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Human Replication Factor C Stimulates Flap Endonuclease 1*

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Il-taeg Cho‡, Do-hyung Kim‡, Young-Hoon Kang‡, Chul-Hwan Lee‡, Tamir Amangyelid‡, Tuan Anh Nguyen‡, Jerard Hurwitz§, and Yeon-Soo Seo‡1

From the ‡Center for DNA Replication and Genome Instability, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea and the §Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Flap endonuclease 1 (FEN1) is the enzyme responsible for specifically removing the flap structure produced during DNA replication, repair, and recombination. Here we report that the human replication factor C (RFC) complex stimulates the nuclease activity of human FEN1 in an ATP-independent manner. Although proliferating cell nuclear antigen is also known to stimulate FEN1, less RFC was required for comparable FEN1 stimulation. Kinetic analyses indicate that the mechanism by which RFC stimulates FEN1 is distinct from that by proliferating cell nuclear antigen. Heat-denatured RFC or its subunit retained, fully or partially, the ability to stimulate FEN1. Via systematic deletion analyses, we have defined three specific regions of RFC4 capable of stimulating FEN1. The region of RFC4 with the highest activity spans amino acids 170–194 and contains RFC box VII. Four amino acid residues (*i.e.* Tyr-182, Glu-188, Pro-189, and Ser-192) are especially important for FEN1 stimulatory activity. Thus, RFC, via several stimulatory motifs per molecule, potently activates FEN1. This function makes RFC a critical partner with FEN1 for the processing of eukaryotic Okazaki fragments.

Faithful genome maintenance is fundamental to the preservation of life. DNA replication, repair, and recombination function together to maintain genome integrity; they are interdependent and share many proteins that interplay in a well orchestrated manner (1–3).

Flap endonuclease 1 (FEN1)[2] is an enzyme that functions in all three processes in eukaryotes and contains structure-specific endonuclease activity that processes flap-structured DNA (4, 5). Inactivation of the *RAD27* gene, which encodes Rad27 (yeast FEN1), led to many anomalies in DNA metabolism. For example, deletion of *RAD27* in *Saccharomyces cerevisiae* produces a temperature-sensitive growth phenotype with a variety of chromosomal instabilities (6–12). Although homozygous FEN1 knock-out is embryonic lethal (13), heterozygotes are prone to develop tumors because of chromosomal instability caused by haplo-insufficiency of FEN1 (14). Genetic and biochemical studies from yeasts and mammals together confirmed that FEN1 has roles in virtually every aspect of major DNA transactions.

For example, FEN1 participates in the maturation of Okazaki fragments by cleaving the flap structure generated by polymerase (pol) δ-catalyzed displacement of DNA synthesis (15, 16). FEN1 is also required for long-patch base excision and nucleotide excision repair because transient short flaps are generated during gap filling (17–19). In nonhomologous end-joining, two single strand DNA ends from resected double strand breaks, upon annealing to each other using micro-homology, can generate 5′ flaps that can be removed by FEN1 (20). The preferred substrate for FEN1 is a double-flap structure containing a 1-nucleotide (nt) 3′ flap in addition to the 5′ flap that is cleaved (21). This double-flap substrate is believed to be a physiological substrate (22). Based upon its enzymatic properties *in vitro*, the primary role of FEN1 *in vivo* is likely the creation of ligatable nicks by removing flap structures generated *in vivo*. The failure to do so is likely to lead to chromosomal instability. Recently, it was reported that FEN1 had gap endonuclease (GEN) activity, which is implicated in restarting stalled replication forks and in apoptotic DNA fragmentation (11, 23).

FEN1 interacts with other proteins to achieve its function more effectively (24, 25). For example, FEN1 is stimulated by proliferating cell nuclear antigen (PCNA) through protein-protein interaction (26–30). A mutation in the PCNA interaction domain of Rad27 decreases stimulation by PCNA, resulting in replication and repair defects *in vivo* (28). There are two Rad27-binding sites in PCNA that are differentially accessible to Rad27 for stimulation, depending upon the binding status of PCNA to substrate DNA. Both Werner syndrome protein (WRN) and Bloom syndrome protein (BLM), members of RecQ helicase family, stimulate FEN1 through physical interaction. The stimulation was independent of ATPase/helicase activity (12, 31–36). A fragment WRN-(949–1092) (144 amino acids) was necessary and sufficient to interact and stimulate human FEN1 (31). Thus, WRN and BLM are likely to prevent genome instability by collaborating with FEN1. Recently, it was reported that the human Rad9-Rad1-Hus1 (9-1-1) checkpoint complex, which resembles PCNA in structure, interacted and stimulated FEN1 (37). They proposed that the 9-1-1 complex serves as a binding platform for FEN1 in DNA repair.

Our findings and the findings of others that several genetic suppressors, which rescued defects of *dna2*, stimulated FEN1 (34, 38, 39) prompted us to examine enzymes or proteins involved in lagging strand synthesis for their ability to stimulate

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[1] To whom correspondence and requests for materials should be addressed. Tel.: 82-42-350-2637; Fax: 82-42-350-2610; E-mail: yeonsooseo@kaist.ac.kr.

[2] The abbreviations used are: FEN1, Flap endonuclease 1; PCNA, proliferating cell nuclear antigen; RFC, replication-factor C; WRN, Werner syndrome protein; BLM, Bloom syndrome protein; nt, nucleotide; BSA, bovine serum albumin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ssc, single-stranded circular; ATPγS, adenosine 5′-(γ-thiotriphosphate).

TABLE 1
Oligonucleotides used to construct DNA substrates in this study

No.	Nucleotide sequences (length in nucleotides)
1.	GAAAACATTATTAATGGCGTCGAGCTAGGCACAAGGCGAACTGCTAACGG (50)
2.	CGAACAATTCAGCGGCTTTAACCGGACGCTCGACGCCATTAATAATGTTTTC (52)
3.	TGCTCGACGCCATTAATAATGTTTTC (26)
4.	TTTTTTTTTTTTTGCTCGACGCCATTAATAATGTTTTC (38)
5.	TTTTTTTTTTTTTTTTTTTTTTTTTTTGCTCGACGCCATTAATAATGTTTTC (52)
6.	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCTCGACGCCATTAATAATGTTTTC (79)
7.	CCGTTAGCAGTTCGCCTTGTGCCTAG (26)
8.	CCGTTAGCAGTTCGCCTTGTGCCTA (25)
9.	GCTCGACGCCATTAATAATGTTTTC (25)
10.	GACGTGCCCATGCGTCTGCGGGCAAGAGAACGCCTTTCGTACGGATCGTTAGTAGACCACTAGTGGAGGTCGCAGCTGGTGCACTCGGGT (90)
11.	CTGCACGGGTACGCAGACGCCCGTTCTCTTGCGGAAAGCATGCCTAG (47)
12.	TCTCTTGCGGAAAGCATGCCTAGCAATCATCTGGTGATCACCTCCAGCGTCGACCACGTGAGCCCA (60)

FEN1 activity. For this purpose, we tested the influence of the human RFC complex on catalytic activity of human FEN1, and we found that it stimulated markedly FEN1 activity via multiple stimulatory motifs per molecule. RFC acts as a loading factor of PCNA and consists of five subunits as follows: RFC1 (140 kDa), RFC2 (40 kDa), RFC3 (38 kDa), RFC4 (37 kDa), and RFC5 (36 kDa). All its subunits share significant homology in seven regions referred to as RFC boxes (box II–VIII) (40). ATP hydrolysis by RFC is required to load PCNA onto a primer-template junction (41–45). PCNA, once loaded, encircles the duplex DNA region in a primed template and tethers a DNA polymerase at the growing end of DNA so that the DNA polymerase can synthesize DNA in a processive manner (46–48). Our findings that RFC has several stimulatory motifs and strongly activates FEN1 indicate that RFC is a critical partner for FEN1 to remove flap structures arising from DNA metabolism, including processing of eukaryotic Okazaki fragments.

MATERIALS AND METHODS

Enzymes and Nucleotides—The oligonucleotides used in this study were commercially synthesized from Genotech (Daejeon, Korea), and their sequences are listed in Table 1. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham Biosciences and Izotop (Budapest, Hungary). Restriction enzymes and T4 polynucleotide kinase were purchased from PerkinElmer Life Sciences and Enzynomics™ (Daejeon, Korea). Adenosine triphosphate and ATPγS were obtained from Roche Applied Science. Rad27, yeast FEN1, was purified as described previously (15). Human replication protein A and PCNA were purified from *Escherichia coli* BL21 (DE3) CodonPlus-RIL (Stratagene) as described (49, 50)

Preparation of Substrates—The oligonucleotide-based partial duplex substrates were prepared essentially as described previously (15) using the synthetic oligonucleotides listed in Table 1. Oligonucleotides used as substrates, the position of radioisotopic label in the substrates, and substrate structures are indicated in each figure. Briefly, an oligonucleotide was first labeled at its 5′-end by incorporating [γ-^{32}P]ATP with T4 polynucleotide kinase and was followed by annealing with upstream and template oligonucleotides in a molar ratio of 1:4:2 (5′-labeled downstream/upstream/template oligonucleotides, respectively). The annealing reaction was performed by using PCR machine (95 °C, 5 min; 65 °C, 30 min; −1 °C/min). An equilibrating flap substrate was prepared as described previously (22) with the following modifications. A downstream oligonucleotide was first labeled at its 5′-end with [γ-^{32}P]ATP by T4 polynucleotide kinase, and the labeled oligonucleotide was then annealed to a template oligonucleotide along with an upstream oligonucleotide in a molar ratio of 1:3:10, respectively, as described above. The resulting equilibrating flap substrate was gel-purified prior to use.

Purification of FEN1 and RFC—The pET-23d(+)-FEN1 plasmid was constructed as described previously (10, 51). FEN1 expressed from this vector contained a C-terminally tagged hexahistidine. The plasmid was transformed into *E. coli* BL21 (DE3) CodonPlus-RIL (Stratagene), and expression of proteins was induced at A_{600} = 0.4 with isopropyl β-D-thiogalactopyranoside (final concentration, 0.4 mM) at 30 °C for 3 h. FEN1 was purified using the same procedure as described (10). Peak fractions from Mono S column chromatography were subjected to glycerol gradient sedimentation as described (52). Active peak fractions from the glycerol gradient were pooled, aliquoted, and stored at −80 °C until use.

