glycerol and stored at −20°C. *Fok*I variants purified via the Impact System have two additional glycine residues at their carboxyl termini.

DNA Cleavage Assays. The cleavage activity of mutant *Fok*I variants was assayed by incubating various amounts of protein at 37°C in either 30 or 50 μl of standard *Fok*I reaction buffer (20 mM

Tris-acetate, pH 7.9/10 mM magnesium acetate/50 mM potassium acetate/1 mM DTT/100 μg/ml BSA) containing 1 μg of λ or plasmid pSKFok1 DNA. The reaction was terminated by addition of 10 μl of stop solution (60 mM EDTA, pH 8.0/50% glycerol/1% SDS/0.02% bromphenol blue), and reaction products were analyzed by electrophoresis in 1% agarose gels.

For quantitative *Fok*I activity assays, plasmid pSKFok1 was first linearized with the restriction endonuclease *Ahd*I to make a linear substrate with the *Fok*I recognition site positioned in the center of the DNA molecule. DNA then was purified by phenol-chloroform extraction followed by isopropanol/ethanol precipitation. DNA concentration was determined spectrophotometrically at 260 nm. *Fok*I was added to either 70 or 200 μl of reaction buffer (see above) containing 6.3 nM substrate DNA. Samples were incubated at 37°C, 10-μl aliquots were removed at various time intervals, and the reaction products were analyzed by agarose gel electrophoresis. The ethidium bromide-stained gels were digitized with IS-500 Digital Imaging System (Alpha Innotech, San Leandro, CA). The intensity of the product band was quantitated by using the NIH IMAGE program v. 1.61.

RESULTS

Rate of Cleavage vs. Enzyme Concentration. The rate of DNA cleavage catalyzed by various *Fok*I concentrations was determined to establish the relationship between the initial velocity of the reaction and the enzyme concentration. Linearized plasmid pSK*Fok*1 DNA containing a single *Fok*I recognition site positioned in the center of the DNA molecule was used as a substrate. Using such a DNA substrate simplified the evaluation of the concentration of reaction products that, because of their same size, run on the agarose gel as a single band.

A limiting amount of *Fok*I restriction endonuclease (from 0.1 to 1.0 nM final concentration) was added last to the reaction mixture, which was preheated at 37°C. The progress of each reaction was monitored by withdrawing 10-μl samples at timed intervals. Typical results of such cleavage assays, performed with 0.2 and 0.6 nM *Fok*I, are shown in Fig. 2. The amount of cleaved DNA was expressed in arbitrary units after the quantitative evaluation of the intensity of the product band in an ethidium bromide-stained agarose gel. The plots showing the progress of the DNA product formation with time at different *Fok*I concentrations are presented in Fig. 3. These plots were used to calculate the initial velocity ($v_o = d[P]/dt$) of each reaction catalyzed by a given *Fok*I concentration. When measured values of v_o were plotted against the corresponding values of [*Fok*I] a nonlinear plot was obtained (Fig. 3, *Inset*). This indicated that, at low *Fok*I concentrations, the initial velocity of the reaction is not directly proportional to the enzyme concentration, suggesting that the *Fok*I-catalyzed reaction is higher than first order with respect to

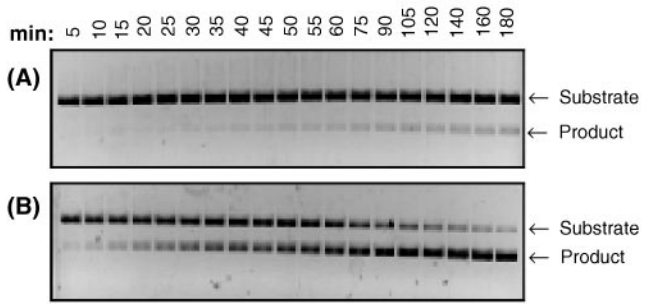

FIG. 2. Time course of the *Fok*I-catalyzed DNA cleavage reaction. Linearized pSKFok1 DNA (6.3 nM) was incubated at 37°C with 0.2 (*A*) or 0.6 nM (*B*) *Fok*I restriction endonuclease in 200 μl of reaction buffer (20 mM Tris-acetate, pH 7.9/10 mM magnesium acetate/50 mM potassium acetate/1 mM DTT/100 μg/ml BSA). At the time points indicated, 10-μl samples were withdrawn, immediately quenched by adding 5 μl of stop solution, and subjected to electrophoresis on 1% agarose-gel.

