Arginine Residues 47 and 70 of Human Flap Endonuclease-1 Are Involved in DNA Substrate Interactions and Cleavage Site Determination

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Running title: Interaction between human FEN-1 and its substrates

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Key words: DNA replication, DNA repair, flap endonuclease-1 (FEN-1), Protein-DNA interaction

Total pages: 33
Total tables: 3
Total figures: 7
Abstract

Flap endonuclease-1 (FEN-1) is a critical enzyme for DNA replication and repair. Intensive studies have been carried out on its structure-specific nuclease activities and biological functions in yeast cells. However, its specific interactions with DNA substrates as an initial step of catalysis are not defined. An understanding of the ability of FEN-1 to recognize and bind a flap DNA substrate is critical for the elucidation of its molecular mechanism and for the explanation of possible pathological consequences resulting from its failure to bind DNA. Using human FEN-1 in this study, we identified two positively-charged amino acid residues, R47 and R70 in human FEN-1, as candidates responsible for substrate binding. Mutation of the R70 significantly reduced flap endonuclease activity and eliminated exonuclease activity. Mutation or protonation of R47 shifted cleavage sites with flap substrate and significantly reduced the exonuclease activity. We revealed that these alterations are due to the defects in DNA-protein interactions. Even though the effect of the single R47 mutation on binding activities is not as severe as R70A, its double mutation with D181 had a synergistic effect. Furthermore, the possible interaction sites of these positively charged residues with DNA substrates were discussed based on FEN-1 cleavage patterns using different substrates. Finally, data were provided to indicate that the observed negative effects of high concentration of Mg$^{2+}$ on enzymatic activity are probably due to the competition between the arginine residues and metal ions with DNA substrate, since mutants were found to be less tolerant.
Introduction

Flap endonuclease-1 (FEN-1) proteins possess a flap endonuclease activity as well as a 5’ to 3’
exonuclease activity (1-5). These nuclease activities allow FEN-1 proteins to remove the RNA primers during
lagging-strand DNA synthesis and damaged DNA fragments in various DNA repair pathways (6-11). Besides
its nuclease activities, it interacts with various proteins involved in DNA metabolic pathways including the
Werner syndrome protein (12) and proliferating cell nuclear antigen (PCNA) (13, 14). FEN-1 localizes into the
nucleus in S-phase during DNA synthesis as well as in response to DNA damage (15). In yeast, loss of FEN-1
functions were determined to cause severely-impaired phenotypes such as conditional lethality, dramatic
increase of cellular sensitivity to alkylating agents, e.g., methyl methanesulfonate (MMS), and significant
increase in the mutation rate. Interestingly, a majority of mutations in the yFEN-1 mutant strains are large
sequence duplications (10). Duplication mutations in humans have been associated with disorders such as
recessive retinitis pigmentosa, lethal junctional epidermolysis bullosa, familial hypertrophic cardiomyopathy,
and cancer. FEN-1 is also a key enzyme for preventing expansion and contraction of bi- or tri-nucleotide
repeats (16-19). More recently, we have demonstrated that FEN-1 nuclease plays an important role in limiting
short sequence recombination for yeast genome stability (20). All of these observations illustrate its critical
roles in genome integrity.

As an essential gene for DNA replication, FEN-1 defects resulting in a complete loss of function are
unlikely to exist in the human population (21, 22). A comprehensive structural and functional analysis of single
amino acid residue mutations will assist in the formulation of models for biological and pathological roles of
this enzyme. Initial domain analysis based on protein sequence comparison and biochemical assays displayed
two major conserved motifs, N (N-terminal) and I (Intermediate) motifs, which are essential for the nuclease
activities of FEN-1 proteins (4, 23). A third motif at the C-terminal end is involved in the interaction of FEN-1
proteins with PCNA (23-25). Even though we have recently demonstrated that stimulation of eukaryotic FEN-1
activities by PCNA is independent of its in vitro interaction via a consensus PCNA binding region (25), the interaction may be crucial for PCNA to recruit FEN-1 onto Okazaki fragment processing and DNA damage sites (24, 26-28). An additional C-terminal motif exists in eukaryotic cells, which is responsible for the localization of FEN-1 protein into the nucleus (15). In recent years, we have performed site-directed mutagenesis on human FEN-1 nuclease coupled with biochemical and yeast genetic analysis. A number of amino acid residues, which are critical for biochemical and biological functions, have been revealed through this ongoing endeavor. The structural and functional relationship of these identified amino acid residues is summarized in Table 1.