The plasmid expressing FEN1-(ΔC337–380), a C-terminal 44 amino acid deletion mutant of FEN1, was constructed with pET-23d(+)-FEN1 as template according to the manufacturer's instruction using EZchange® mutagenesis kit (Enzynomics™, Korea). The FEN1-(ΔC337–380) mutant enzyme was expressed and purified essentially as the same procedure used for wild type FEN1 described above. Recombinant human RFC complex containing a truncated version of p140 lacking the N-terminal 555 amino acid residues was purified from baculovirus-infected Sf9 insect cells as described previously (53, 54) and is referred to as RFC in this study.

Preparation of RFC4 and Its Derivatives—DNA sequences encoding full-length RFC4 and its truncated derivatives (see Fig. 7*A*) were amplified by PCR and inserted into BamHI/NotI sites of pET28a(+) (Novagen), resulting in pET28a(+)-RFC4$_{x\text{-}y}$ (x and y indicate the position of amino acid at its beginning and end, respectively). All polypeptides expressed from this construct contained a His$_6$ tag and 24-amino acid linker fused to RFC4 and its derivatives to facilitate purification. The expression vectors constructed were introduced into *E. coli* BL21(DE3) CodonPlus-RIL (Stratagene), and expression of proteins was induced at A_{600} = 0.5 with isopropyl β-D-thiogalactopyranoside (final concentration, 0.5 mM) at 30 °C for 3 h. Cells from a 0.2-liter culture were col-

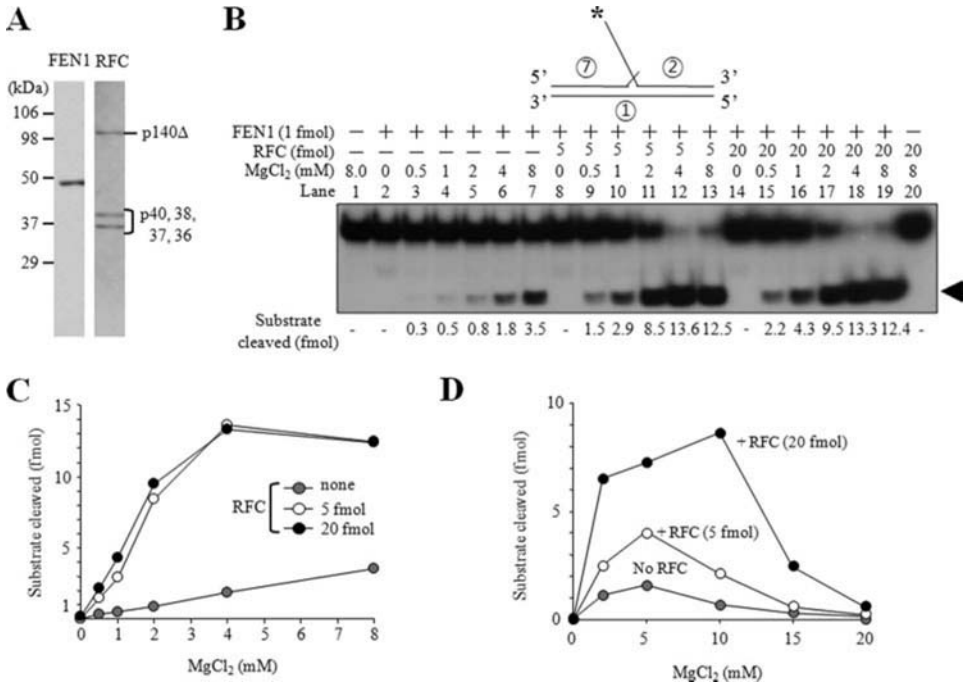


FIGURE 1. **Human RFC stimulates the activity of FEN1 on a double-flap substrate.** *A*, SDS-PAGE analysis of human FEN1 and RFC complex. Purified proteins were subjected to 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250. The sizes of molecular mass markers are as indicated in kDa. *B,* FEN1 endonuclease assays were performed as described under "Materials and Methods" with indicated amounts of FEN1 and RFC in the presence of increasing concentrations of Mg^{2+}. The double-flap substrate shown at the top of the gel was prepared as described under "Materials and Methods" and contains a 27-nt 5′ flap and a 1-nt 3′ flap. We refer to this double-flap substrate as standard substrate. The *circled numbers* denote the oligonucleotide listed in Table 1. Reaction products (indicated with an *arrowhead*) were separated in a 10% polyacrylamide gel, and quantified as described under "Materials and Methods." The amount of cleavage products is indicated at the bottom of the gel. The *asterisk* in the substrate indicates the position of ^{32}P-radioactive label. *C,* amount of cleavage products formed in reactions described in *B* was plotted against Mg^{2+} concentrations. The amount of RFC used is as indicated. *D,* RFC allows FEN1 to overcome the inhibitory effects of high Mg^{2+} concentrations. FEN1 endonuclease assays were performed in the standard reaction mixtures with a fixed amount (0.5 fmol) of FEN1 and varying levels of RFC in the presence of increasing concentrations of Mg^{2+}. The amount of cleavage products formed in reactions was plotted against Mg^{2+} concentrations.

lected by centrifugation, resuspended in 15 ml of buffer N_{300} (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 1.0 μg/ml leupeptin, and 1.0 μg/ml pepstatin A), and sonicated (three cycles of 60 s with a 3-min cooling interval). The subscript in buffer N_{300} denotes NaCl in mM. Extracts were centrifuged at 13,000 × *g* for 40 min at 4 °C, and the cleared lysate was loaded onto a Ni^{2+}-nitrilotriacetic acid column (300 μl, Qiagen), pre-equilibrated with buffer N_{300}. The column was successively washed with 10-column volumes of buffer N_{300} and with buffer N_{300} plus 25 mM imidazole. Proteins were eluted stepwise with buffer N_{300} plus 50, 100, 200, and 300 mM imidazole. Fractions containing most pure proteins were collected and used to test whether it possessed FEN1 stimulation activity. Protein concentrations were measured using Bradford solution with BSA standard. To measure concentrations of small proteins (with <100 amino acid residues), purified aliquots of the fractions were subjected to 16% Tricine/SDS-PAGE (55), and the gel was run until the dye front reached the bottom of the gel. The concentrations of small proteins were measured by comparing intensities of Coomassie-stained bands with those of BSA control. For single amino acid substitution in RFC4-(170–194), pET-28a(+)-RFC4-(170–194) prepared as described above was used as template, and alanine substitution was performed using the EZchange® mutagenesis kit (Enzynomics™, Korea). All oligonucleotides used for construction of expression plasmids or site-directed mutagenesis are available upon request.

Standard Endonuclease Assays—Standard endonuclease assays were performed in reaction mixtures (20 μl) containing 50 mM Tris-Cl, pH 8.0, 4 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 mg/ml BSA, and 15 fmol of the standard double-flap substrate (see Fig. 1*B*). Reactions were incubated for 15 min at 37 °C, followed by the addition of 4 μl of 6× stop solution (40% sucrose, 60 mM EDTA, 1.2% SDS, 0.05% bromphenol blue, and 0.05% xylene cyanol). The cleavage products were separated on a 10% polyacrylamide gel (plus 0.1% SDS) for 40 min at 140 V in 1× TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). The gels were dried on DEAE-cellulose paper (DE81, Whatman) and autoradiographed. Labeled DNA products were quantified with the use of a PhosphorImager (GE Healthcare).

Determination of Kinetic Parameters—To measure kinetic parameters, kinetic analyses were repeated in triplicate using increasing amounts (0, 20, 40, and 80 fmol) of a double-flap substrate; the substrate was prepared with oligonucleotides 1, 7, and 5 (upstream primer, template, and labeled downstream primer, respectively; refer to Fig. 5 for its structure) as described above. Reactions were carried out with 1 fmol of FEN1 and saturating levels (40 fmol) of RFC per reaction, and under these reaction conditions we were able to obtain reliable amounts of products at the early time point of incubation. Reaction mixtures (120 μl) were assembled on ice, followed by preincubation at 37 °C for 5 min, and initiated by the addition of 4 mM $MgCl_2$. Aliquots (20 μl) were withdrawn at 1, 2, 4, and 8 min after incubation and transferred to a tube containing 4 μl of 6× stop buffer. The amounts of products were analyzed as described above. Kinetic parameters were obtained based on the Michaelis-Menten equation. $V = dt[P]/dt$, where [P] is the amount of products in nM. The concentration of [P] was calculated using the equation, $[P] = I_{cleaved}/(I_{uncleaved} + I_{cleaved}) \times [S]$, where [S] is concentration of substrate used, and $I_{cleaved}$ and $I_{uncleaved}$ are band intensities of products and substrate left, respectively. The initial velocity was plotted against [S], and the values K_m and V_{max} were calculated by nonlinear regression using SigmaPlot

(Systat Software Inc.) to avoid distortion of the experimental errors, which can occur during reciprocal transformation of the data.