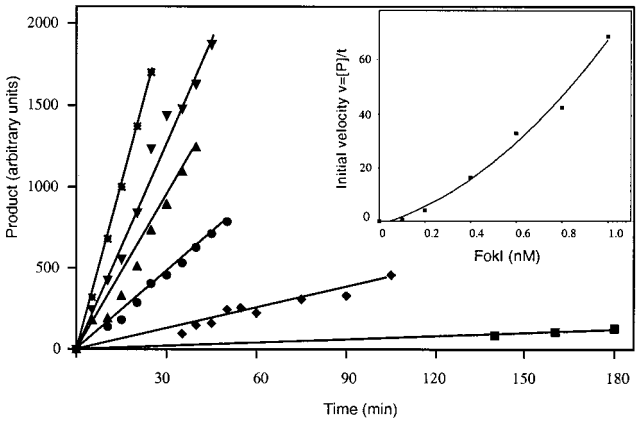

FIG. 3. Rate of DNA cleavage with various *Fok*I concentrations. The conditions of DNA cleavage reaction are described in Fig. 2. Samples were withdrawn at the indicated times from the reactions that contained 0.1 nM *Fok*I (■), 0.2 nM *Fok*I (♦), 0.4 nM *Fok*I (●), 0.6 nM *Fok*I (▲), 0.8 nM *Fok*I (▼), and 1.0 nM *Fok*I (∗). The amount of cleaved DNA was determined as in *Experimental Procedures*. (*Inset*) Initial velocity vs. *Fok*I concentration.

[*Fok*I]. Among the possible interpretations, the nonlinear relationship of v_o vs. [*Fok*I] can be explained by a cooperative binding of two *Fok*I molecules to cleave both strands of DNA.

Characterization of *Fok*N13Y Variant. If the catalytically active *Fok*I complex requires two *Fok*I molecules, i.e., two catalytic domains, for double-stranded DNA cleavage, then the reaction at low concentrations of *Fok*I protein may be stimulated by a binding-deficient variant of *Fok*I that contains an active catalytic domain. The three-dimensional structure of the *Fok*I endonuclease bound to the DNA substrate revealed that Asn13 makes bidentate hydrogen bonds with the central adenine base of the recognition sequence GGATG (14). Substitution of this residue with the bulky amino acid tyrosine should destroy base-specific contacts at the recognition interface. Such a mutant protein would be incapable of binding to the target DNA and therefore would be impaired for DNA cleavage. To test this, the pTYB4/FokN13Y derivative was constructed as described in *Experimental Procedures*. The mutant *Fok*N13Y protein then was expressed and purified by a single chromatographic step from a chitin column. The purified protein was >95% pure as determined by SDS/PAGE. *Fok*N13Y (200 nM) was incubated with supercoiled and linearized pSKFok1 DNA for 1 h at 37°C. At these conditions, no detectable double-strand or single-strand cleavage of DNA substrate was observed.

Stimulation of *Fok*I Activity with *Fok*N13Y. To test the *Fok*N13Y variant for its ability to stimulate *Fok*I restriction endonuclease, the substrate DNA was incubated with either 1 nM *Fok*I alone, 500 nM *Fok*N13Y protein alone, or the premixed *Fok*I (1 nM) and *Fok*N13Y (500 nM) protein combination. Fig. 4*A* reveals that the DNA cleavage reaction catalyzed by 1 nM *Fok*I alone is slow. After 12 min only a small portion (≈5%) of the substrate DNA is cleaved. Under the same reaction conditions, 500 nM *Fok*N13Y did not show any detectable DNA cleavage activity (Fig. 4*A*). However, when *Fok*I endonuclease was supplemented with 500 nM *Fok*N13Y protein, a 10–20-fold increase in the rate of DNA cleavage was observed relative to *Fok*I alone (Fig. 4*A*). This suggests that wild-type *Fok*I protein interacts with the *Fok*N13Y molecule to facilitate site-specific cleavage.