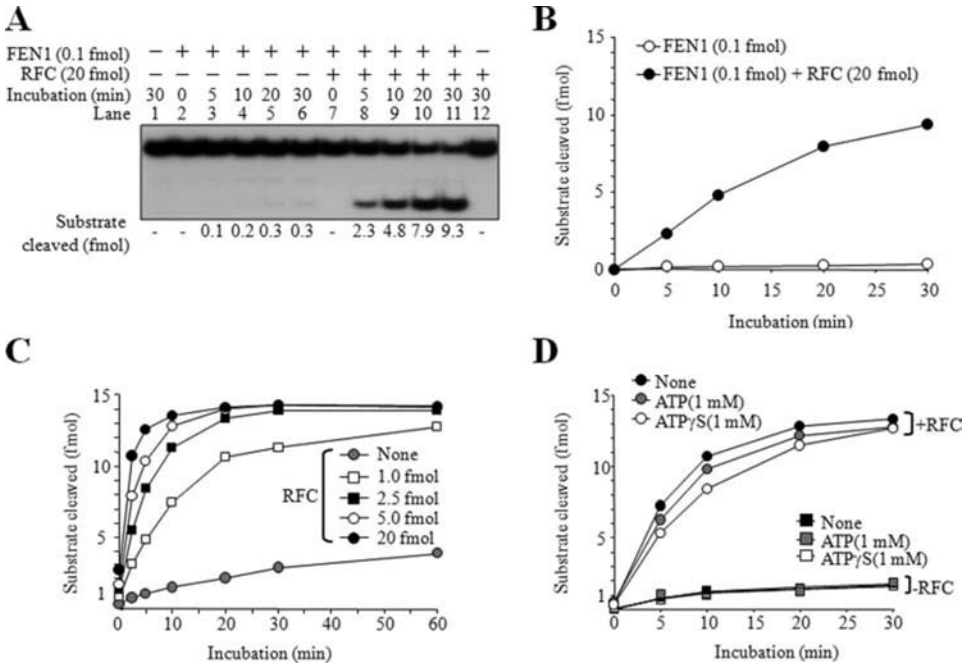


FIGURE 2. **RFC accelerates FEN1 activity in an ATP-independent manner.** *A,* endonuclease activity of FEN1 measured in the standard reaction mixtures in the presence of low levels of FEN1 and saturating levels of RFC. The amount of substrate cleaved is indicated at the *bottom* of the gel. *B, graph* of the data obtained in *A. C,* a time course experiment in the presence of varying amounts of RFC. Five sets of standard reaction mixtures (160 μl each) containing 8 fmol of FEN1, 120 fmol of the standard double-flap substrate, and varying levels of RFC, as indicated in the graph, were assembled on ice and prewarmed at 37 °C for 5 min, and reactions were initiated by adding Mg^{2+} to 4 mM (final concentration). Aliquots (20 μl) were withdrawn at times indicated after initiation of reaction. *D,* rates of FEN1 endonuclease activity in the absence or presence of 1 mM ATP or ATPγS. The amount of FEN1 and RFC used is as indicated.

RESULTS

Human RFC Stimulates Human FEN1 on Double-flap Substrate—To test the influence of the human RFC complex on catalytic activity of human FEN1, we purified both enzymes to near homogeneity as shown in Fig. 1*A*. The RFC complex purified contained the N-terminally deleted version of RFC1, which supported DNA pol δ *in vitro* as efficiently as wild type (42, 54). We found that the RFC complex markedly stimulated FEN1 activity (Fig. 1*B*) with a double-flap DNA substrate containing a 5′ 27-nt and a 3′ 1-nt flap, a physiological form of substrate (22). We refer to this double-flap substrate as standard substrate in this study. Unless otherwise stated, this double-flap substrate was used to measure FEN1 endonuclease activity. Additions of 5 or 20 fmol (Fig. 1*B*, *lanes 8–13* and *14–19*, respectively) of RFC to reaction mixtures containing 1 fmol of FEN1 resulted in a significant (5–10-fold) stimulation of FEN1-catalyzed cleavage of the 5′ flap in response to concentrations of Mg^{2+} used (Fig. 1*B*). Increase in Mg^{2+} concentrations resulted in a

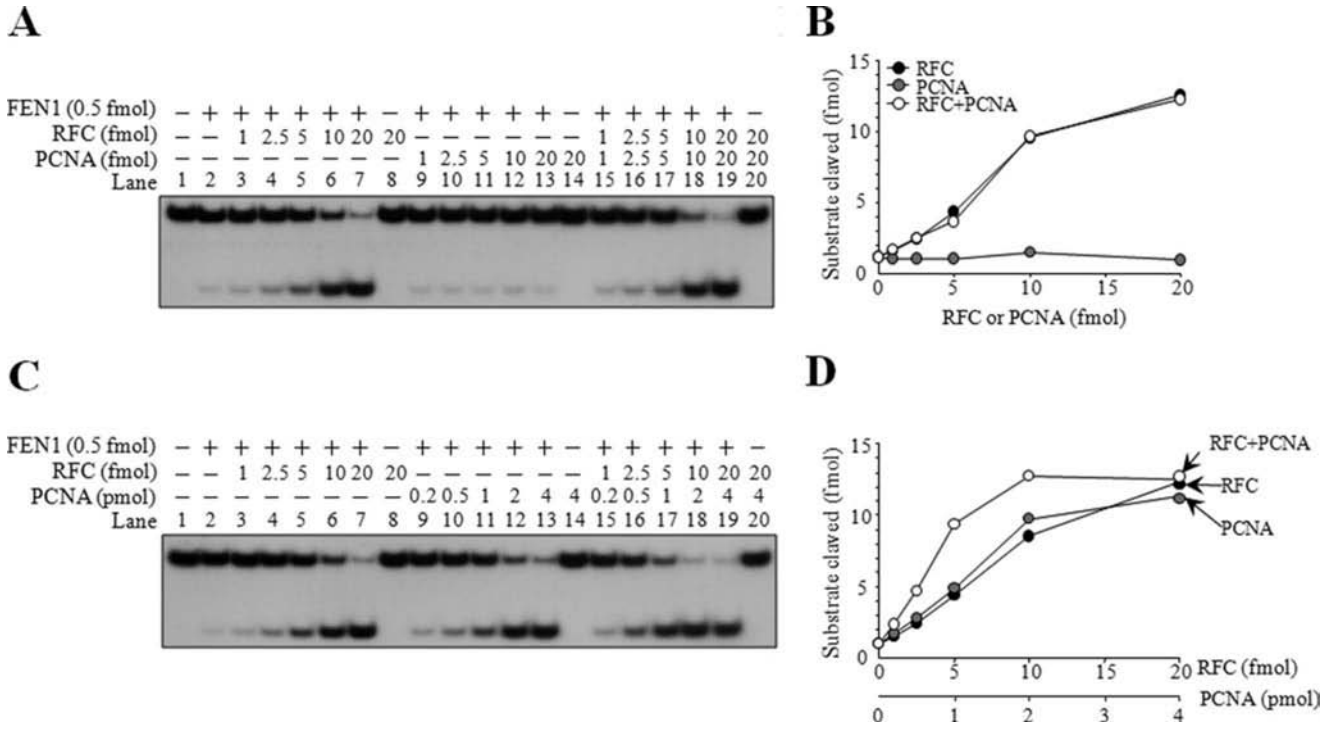


FIGURE 3. **RFC stimulates FEN1 activity in a manner distinct from PCNA.** *A,* influence of RFC and PCNA on the nuclease activity of FEN1. Standard endonuclease assays were carried out with amount of enzymes as indicated. *B,* quantitation of products formed in *A. C,* experiment described in *A* was repeated with elevated levels of PCNA. The amount of PCNA added was considerably greater than that added in *A. D,* results of quantification of cleavage products formed in *C.*

linear increase in cleavage of flap DNA by FEN1 in the absence of RFC (Fig. 1, *B*, *lanes 2–7,* and *C*, *gray circles*). The addition of RFC (5 fmol) increased FEN1-catalyzed cleavage of flap dramatically at all Mg^{2+} concentrations tested (Fig. 1*B*, compare *lanes 2–7* with *lanes 8–13*), reaching a plateau at 4 mM of Mg^{2+} (Fig. 1*C*, *open circles*). The addition of more (20 fmol) RFC did not further increase the stimulation (Fig. 1*B*, compare *lanes 8–13* with *14–19*). Thus, increases in Mg^{2+} concentrations not only enhance FEN1 activity but also increase the extent of FEN1 stimulation by RFC. With RFC (20 fmol) alone, no cleavage products were detected (Fig. 1*B*, *lane 20*). We next decided to determine optimal concentration of Mg^{2+} for stimulation of FEN1 by RFC. For this purpose, we tested effects of increasing concentrations (2, 5, 10, 15, and 20 mM) of Mg^{2+} on FEN1 activity in the presence of increasing levels (0, 5, and 20 fmol) of RFC (Fig. 1*D*). In this experiment, we reduced the amount of FEN1 to 0.5 fmol. We found that higher concentrations (>10 mM) of Mg^{2+} were inhibitory to FEN1 activity. In addition, lower levels (5 fmol) of RFC stimulated FEN1 activity, most effectively in Mg^{2+} concentrations less than 10 mM (Fig. 1*D*).

TABLE 2
Kinetic parameters for FEN1 endonuclease activity

	V_{max}	K_m	K_{cat}	K_{cat}/K_m
	$\times 10^{-3}$ nM/s	nM	$\times 10^{-1}\ s^{-1}$	$\times 10^{7}\ M^{-1}s^{-1}$
FEN1 alone	6.35 ± 1.1	7.28 ± 1.20	1.27 ± 0.22	1.75 ± 0.09
FEN1 + RFC	27.00 ± 2.0	6.48 ± 0.47	5.40 ± 0.39	8.34 ± 0.02

The addition of 20 fmol of RFC resulted in robust stimulation of FEN1 in Mg^{2+} concentrations at 10 mM or above, indicating that RFC allows FEN1 to overcome the inhibitory effect of high concentrations of Mg^{2+} (Fig. 1*D*). Based upon these findings, we decided to use 4 mM of Mg^{2+} in all subsequent reactions.

RFC Accelerates FEN1 Activity in an ATP-independent Manner—Next, we examined whether RFC could stimulate FEN1 in its low concentrations. To this end, we used a very low level (0.1 fmol) of FEN1 and excess RFC (20 fmol). As shown in Fig. 2*A*, the amounts of products formed were barely detectable (~0.3 fmol) after 30 min of incubation in the absence of RFC. In the presence of RFC, however, the amounts of products formed were 9.3 fmol (~30-fold more than in its absence) after 30 min of incubation (Fig. 2, *A* and *B*). We also investigated the influence of RFC concentration on FEN1 activity in a time course experiment, and we found that increasing concentrations (0, 1, 2.5, 5, 20 fmol) of RFC increased the rate of FEN1-catalyzed cleavage (Fig. 2*C*). This kinetic analysis revealed that the increase in RFC concentrations enhanced markedly (>5-fold) the rate of substrate cleavage by FEN1 (Fig. 2*C*). In the absence of RFC, the FEN1-alone reaction continued to accumulate cleavage products up to 1 h at 37 °C, indicating that FEN1 was not inactivated during the incubation period (Fig. 2*C*). However, the substrate cleavage rate increased proportionally to the amount of RFC added (Fig. 2*C*). Thus, we concluded that RFC accelerates the FEN1-catalyzed cleavage reaction. These results indicate that the increase in FEN1 activity is because of RFC stimulation of FEN1, not because of stabilization of the FEN1 protein.

Because RFC is a weak DNA-dependent ATPase (53), we examined whether ATP binding or ATP hydrolysis could alter the ability of RFC to stimulate FEN1. Neither ATP nor its nonhydrolyzable analog, ATPγS (1 mM, each), affected the stimulation of FEN1 activity by RFC (Fig. 2*D*), indicating that FEN1 stimulation by RFC requires neither ATP binding nor hydrolysis.

RFC Stimulates FEN1 Activity in a Manner Distinct from PCNA—Because FEN1 and RFC interact with PCNA, we performed mixing experiments to determine whether stimulation of FEN1 by RFC and PCNA is mutually exclusive (Fig. 3). First, we used the same concentrations (0–20 fmol; 0–1 nM) of RFC and PCNA in the presence of a fixed amount (0.5 fmol) of FEN1 as shown in Fig. 3*A*. The addition of increasing concentrations of RFC (Fig. 3, *A*, *lanes 3–7,* and *B, closed circles*) resulted in significant stimulation, whereas the addition of PCNA

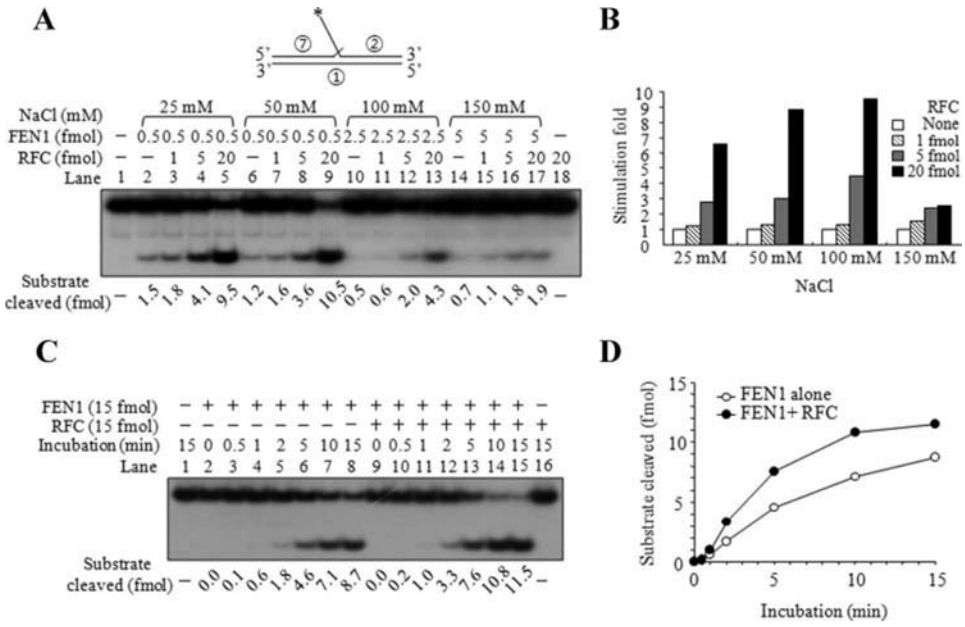


FIGURE 4. **RFC stimulates FEN1 at physiologic salt concentrations.** *A,* FEN1 stimulation activity of RFC was examined in a standard reaction condition in the presence of increasing concentrations (25, 50, 100, and 150 mM) of NaCl. Note that different amounts of FEN1 were used because of inhibitory effect of high salt concentrations. The amount of cleavage products is indicated at the *bottom* of the gel. *B,* amount of each products formed in *A* was measured and normalized with respect to that obtained in the absence of RFC. Fold stimulation observed in the presence of increasing amounts (0, 1, 5, and 20 fmol) of RFC is shown in the *bar graph*. The amount of RFC used is indicated in the *graph*. *C,* time course experiment was performed with FEN1 in stoichiometric amount of substrate DNA in the presence of 100 mM NaCl. Two sets of reaction mixtures (160 μl each) containing 120 fmol of FEN1 and 120 fmol of the standard double-flap substrate in the absence and presence of 120 fmol of RFC were assembled on ice and prewarmed at 37 °C for 5 min, and reactions were initiated by adding Mg^{2+} to 4 mM (final concentration). Aliquots (20 μl) were withdrawn at times indicated after initiation of reaction. The amount of substrate cleaved is shown at the *bottom* of figure. *D,* amount of cleavage products formed in *C* was plotted against incubation time.

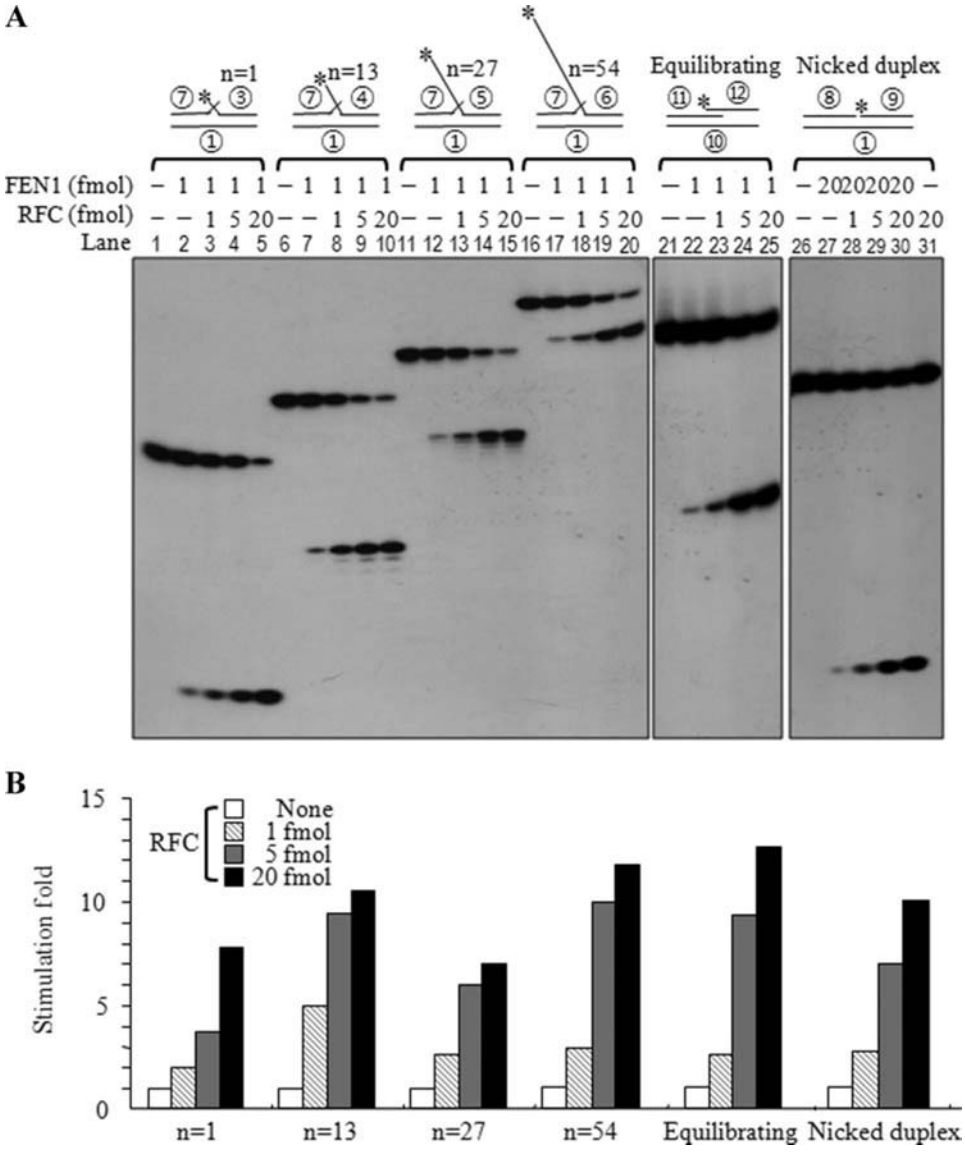


FIGURE 5. **RFC stimulates FEN1 activity using various substrates.** *A,* standard FEN1 endonuclease assays were performed as described under "Materials and Methods" with various structurally different substrates indicated. Schematic representation of each substrate used is shown at the *top* of the gel. The first four double-flap substrates differ in length of the 5′ flap with *n* indicating the oligs(dT) nucleotide length of the 5′ flap. Reactions were stopped by the addition of 20 μl of 2× stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue), and cleavage products, after boiled for 1 min, were subjected to electrophoresis in 15% denaturing polyacrylamide gel (plus 7 M urea) for 1.5 h at 35 watts in 1× TBE. *B,* amount of each substrate cleaved in reactions described in *A* was measured and normalized with the respect to that obtained in the absence of RFC for each substrate. Fold stimulation observed in the presence of increasing levels of RFC is shown in a *bar graph*.

equivalent to RFC failed to give rise to any detectable stimulation (Fig. 3, *A*, *lanes 9–13,* and *B*, *gray circles*). We then determined concentrations of PCNA required to stimulate FEN1 activity to that comparable with RFC (data not shown), and we found that ~200-fold more PCNA was required than RFC to obtain comparable stimulation (Fig. 3*C*, compare *lanes 3–7* with *9–13,* and *D*, *closed* and *gray circles*). Amounts of products formed in the presence of both RFC and PCNA (Fig. 3*C*, *lanes 15–19*) were close to the combined amounts of products formed in the presence of PCNA or RFC alone (Fig. 3*D*). Our results suggest that the mechanisms by which FEN1 is stimulated by RFC and PCNA are different. We also investigated whether ATP could affect stimulation of FEN1 by RFC in the presence of PCNA, but we found that it did not affect the stimulation extent (data not shown). This result indicates that stimulation of FEN1 by RFC occurs independently of the RFC loading function of PCNA.