Characterization of *Fok*CD and *Fok*CD/D450A Variants. The pTYB2/FokCD and pTYB2/FokCD/D450A derivatives code for the C-terminal 196 aa of *Fok*I restriction endonuclease, constituting the 25-kDa catalytic domain. In addition, the derivative pTYB2/FokCD/D450A codes for a catalytically defective *Fok*CD protein, *Fok*CD/D450A. The purified *Fok*CD and *Fok*CD/D450A proteins were tested for the ability to cleave λ

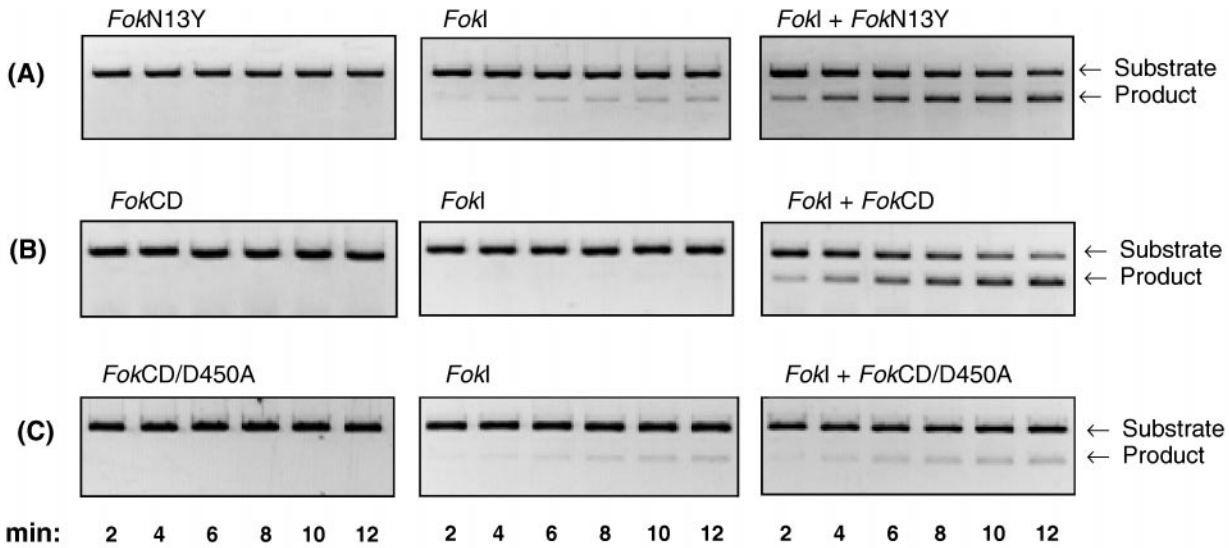

FIG. 4. Stimulation of *Fok*I endonuclease by (*A*) *Fok*N13Y, (*B*) *Fok*CD, or (*C*) *Fok*CD/D450A. Reactions were performed at 37°C in 70 μl of 20 mM Tris-acetate buffer (pH 7.9), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, 100 μg/ml BSA, and 6.3 nM linearized pSKFok1 DNA; 10-μl samples were withdrawn at the indicated times from the reactions that contained: (*A*) either 500 nM *Fok*N13Y, 1.0 nM *Fok*I, or 1.0 nM *Fok*I + 500 nM *Fok*N13Y; (*B*) either 500 nM *Fok*CD, 0.5 nM *Fok*I, or 0.5 nM *Fok*I + 500 nM *Fok*CD; and (*C*) either 500 nM *Fok*CD/D450A, 1.0 nM *Fok*I, or 1.0 nM *Fok*I + 500 nM *Fok*CD/D450A. Samples were quenched by adding 5 μl of stop solution and were analyzed by electrophoresis on 1% agarose gel.