Effect of RFC on the Kinetic Parameters for FEN1 Cleavage—We next determined the reaction kinetic parameters, K_m and V_{max}, as described under "Materials and Methods." The addition of RFC results in significant increase (>4-fold) in V_{max} (and thus K_{cat}), although hardly affecting K_m (Table 2). This result indicates that the addition of RFC increases K_{cat}/K_m, the catalytic efficiency of FEN1 ~4.8-fold (Table 2). The results of our kinetic analysis indicate that RFC increases catalytic power by increasing the turnover rate of FEN1. The two kinetic parameters also support the idea that the mechanism by which RFC stimulates FEN1 is distinct from that of PCNA, which increased V_{max} 2-fold, but decreased K_m ~12-fold (29). It has been suggested that the direct physical interaction of PCNA with FEN1 results in tethering FEN1 at the cleavage site, contributing to the increased affinity of FEN1 for DNA substrates (29). These findings are in support of our conclusion above that RFC stimulates FEN1 activity in a manner distinct from PCNA (Fig. 3).

RFC Stimulates FEN1 at Physiological Salt Concentrations—Because we wondered why stimulation of FEN1 by RFC was not noticed before by others, we compared other reaction conditions. The other reaction conditions were not significantly different from ours (see Refs. 4, 10, 11, 26–32, 36, 37, 51, 56). Briefly, they used either 50 mM Tris-Cl, pH 8.0, or 30 mM Hepes-KOH, pH 7.5, as buffer, salt concentrations were less than 50 mM NaCl or KCl and Mg^{2+} from 8 to 10 mM. When 30 mM Hepes-KOH, pH 7.5, was used, 40 mM KCl was added. To establish stimulation of FEN1 by RFC in a condition closer to a physiological one, we examined whether RFC could stimulate FEN1 at higher salt concentrations. For this purpose, we examined the ability of RFC to stimulate FEN1 in the presence of increasing concentrations (25, 50, 100, and 150 mM) of NaCl. As shown in Fig. 4*A*, we found that the increase in salt concentrations resulted in the inhibition of FEN1 activity (compare *lanes 2, 6, 10,* and *14*). For this reason, we used increased amounts

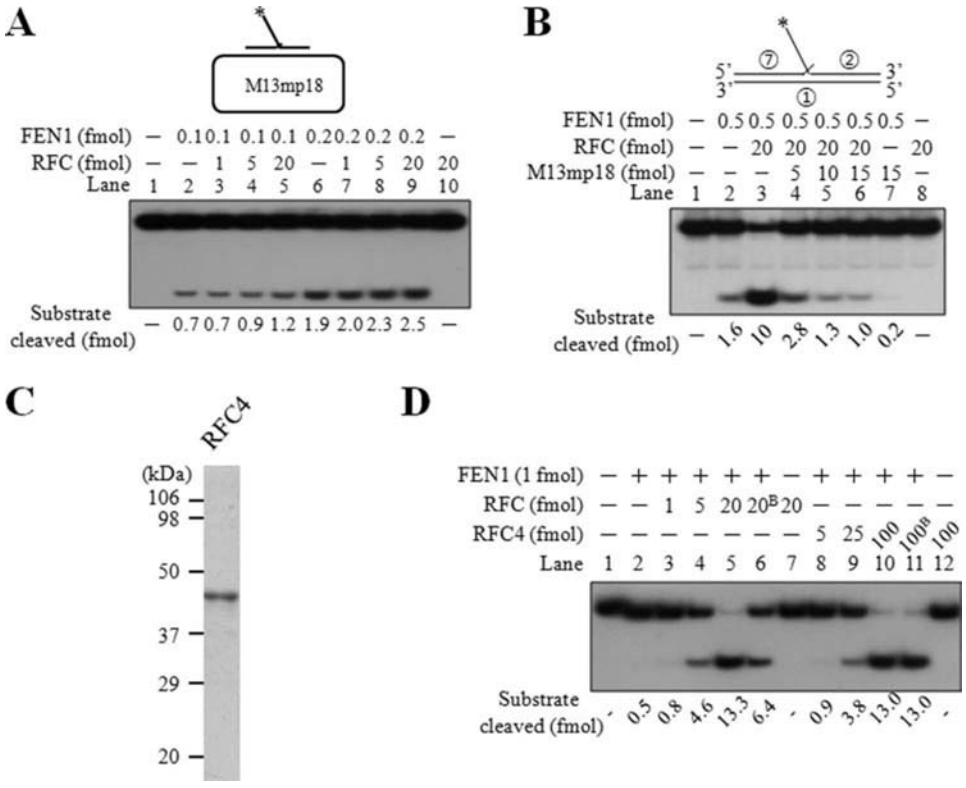


FIGURE 6. **Bulk single-stranded DNA interferes with RFC stimulation of FEN1, and the RFC4 subunit is able to stimulate FEN1.** *A,* standard endonuclease assays were performed with a double-flap substrate prepared by annealing two oligonucleotides to a single-stranded circular M13mp18 DNA in the presence of varying amounts of RFC (1, 5, and 20 fmol). The sequence of upstream and downstream primers are as follows: 5′ CAT CAA TAT GAT ATT CAA CCG TTC TAG-3′ (27 nt) and 5′-TTT TTT TTT TTT TTT TTT TTT TTT TTT GCT GAT AAA TTA ATG CCG GAG AGG G-3′ (52 nt). The underlined sequences are complementary to the nucleotide positions of 6978–7004 and 6954–6978, respectively, in single-stranded circular M13mp18 DNA (PerkinElmer Life Sciences). The long oligonucleotide (10 pmol) was 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and then annealed to M13mp18 DNA (2 pmol) together with the short one (20 pmol). The M13mp18-based double-flap DNA was separated from unannealed oligonucleotides and unincorporated [γ-^{32}P]ATP through a Sepharose CL-4B column (Amersham Biosciences). *B,* endonuclease assays were carried out with a standard substrate in the presence of increasing amounts (5, 10, and 15 fmol) of unlabeled M13mp18 DNA. *C,* SDS-PAGE analysis of the RFC4 subunit. The purified RFC4 subunit prepared as described under "Materials and Methods" was subjected to 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250. *D,* standard endonuclease assays were performed with varying levels of RFC or RFC4 as indicated. The *superscript B* indicates that the protein was heat-denatured by boiling for 5 min (*lanes 6* and *11*). The boiled proteins were chilled immediately by placing it on ice just prior to their addition to reaction mixtures. The amount of substrate cleaved is presented at the *bottom* of the gel.

(2.5 and 5 fmol) of FEN1 in the presence of 100 and 150 mM NaCl, respectively. In the presence of 100 mM NaCl, the addition of RFC resulted in stimulation of FEN1 activity to comparable levels obtained in the presence of 25 or 50 mM NaCl (Fig. 4, *A* and *B*), Stimulation was markedly reduced when we further increased salt to 150 mM. The ionic strength of 100 mM NaCl together with 50 mM Tris-Cl, pH 8.0, renders our reaction condition close to a physiological one. Thus, we conclude that RFC is able to stimulate FEN1 not only at low salt concentrations but also at the physiological salt concentration.

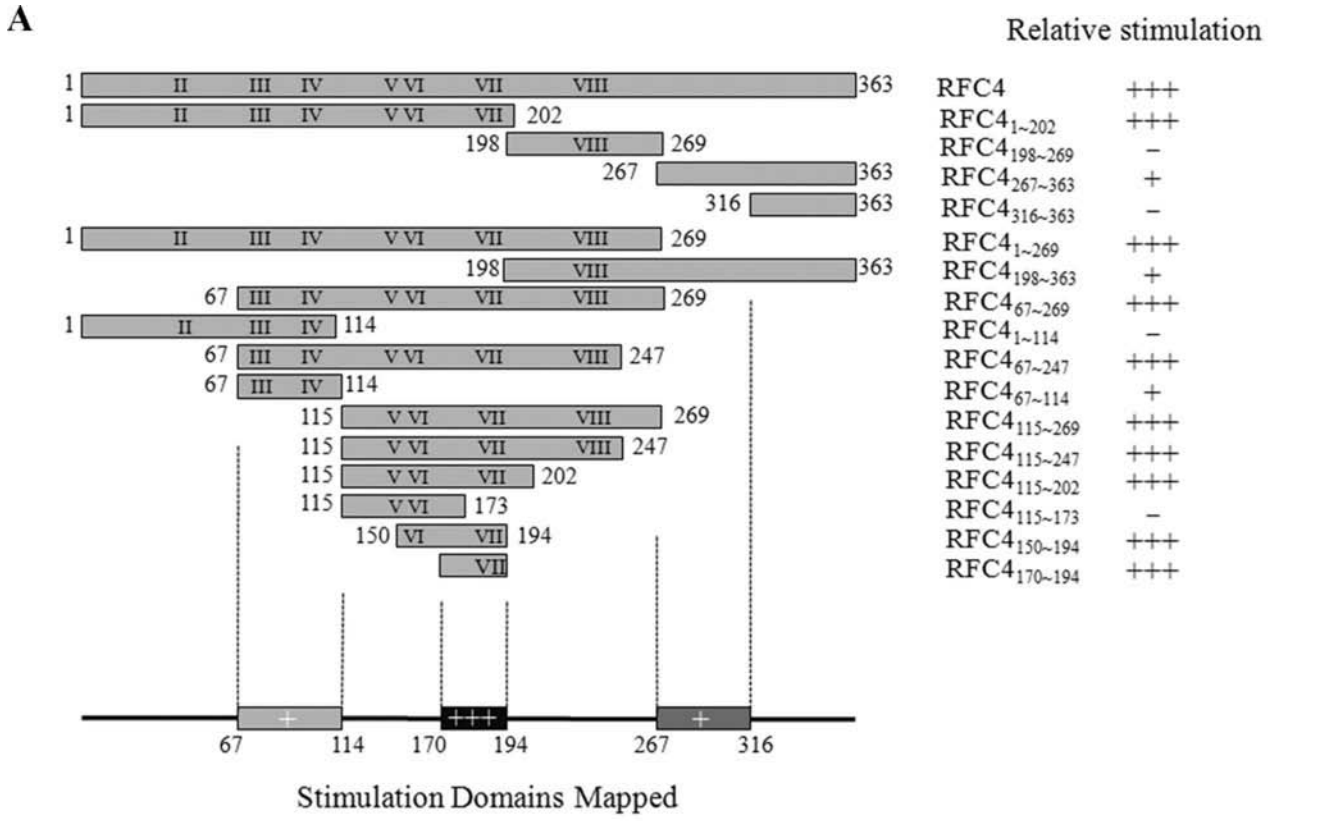
Our finding that FEN1 was stimulated by RFC at 100 mM NaCl allowed us to investigate the influence of RFC on FEN1 activity when FEN1 is present in a stoichiometric amount with substrate DNA. As shown in Fig. 4*C*, we carried out a time course experiment in which equimolar amounts (15 fmol each) of FEN1 and standard substrates were used. Under this condition, the recycling effect of FEN1 is minimized because the amount of enzyme is close to the amount of the substrate. We found that RFC was still able to stimulate FEN1 activity under this condition (Fig. 4, *C* and *D*). Therefore, this result indicates that RFC is capable of stimulating FEN1 in a single catalytic cycle. Because RFC stimulation of FEN1 decreased in the presence of high salt and/or in the presence of excess amounts of FEN1, we cannot formally exclude the possibility that RFC could stimulate FEN1 by a nonspecific mechanism under these conditions. For example, RFC could stimulate FEN1 by dissociating FEN1 from the cleavage products using the nonspecific DNA binding activity of RFC, and thereby recycling it. However, we prefer the possibility that RFC stimulates FEN1 activity in a specific manner under physiological conditions because of the presence of specific stimulatory motifs in RFC required for FEN1 stimulation (see below).

RFC Supports FEN1 to Utilize a Variety of Known Substrates—The 9-1-1 complex and PCNA were both reported to stimulate FEN1 irrespective of flap length (29, 37). To determine stimulation activity of RFC in this regard, FEN1 endonuclease assays were performed with a number of substrates (Fig. 5). We first tested double-flap substrates with varying 5′ flap lengths (1–54 nt) (Fig. 5*A*, *lanes 1–20*). All single strand DNA flaps contained homogeneous oligo(dT) to prevent, as their length increases, the formation of secondary structure that may interfere with FEN1 activity. As shown in Fig. 5*A*, flap length did not cause any noticeable difference in the extent of FEN1 stimulation by RFC. We obtained 8–12-fold stimulation in the presence of 20 fmol of RFC (Fig. 5, *A* and *B*). Like the 9-1-1 complex and PCNA, stimulation of FEN1 by RFC was not affected by flap length. Like the double-flap substrates, an equilibrating flap substrate, in which the double flaps compete with each other to anneal to the template, was more efficiently cleaved by FEN1 in the presence of RFC (Fig. 5*A*, *lanes 21–25*). Note that we used low levels (1 fmol) of FEN1 for the double-flap (*lanes 1–20*) and equilibrating flap substrates (Fig. 5*A*, *lanes 21–25*) as they are efficiently cleaved by FEN1.

We also tested nicked duplex substrate (*lanes 26–31*) as shown in Fig. 5*A*. Consistent with previous reports (22, 56), the level of FEN1 required to obtain the same level of cleavage with these substrates was greater (20-fold) than with either double-flap or equilibrating substrates. The 5′–3′-exonuclease activity

of FEN1 was markedly stimulated (up to 9-fold) by RFC (Fig. 5, *A*, *lanes 26–31*, and *B*). This result, taken together with the results above, indicates that RFC potentiates catalytic activities tested of FEN1.

Bulk Single-stranded DNA Interferes with RFC Stimulation of FEN1—Previously, the ability of PCNA to stimulate FEN1 was investigated with an oligonucleotide-based substrate in the presence and absence of RFC (26). In this study, however, the



influence of RFC alone on FEN1 activity was not examined. Another study showed that RFC did not stimulate FEN1 activity with a flap substrate containing bulk single-stranded DNA (51). The substrate used in this study was prepared by annealing two oligonucleotides to a single-stranded circular (ssc) DNA. We repeated this experiment by preparing an M13sscDNA-based substrate. As shown in Fig. 6*A*, the addition of increasing amounts of RFC hardly stimulated FEN1-catalyzed cleavage of flap structure in this substrate. To assess the influence of bulk single strand DNA on stimulation of FEN1 by RFC, we performed a challenge experiment in which cold M13sscDNA was added to standard reaction mixtures. As expected, the addition of RFC stimulated FEN1 activity (Fig. 6*B*, *lanes 2* and *3*). However, addition of increasing amounts of M13sscDNA resulted in marked decrease in product formation (Fig. 6*B*, *lanes 4–6*). For example, the addition of 10 fmol of M13sscDNA reduced cleavage products to the level of FEN1 alone (Fig. 6*B*, *lanes 2* and *5*). We noted that the addition of M13sscDNA also inhibited FEN1 activity significantly (Fig. 6*B*, *lanes 2* and *7*). This inhibition was relieved by the addition of 20 fmol of RFC (Fig. 6*B*, *lanes 6* and *7*), most likely through stimulation of FEN1. Consistent with this, we found that excess RFC (>0.1 pmol) was required to obtain 5-fold stimulation of FEN1 activity with M13sscDNA-based substrates (data not shown). Therefore, we conclude that the amount of RFC required to stimulate FEN1 activity varies depending on types of substrate used.

Multiple Stimulatory Motifs in RFC4—As an initial attempt to define which subunit of RFC has FEN1 stimulation activity, we purified the subunits and found that all five were capable of stimulating FEN1 activity (data not shown). We first isolated the full-length RFC4 subunit to near homogeneity because this subunit was well expressed in soluble form in *E. coli* (Fig. 6*C*). We compared its ability to stimulate FEN1 activity with that of the five-subunit RFC complex. Interestingly, we discovered that the amount of RFC4 required for comparable stimulation was ~5-fold greater than that of the full RFC complex (Fig. 6*D*, compare *lanes 3–5* and *8–10*).

Unexpectedly, we found that RFC and RFC4 did not completely lose FEN1 stimulation activity even after being boiled for 5 min (Fig. 6*D*, *lanes 6* and *11*). Although the RFC complex, upon heat treatment, lost approximately two-thirds of its stimulatory activity, the RFC4 subunit is highly heat-resistant. This finding immediately suggested to us that stimulation activity is likely to reside in a small motif that could easily fold back into a functional structure after heat denaturation. This finding is consistent with the fact that stimulation activity of RFC was independent of ATP hydrolysis and binding, which would require structural integrity of RFC (see Fig. 2*D*). This hypothesis encouraged us to define the stimulatory motif(s) of the RFC4 subunit.

To map the stimulatory motif(s) for FEN1 in RFC4, various truncated derivatives of RFC4 were expressed and purified as described under "Materials and Methods." Each truncated derivative of RFC4 was prepared as illustrated in Fig. 7*A*, and some of the proteins purified are shown in Fig. 7*B*. We first prepared three truncated derivatives of RFC4 as follows: the N-terminal RFC4-(1–202) (numbers in parentheses indicate the amino acid positions), the central RFC4-(198–269), and the C-terminal RFC4-(267–363) fragments. We found that the N-terminal RFC4-(1–202) fragment retained FEN1 stimulation activity (Fig. 7*A*). The C-terminal fragment RFC4-(267–363) (Fig. 7*B*, *lane 1*) weakly stimulated FEN1 activity at low protein levels (<0.1 pmol) (Fig. 7*C*, *lanes 18–20*) but greatly stimulated FEN1 activity at high protein levels (>1 pmol) (Fig. 7*C*, *lane 21*). In contrast, RFC4-(316–363) (Fig. 7*B*, *lane 2*) did not stimulate FEN1 at either low (25–100 fmol; Fig. 7*C*, *lanes 11–13*) or high (500 fmol, Fig. 7*C*, *lane 17*) protein levels. RFC4-(1–202) was further fragmented into RFC4-(1–114) (Fig. 7*B*, *lane 3*) and RFC4-(115–202) (Fig. 7*B*, *lane 4*). RFC4-(1–114) lacked any stimulatory activity (Fig. 7*C*, *lanes 3–5*), but RFC4-(115–202) activity (Fig. 7*D*, *lanes 8–10*) was comparable with full-length RFC4 (Fig. 7*D*, *lanes 3–5*). Unlike RFC4-(1–114) that did not stimulate FEN1 activity at any protein levels (Fig. 7*C*, *lanes 3–5* and *15*), RFC4-(67–114) (Fig. 7*B*, *lane 5*), although not stimulatory at low protein levels (Fig. 7*C*, *lanes 7–9*) was stimulatory at high protein levels (500 fmol; Fig. 7*C*, *lane 16*). This finding suggests that RFC4-(1–66) inhibits or interferes with the interaction of RFC4-(67–114) and FEN1. Further deletion analyses of RFC4-(115–202) led to the finding that RFC4-(170–194) (Fig. 7*B*, *lane 6*), the region comprising RFC box VII, retained strong FEN1 stimulation activity (Fig. 7*D*, *lanes 18–21*), whereas fragment RFC4-(115–173), which contains RFC box V and VI, had no stimulation activity (Fig. 7, *B*, *lane 7*, and *D*, *lanes 13–16*). FEN1 stimulation activities of RFC4-(115–202) and RFC4-(170–194) were not abolished by heat denaturation (Fig. 7*D*, *lanes 11* and *21*, respectively) like full-length RFC4 (Fig. 7*D*, *lane 6*). Thus, the main stimulatory activity of RFC4 resides in the RFC4-(170–194) fragment.