DNA. When <1 μM *Fok*CD protein was incubated with 1 nM λ DNA for 1 h at 37°C, there was no detectable DNA cleavage. At >1 μM *Fok*CD concentrations, nonspecific DNA cleavage was observed. In contrast, incubating 2.5 μM *Fok*CD/D450A as long as 20 h with 1 nM λ DNA at 37°C did not show any detectable DNA cleavage.

Stimulation of *Fok*I Activity with the Catalytic Domain. Stimulation of *Fok*I with the *Fok*CD protein was performed as described for the *Fok*N13Y variant. After 12 min, <1% of DNA substrate was cleaved by 0.5 nM *Fok*I alone (Fig. 4*B*). No detectable DNA cleavage was observed with 500 nM *Fok*CD alone (Fig. 4*B*). When 0.5 nM *Fok*I was supplemented with 20 nM *Fok*CD, no increase in the rate of DNA cleavage was observed (data not shown). However, with 0.5 nM *Fok*I plus 500 nM *Fok*CD, ≈50% of substrate DNA was cleaved after 8 min (Fig. 4*B*). This rate is ≈100-fold higher than with 0.5 nM *Fok*I alone. In contrast, the catalytically inactive *Fok*CD variant *Fok*CD/D450A failed to activate *Fok*I (Fig. 4*C*). These results indicate that two separate catalytic domains are necessary for site-specific, double-strand DNA cleavage by *Fok*I.

Dimerization-Defective *Fok*I Variant Displays Diminished Cleavage of Both DNA Strands. In an accompanying paper in this issue of *Proceedings* (15), we report the structure of *Fok*I in the absence of DNA. The structure reveals protein–protein interactions between two catalytic domains that mimic a *Bam*HI homodimer. Close inspection of this interface shows that amino acids Asp-483 and Arg-487 are located within hydrogen bond distance with the corresponding residues in the other catalytic domain subunit. These residues were considered to be good candidates for disrupting the dimer interface while not affecting folding of the protein for the following reasons: (*i*) the residues are located on the surface of the domain; and (*ii*) these residues do not contact neighboring amino acid residues within the same subunit.

The pTYB4/FokD483A, R487A construct was made and the protein was expressed and purified as described in *Experimental Procedures*. Cleavage of supercoiled pSKFok1 plasmid by *Fok*I endonuclease and *Fok*D483A, R487A protein was compared. As shown in Fig. 5*A*, after 1 h at 37°C, 6 nM *Fok*I completely converted 20 nM supercoiled pSKFok1 plasmid DNA to a full length linear product. Incubation of *Fok*I with a plasmid DNA that does not contain a *Fok*I recognition sequence, pSKFok0, showed that *Fok*I cleavage reaction is sequence-specific (Fig. 5*B*). In contrast to the wild-type endonuclease, the *Fok*D483A, R487A variant showed no detectable DNA cleavage at 9 nM (Fig. 5*C*). At a 10- to 80-fold higher concentration of *Fok*D483A, R487A variant, the major product observed was the open circular form of plasmid DNA. The nicking activity was site-specific because the *Fok*D483A, R487A variant did not convert plasmid pSKFok0 DNA to the open circular form (Fig. 5*D*). The results indicate that double-strand cleavage activity of the *Fok*D483A, R487A variant is reduced three orders of magnitude as compared with the double-strand cleavage activity of wild-type enzyme (Fig. 5*C*).

DISCUSSION

Typical type II restriction endonucleases exist in solution as homodimers and are known to bind to and cleave DNA as homodimers. The molecular mass of the monomers are generally in the 20- to 40-kDa range. Each monomeric subunit is responsible for half of the DNA base-specific contacts, and each subunit contains one catalytic center that cleaves one strand of the duplex. The sites of cleavage are always found symmetrically positioned within the recognition sequence. The three-dimensional structures of the type II endonucleases show that the recognition and catalytic functions are well integrated into a single protein domain (21). On the other hand, the type IIs restriction endonuclease *Fok*I is a larger, 65-kDa protein. It is monomeric in solution (4). The asymmetric recognition sequence and sites of cleavage are separated by one turn of the DNA helix. The DNA recognition function and single catalytic center of *Fok*I reside on two separate domains of the protein. How does *Fok*I cleave both strands of the DNA?