Mapping of Amino Acid Residues Critical for FEN1 Stimulation—Because all five RFC subunits have FEN1 stimulation activity and all subunits contain the box VII motif, we expressed and purified the truncated forms (corresponding to RFC4-(115–202)) of the four subunits (RFC1–3 and -5). The truncated forms of each subunit all were capable of stimulating FEN1, confirming our mapping results (data not shown).

FIGURE 7. **Multiple stimulatory motifs in RFC4.** *A,* RFC4 and its derivatives, prepared as described under "Materials and Methods," were schematically shown with the location of RFC homology boxes indicated in *roman numerals* in each derivative. *Arabic numbers* indicate the position of amino acids at the beginning and end of each derivative. The FEN1 stimulation by the different RFC4 derivatives is indicated on the *right*. The values are presented as follows: +++, stimulation equivalent to wild type RFC4; +, stimulation observed in the presence of 3–5-fold excess of proteins; −, no detectable stimulation. The positions of the three stimulatory motifs in RFC4 are shown at the *bottom* of the figure (see text for details). *B,* purity of RFC4 derivatives used for experiments described in *C* and *D*. The purified proteins were analyzed in 16% Tricine/SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250. Prior to use, the concentrations of the small proteins were estimated by comparing their band intensities with BSA controls. *C,* FEN1 endonuclease assays were carried out in the presence of increasing concentrations of RFC4 derivatives; RFC4-(1–114), RFC4-(67–114), RFC4-(316–363), and RFC4-(267–363). The amount of substrate cleaved is presented at the *bottom* of the gel. *D,* standard endonuclease assays were carried out with RFC4-(115–202), RFC4-(115–173), and RFC4-(170–194) as well as full-length RFC4. The amount of substrate cleaved is shown at the *bottom* of the gel. The *superscript B* indicates heat treatment of protein as described in Fig. 6*D*.

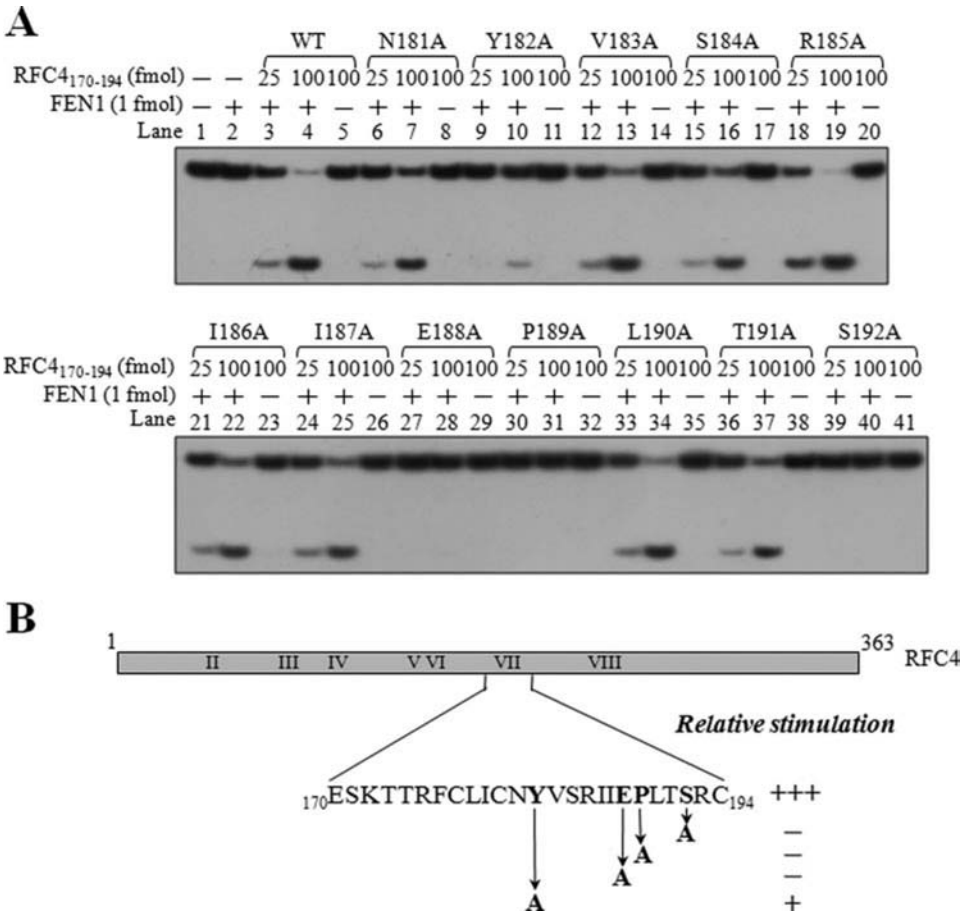


FIGURE 8. **Mapping of amino acid residues in RFC4 box VII required for the stimulation of FEN1.** *A,* effect of mutated RFC4-(170–194). Indicated levels of the alanine-mutated RFC box VII peptides were analyzed as described under "Materials and Methods." Standard endonuclease assays were performed with 1 fmol of FEN1 in the presence of each alanine-substituted RFC4-(170–194) as indicated at *top* of the gel. *B,* positions of amino acids critical for stimulation of FEN1 in RFC box VII are shown. See the legend of Fig. 7*A* for relative stimulation. *WT*, wild type.

We next used site-directed mutagenesis to further confirm that motif VII of RFC4 is responsible for its FEN1 stimulation activity. For this purpose, we performed alanine substitution from amino acids 181–192 of RFC4-(170–194). The mutant polypeptides were expressed and purified from *E. coli* (data not shown). All polypeptides contained a His_6 tag and a 24-amino acid linker fused to the stimulatory motif of RFC4-(170–194) to facilitate purification. The protein bands of each purified polypeptide were quantified with a BSA standard. As shown in Fig. 8*A*, we found that the change of four amino acids, Tyr-182, Glu-188, Pro-189, and Ser-192, into alanine affected FEN1 stimulation by RFC. The change of Tyr-182 into alanine resulted in partial loss of stimulation activity. These results are summarized in Fig. 8*B*. The finding that a single amino acid change in RFC4-(170–194) resulted in loss of stimulatory activity clearly demonstrates that RFC4-(170–194) contains a definable FEN1 stimulation motif.

Human RFC Stimulates Yeast Rad27 as Well as Human FEN1—FEN1 is structurally and functionally conserved from yeast to humans. It was reported that expression of human FEN1 in *S. cerevisiae* complemented several *RAD27*-deletion phenotypes as follows: allowing *rad27*Δ mutant cells (i) to grow at restrictive temperature (37 °C), (ii) to restore sensitivity to DNA damaging agents, and (iii) to suppress synthetic lethality of *rad27* with *rad51* or *pol3-01* (7, 11, 57). For this reason, it is expected that human RFC should stimulate Rad27, yeast FEN1. To confirm this, we purified yeast Rad27 to near homogeneity as shown in Fig. 9*A* and examined cross-species FEN1 stimulation by human RFC. Not surprisingly, human RFC significantly stimulated Rad27 (Fig. 9*B*, *lanes 8–12*), although less efficiently than it stimulated human FEN1 (compare *lanes 2–5* to *8–11*). Heat-denatured human RFC retained the ability to stimulate both human FEN1 and yeast Rad27 (Fig. 9*B*, *lanes 6* and *12*). For both human FEN1 and yeast Rad27, heat denaturation of RFC (20 fmol) reduced stimulation to the level obtainable with 5 fmol of RFC (Fig. 9*B*, compare *lanes 4* and *6*, for example). Yeast Rad27 was also stimulated by the human RFC4 subunit (data not shown), suggesting that the stimulatory motifs present in RFC4 also function to stimulate yeast Rad27. It is worthwhile to mention that the human RFC complex did not stimulate yeast Dna2 endonuclease activity under conditions used for FEN1 stimulation (data not shown).

C-terminal Region of FEN1 Is Essential for Stimulation by RFC—Although FEN1 is evolutionarily well conserved from archaea to mammals, eukaryotic FEN1 has an extended and unstructured C terminus, known to be involved in nuclear localization, substrate binding, and protein-protein interaction (51, 58). Recently, it was shown that the interaction of WRN with the C terminus of FEN1 stimulated FEN1 activity *in vitro* (58). To discover how RFC stimulates FEN1, we purified a FEN1 C-terminal deletion mutant FEN1-(ΔC337–380) (Fig. 10*A*). As shown in Fig. 10*B*, we found that C-terminal deletion of FEN1 results in lower (5-fold) activity compared with full-length FEN1 (Fig 10, *B,* compare *lanes 2–7,* with *C, lanes 8–13*). This is likely due to the loss of the substrate binding domain (51). Thus, we used 5-fold more FEN1-(ΔC337–380) than wild type FEN1 to assess the stimulatory effect of RFC on FEN1-(ΔC337–380) endonuclease activity. Wild type FEN1 was dramatically enhanced by RFC (Fig. 10*B*, *lanes 15–18*), whereas FEN1-(ΔC337–380) was not stimulated at all (Fig. 10, *B*, *lanes 19–22,* and *D*). The RFC4 subunit also failed to stimulate FEN1-(ΔC337–380) (data not shown). Therefore, we concluded that the stimulatory effect of RFC is mediated through the C-terminal region of FEN1.

DISCUSSION

In this study, we report for the first time that the human RFC complex markedly stimulates human FEN1 via its multiple stimulatory motifs per subunit, thus empowering FEN1 to cleave more efficiently all its known substrates. FEN1 stimulation by RFC is independent of ATP binding and hydrolysis, suggesting that this function of RFC is novel and separable from ATP-dependent PCNA loading to primer ends. Our findings demonstrate that RFC is involved throughout lagging strand metabolism, playing roles not only in loading PCNA but also in generating ligatable nicks through FEN1 stimulation.