Unlike the type II endonucleases, the specific recognition of DNA duplex by *Fok*I is mediated clearly by the single *Fok*I recognition domain (14). However, the nature of the termini of the *Fok*I-cleaved DNA is comparable to the termini of *Bam*HI or *Eco*RI cleaved DNA because all three endonucleases cleave double-stranded DNA to leave a 4-base, 5′-extension. The three-dimensional structures of *Bam*HI and *Eco*RI show a similar common core and positioning of the catalytic residues relative to the targeted scissile phosphate groups (22). The structural organization of the *Fok*I catalytic domain displays a striking similarity to the monomer of *Bam*HI (14). The two structures share a similar β-sheet core surrounded by α-helices. The active site

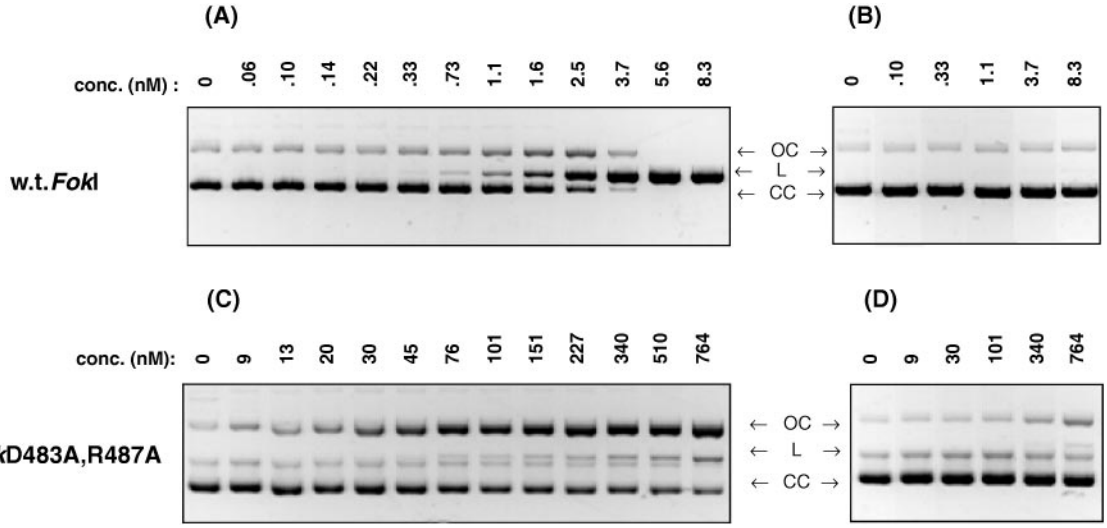

FIG. 5. Comparison of DNA cleavage by *Fok*I and *Fok*D483A, R487A. The cleavage reactions were performed for 1 h at 37°C in 30 μl of 20 mM Tris-acetate buffer (pH 7.9), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT, and 100 μg/ml BSA, containing 20 nM of (*A* and *C*) supercoiled plasmid pSKFok1 or (*B* and *D*) supercoiled plasmid pSKFok0 and either (*A* and *B*) *Fok*I or (*C* and *D*) *Fok*D483A, R487A. The concentrations of *Fok*I and *Fok*D483A, R487A are indicated. (CC), covalently closed form of plasmid DNA; (OC), open circle form; (L), linear form.

residues of both endonucleases occur at one end of the β-sheet and are superimposed easily on each other (14). This data, together with the structural organization of *Fok*I in the absence of DNA (15), led us to propose that *Fok*I endonuclease may use a strategy similar to *Eco*RI and *Bam*HI for DNA cleavage. From this perspective, we have postulated that (*i*) two *Fok*I molecules are involved in double-stranded DNA cleavage; (*ii*) DNA cleavage reaction by *Fok*I proceeds via an enzyme dimerization step that takes place in the presence of both the specific DNA and divalent metal ion; and (*iii*) dimerization occurs at the interface of two catalytic domains of *Fok*I.