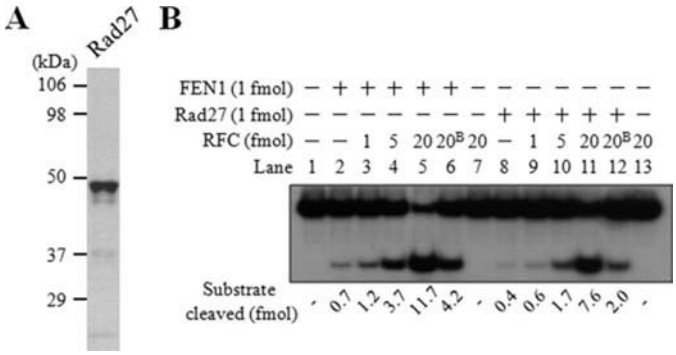


FIGURE 9. **Human RFC stimulates yeast Rad27.** *A,* SDS-PAGE analysis of yeast Rad27 used. Purified protein was subjected to 10% SDS-PAGE, and the gel stained with Coomassie Brilliant Blue R-250. *B,* FEN1 endonuclease assays were carried out with human FEN1 and yeast Rad27 in the presence of increasing levels of human RFC. The amount of substrate cleaved is indicated at the *bottom* of the gel.

Our efforts to define the stimulatory domain of RFC4 revealed that RFC4-(170–194) was able to stimulate FEN1 activity and that the four amino acid residues Tyr-182, Glu-188, Pro-189, and Ser-192 were critical for FEN1 stimulation. According to the three-dimensional structure of yeast RFC, the side chains of these four conserved amino acid residues are located on the exterior of the 5th α-helix of yeast RFC4 (46), and thus are accessible for interaction with FEN1. Unexpectedly, RFC4 carrying the deletion of RFC4-(170–194) retains approximately 2/3 of stimulation activity when compared with wild type RFC4, allowing us to find the two additional weak stimulatory motifs located near the N and C termini (Fig. 7). These two weak motifs may function when the strong RFC4-(170–194) motif is unavailable, particularly due to conformational changes. It is also possible that inactivation of one strong motif may lead to activation of the other weak motifs so that FEN1 stimulation is possible under all circumstances.

The fate of RFC after PCNA loading is still controversial. Fanning and co-workers (59) showed that RFC disengaged from DNA after PCNA loading. In contrast, others reported that RFC remained attached to the primer terminus throughout elongation and behaved as a processive factor (60, 61). One possible explanation for this discrepancy is a difference in the RFC preparations; when a mutant RFC1 subunit lacking the first 273 amino acid N-terminal domain was used, the complex disengaged after PCNA loading (59), whereas full-length RFC moved along DNA with PCNA and pol δ (60, 61). The 273-amino acid N-terminal region of RFC1 was not required for interaction with PCNA or DNA replication *in vitro* (54), but its nonspecific DNA binding activity may prevent RFC from dissociating after PCNA loading. Even if RFC is not an integral part of the DNA synthesis machinery, RFC could stimulate FEN1 as it moves between Okazaki fragments. Despite this, our finding that RFC is a potent stimulator of FEN1 better supports the scenario that RFC moves along with PCNA and pol δ, so that RFC is available to stimulate FEN1 to cleave flap structures. In this way, RFC could contribute to more rapid formation of ligatable nicks as an integral part of the replication machinery. The multiple stimulatory motifs present in one molecule of RFC may account for why much less (~200-fold) RFC is required than PCNA for a comparable level of FEN1 stimulation. The fact that RFC is intrinsically more potent than PCNA in stimulating FEN1 makes it likely that RFC is a more physiologically relevant stimulator of FEN1. It is important to note the difficulty of demonstrating that FEN1 stimula-

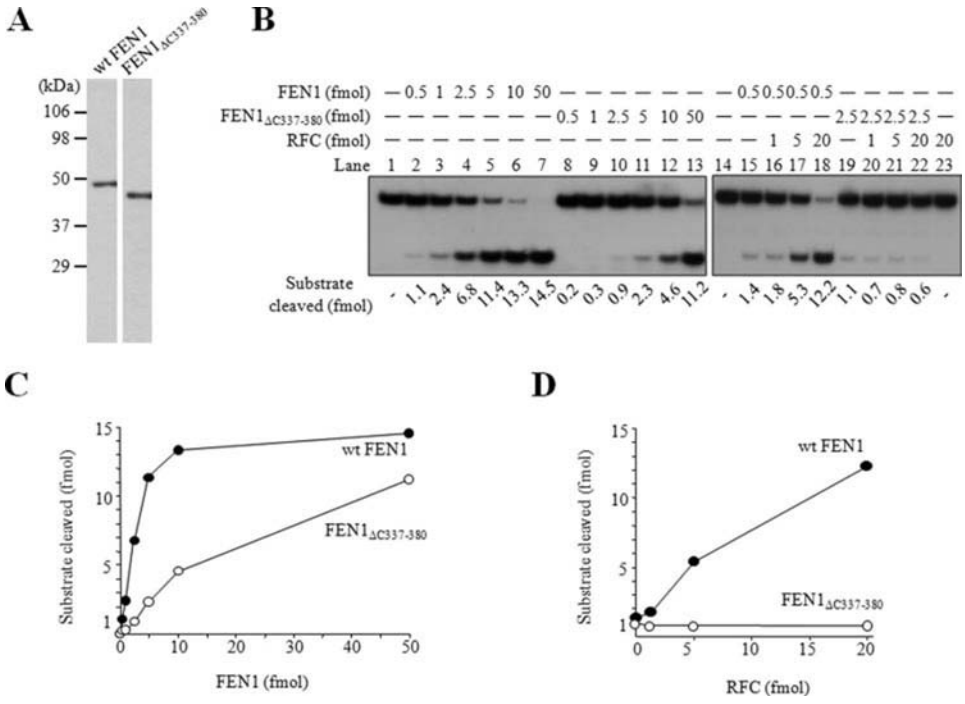


FIGURE 10. **C-terminal region of FEN1 is required for the stimulation of FEN1 by RFC.** *A,* both wild type (*wt*) and mutant FEN1 ($FEN1_{\Delta C337-380}$) lacking the C-terminal 44 amino acids were purified and subjected to 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250. *B,* FEN1 endonuclease assays were carried out with wild type FEN1 and FEN1-(ΔC337–380) in the presence of increasing amounts of human RFC complex. The amount of substrate cleaved is presented at the *bottom* of the gel. *C,* quantification of products formed from reactions performed in the absence of RFC (*lanes 1–13*) in *B*. Note that level of wild type FEN1 used was the same as FEN1-(ΔC337–380). *D,* quantification of products formed in the presence of RFC (*lanes 14–23*) in *B*. Note that level of FEN1-(ΔC337–380) was 5-fold higher than that of wild type FEN1.

tory function of RFC is essential for Okazaki fragment maturation *in vivo*, because (i) there are at least five stimulatory motifs in the RFC complex, and (ii) all subunits of RFC are essential. Thus, it will be difficult to obtain mutant RFC free of FEN1 stimulatory activity without affecting DNA replication.

Determination of kinetic parameters, including V_{max} and K_m, revealed that RFC increased V_{max} ~4-fold without affecting K_m. This change resulted in a significant increase in the catalytic efficiency (K_{cat}/K_m) of FEN1 (Table 2). Like RFC, the addition of WRN increased V_{max} 6-fold compared with that of FEN1 alone, but it did not affect K_m (32). These kinetic results suggest that stimulation mechanisms by which RFC and WRN are similar, but distinct from that of PCNA, increased V_{max} 2-fold and decreased K_m 12-fold (29). PCNA physically interacts with FEN1 in two distinct modes depending on its interaction status with DNA (28). The direct physical interaction of PCNA with FEN1 may result in tethering FEN1 at the cleavage site, effectively increasing FEN1 substrate affinity (28). In contrast, our finding that RFC did not alter K_m but increased V_{max} suggests that RFC promotes the formation of the FEN1-substrate complexes, their turnover, or both. This may be achieved by conformational changes in FEN1 induced by its transient interaction with RFC or WRN. Although kinetic parameters have not been determined with BLM, we believe that BLM may also stimulate FEN1 in a similar way.

Physical interactions of WRN and BLM with FEN1 were demonstrated (31, 35), but we failed to detect any physical interaction *in vitro* using purified proteins (data not shown). Consistent with this, genome-wide analyses did not reveal any evidence that yeast Rad27 physically interact with components of the RFC complex. Therefore, we believe that there is weak and transient interaction between FEN1 and RFC that allows rapid catalytic action of FEN1. The interaction between the box VII motif in each RFC subunit and the C terminus of FEN1 may induce a conformational change in FEN1 that increases its catalytic efficiency.

The stimulation of FEN1 by RFC may provide cells with an effective means of preemptively removing DNA flaps before they grow too long. Excessively long flaps are likely to form secondary structure that would inevitably require additional enzymatic activities to resolve. Long flaps can form even higher ordered structure or bind to replication protein A, both of which inhibit FEN1 activity.

We also found that RFC could stimulate FEN1-catalyzed cleavage of a hairpin-containing flap or a flap containing a partial duplex at its 5′-end (data not shown). In other words, RFC can stimulate the GEN activity of FEN1. Thus, RFC might have other biological importance during Okazaki fragment processing. Repeat-induced secondary flap structures might be more effectively processed by the GEN activity of FEN1 in the presence of RFC than by the Dna2 pathway. If this is the case, FEN1 together with RFC may contribute to stabilizing repeat sequences. Alternatively, the GEN activity of FEN1, when assisted by RFC, may be more effective at resolving stalled replication forks.

There is a growing list of proteins, all involved in DNA metabolism, that can stimulate FEN1 activity. These include WRN, BLM, Mgs1, PCNA, and Mph1 (62). The acquisition of FEN1 stimulation activity by these proteins may have conferred evolutionary benefits, because such an ability may permit faster generation and sealing of DNA nicks. If this is the case, the stimulation of FEN1 by RFC and other proteins reflects the importance of the generation and sealing of ligatable nick. This may act as the first line of defense in the preservation of genome integrity.

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