Measurement of the rate of DNA cleavage at different enzyme concentrations suggests that the *Fok*I catalyzed reaction is higher than first order with respect to *Fok*I. This is consistent with the cooperative binding of two *Fok*I molecules. On the other hand, the nonlinear relationship between the initial velocity of the reaction and *Fok*I at low enzyme concentrations also could be explained by other factors; for example, at low protein concentration, the enzyme may first be adsorbed to the side of the reaction vessel. Although the reaction buffer used in the DNA cleavage experiments contained 100 μg/ml BSA, which should alleviate such undesirable nonspecific binding of *Fok*I, we cannot rule out that some analogous interaction did not take place.

To obtain additional evidence for *Fok*I cooperativity, *Fok*I variants with mutated (*Fok*N13Y) or deleted (*Fok*CD) recognition domains were constructed and were shown to stimulate *Fok*I activity. When incubated alone with DNA, these variants were shown to be incapable of cleaving DNA. However, when *Fok*N13Y was incubated together with a limiting amount of *Fok*I, the rate of DNA cleavage was increased 10-fold. Likewise, in the presence of *Fok*CD, *Fok*I activity increased 100-fold. The inactive *Fok*I catalytic domain *Fok*CD/D450A failed to stimulate *Fok*I endonuclease. Furthermore, the inactive *Fok*I catalytic domain did not appear to inhibit the low level of *Fok*I activity, indicating that the inactive catalytic domain is incapable of forming a complex with *Fok*I. A precleavage complex probably relies on the binding energy derived from contributions of the appropriate ionic interactions between the magnesium coordinated with residues D450, D467, and the scissile phosphate. In the catalytically defective domain, D450 is replaced by alanine. It is expected that this will upset the coordination of the magnesium, hence the network of interactions that afford the transient binding energy to form a pre-cleavage complex. The stimulation of *Fok*I by *Fok*CD suggests that a *Fok*I monomer must interact with a second catalytic center for double-stranded DNA cleavage to occur. Thus, during cleavage by wild-type *Fok*I endonuclease, two monomeric *Fok*I molecules interact with each other to align two catalytic centers for the cleavage of both DNA strands.

The cooperativity that *Fok*I displays for DNA cleavage is apparent at a concentration range below 1 nM. Above 1 nM, the rate of DNA cleavage increases proportionately to the amount of *Fok*I added to the reaction. This suggests that the equilibrium binding constant of *Fok*I for a *Fok*I/DNA complex is <1 nM. In the stimulation experiments, a >100 nM concentration of mutant *Fok*I variant is required to observe the same rate of DNA cleavage as 3 nM *Fok*I alone. These results indicate that the full length, wild-type *Fok*I molecule is two orders of magnitude more efficient at facilitating DNA cleavage than the mutant proteins that had their DNA recognition domain mutated (*Fok*N13Y) or deleted (*Fok*CD). This suggests that the second recognition domain also plays a role in the formation and/or stability of the catalytically active *Fok*I dimer/DNA complex.

The phenomenon of "sequestration" of the *Fok*I catalytic domain by the recognition domain that was observed in the three-dimensional structure (14) may exist to prevent the catalytic domain from interacting with the DNA while the *Fok*I molecule scans the DNA in search of a new recognition site or searches for another monomer that already has bound to a *Fok*I recognition sequence. The sequestering of the catalytic domain may explain how *Fok*I manages to regulate its cleavage activity. The catalytic domain remains sequestered during binding to its DNA recognition sequence and is triggered for release by dimerization. When wild-type *Fok*I is incubated with supercoiled DNA at low enzyme concentration and there is sufficient enzyme to bind only as monomer, it may be expected to only nick supercoiled DNA. This would result in the accumulation of the open circular form of DNA. However, the experimental results do not support such a model but rather support the view that the catalytic domain remains inactive. At a molar ratio of 1:20 of *Fok*I:DNA, a slight excess of the nicked intermediate over the linear DNA was observed. At a 1:8 ratio of *Fok*I:DNA, the linear product was predominant in the reaction (Fig. 5*A*). The behavior of the double mutant *Fok*D483A, R487A also supports the view that the catalytic domain is inactive until dimerization occurs. The D483A+R487A mutations targeted the catalytic domain dimerization interface while leaving the recognition domain and catalytic center of the enzyme unchanged. However, by altering the catalytic domain dimerization interface, the *Fok*I cleavage of

either strand of the DNA duplex was impaired. Thus, neither the results for *Fok*I or the *Fok*D483A, R487A variant indicate that the *Fok*I monomer can cleave one DNA strand. More likely, the *Fok*I monomer complexed to the specific DNA is inactive because its catalytic domain is sequestered. Alternatively, the *Fok*I catalytic domain may be able to dissociate from the recognition domain after the *Fok*I makes specific contacts with its DNA substrate but may not be able to properly align its catalytic center for the phosphodiester bond cleavage without dimer formation.

Based on this work and previous reports, the following view of *Fok*I endonuclease emerges:

*Fok*I exists in solution as a monomer, and first binds to DNA as a monomer. The complex is catalytically inactive.

A second *Fok*I monomer arrives either when: (*i*) DNA is scanned until the monomer collides with the first *Fok*I molecule. Presumably, only monomers arriving in one of two orientations at the 3′ end of the recognition sequence can dimerize successfully; (*ii*) the monomeric *Fok*I/DNA complex interacts with another monomeric *Fok*I/DNA complex; or (*iii*) *Fok*I monomer from solution collides with a *Fok*I/DNA complex. This alternative seems the least likely because the binding-deficient *Fok*I mutant proteins are relatively poor stimulators on a molar basis.

The catalytic domain of each *Fok*I molecule swings away from the recognition domain to position its catalytic sites opposite the targeted phosphodiester bond. The residues at the catalytic domain dimerization interface interact with each other, forming a dimer that resembles a structure not unlike a prototypical type II restriction endonuclease bound to DNA. The formation of the dimer may require magnesium ion(s).

Cleavage of both DNA strands occurs after *Fok*I dimerization in the presence of magnesium. The dimerization model offers two levels of control to prevent *Fok*I from cleaving DNA nonspecifically. First, the release of the catalytic domain from its sequestered position may depend on sequence-specific DNA binding. Second, the dimerization of the catalytic domain may be required to achieve proper alignment of the two catalytic domains for phosphodiester bond cleavage.

Dimerization in the presence of specific DNA may be a strategy other type IIs restriction endonucleases use. To date, all type IIs endonucleases studied (*Mbo*II, *Mme*I, *Tth*111II, *Eco*57I) are monomers in solution (23–26). *Mbo*II binds to its recognition sequence as a monomer (27).

Several chimeric enzymes have been created by fusing the *Fok*I catalytic domain with site-specific DNA binding domains. *Fok*I catalytic domain was fused genetically to *Drosophila Ubx* homeodomain (9) and with Sp1-QNR and CP-QDR zinc finger proteins (10, 11). Recently, a Z-DNA-specific nuclease was constructed following the same strategy (12). However, the rate and efficiency of DNA cleavage by the hybrid endonucleases are much lower compared with the wild-type *Fok*I (10). It is difficult to achieve complete site-specific cleavage with hybrid nucleases because high protein concentration and/or long incubation times usually lead to a nonspecific DNA cleavage. The dimerization of *Fok*I suggests that cleavage rates and the specificity of chimeric endonucleases may be improved by adding excess exogenous *Fok*I catalytic domain or by constructing a triple fusion where the heterologous DNA binding motif is linked to the covalently fused *Fok*I catalytic domain dimer.

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