Even though relatively abundant information on the structural and functional relationship of some FEN-1 proteins is available, identification of structural elements for DNA substrate binding has been a difficult task without a 3-D structure of the protein/DNA complex. In order to identify amino acid residues directly involved in substrate binding, we constructed a 3-D molecular model for human FEN-1 based on the available homologous crystal structures (29-33). We have identified 10 amino acid residues including R29, R47, R70, R73, K80, K93, K99, R100, R103 and R104 as candidate residues that directly interact with DNA substrates. These residues are conserved in eukaryotes and are located on the surface of the molecule. We have performed site-directed mutagenesis on these residues. Indeed, individual mutations of many of these residues affect biochemical activities. The first five residues are located in the N-terminal α-helix and β-sheet region near the active center of the enzyme while the other five residues are exclusively in the loop region, which has been proposed to interact with the single-stranded flap of the DNA substrate (32, 33). In this report, we focused on the first five residues (R29, R47, R70, R73, and K80) and determined that R47 and R70 are important for the interaction with the DNA substrates. R70 might interact with the upstream double-stranded region of the DNA substrates; and R47, possibly interacting with the upstream template region of DNA substrate, was revealed to play a role in determining cleavage sites via mutational analysis, biochemical assays, protonation and competition experiments.
Experimental Procedures

Site-directed mutagenesis, overexpression, and purification of FEN-1s. All mutant proteins created for this study were prepared using the QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). Mutagenic primers were synthesized at the City of Hope DNA/RNA/peptide synthesis core facility. Mutations and corresponding oligo sequences for primers are listed in Table 2 for clarity. Site-directed mutagenesis, overexpression, and purification of the wild type and mutant FEN-1 and Rad27 proteins were carried out based on our previously published procedures (15, 34-36). Mutagenesis reactions contain 50 ng of template pET28-derived plasmids harboring the wild type human FEN-1 sequence (37) so that the isolated plasmids containing a mutation can be directly expressed in E. coli.

DNA substrate preparation and FEN-1 nuclease activity assays. The oligos used to formulate nuclease substrates are also listed in Table 2. Protocols for DNA substrate preparation and nuclease activity assays were based on the previous publication (34). Briefly, Flap-G1 (labeled oligo of the flap substrate), Flap-ND [labeled oligo for single flap substrate (SFS) and double flap substrate (DFS)], or Exo-3PT (labeled oligo of exonuclease substrate) were individually phosphorylated at the 5’ end. This was done by incubating 40 pmol of the oligo with 10 μCi of (γ-32P) ATP and 1 μL (10 U/μL) of polynucleotide kinase (PNK) at 37 °C for 60 min. PNK was then inactivated by heating at 72 °C for 10 min. 80 pmol each of the Temp-3B and Prim-1G oligos were added to the labeled oligos, respectively. The samples were incubated at 70 °C for 5 min and then followed by slow cooling to 25 °C, allowing the oligos to slowly anneal and form the flap endonuclease and exonuclease substrates as shown in Fig. 2B and 2C. Substrates were precipitated at −20 °C overnight after adding 20 μL of 3M NaOAc and 1 mL of 100% ethanol. Substrates were collected by centrifugation and washed once with 70% ethanol and resuspended in sterile water.

Reactions were carried out with the amount of hFEN-1 protein as indicated and 500 fmol of flap or exonuclease substrate with reaction buffer containing 50 mM Tris (pH 8.0) 10 mM MgCl₂ and 100 μg/ml BSA.
Each reaction was then brought to a total volume of 13 µl with water. All reactions were incubated at 30 °C for 15 minutes and terminated by adding an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). An aliquot of each reaction was then run on a 12% denaturing PAGE at 1900 V for 1 hour. The gel was dried at 70°C for 50 minutes, and then visualized by autoradiography.

**Kinetic analysis.** FEN-1 cleavage kinetics were performed employing various concentrations of DNA substrates (31.25 to 500 nM) and constant amounts of FEN-1s (92 nM) following the procedures described in Hosfield, et al. (35). Briefly, reactions were initiated by combining standard reaction buffer, substrate, and enzyme in the order. Samples were mixed and incubated for 10 min. The products and substrate were separated by a denaturing gel electrophoresis. The initial velocity was calculated by measuring products and substrate intensity on the gel by using IP Lab Gel program and by using the equation \( \nu = \frac{I_1}{(I_0 + 0.5I_1)t} \times [\text{substrate}] \), where \( t = \) time in seconds, \( I_1 = \) product intensity, \( I_0 = \) final substrate concentration. The substrate concentration was expressed in nM. \( V_{\text{max}} \) and \( K_m \) values were calculated by directly fitting the data to Michaelis-Menten equation and \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) were then derived.

**Nuclease activity competition assay.** The experiment was based on a constant amount of wild type protein with various concentrations of each mutant protein. Three mutants were employed for this analysis, including D181A, R47A/D181A and R70A/D181A. A plasmid overexpressing D181A mutant gene (D181A/pET) was constructed previously (38). Based on this plasmid, two other mutants, R47A/D181A and R70A/D181A, were constructed, overexpressed, and purified. The competition assay was conducted with standard reaction buffer and \(^{32}\)P labeled substrates as described above. The amount of wild type hFEN-1 and mutant proteins were specified the Fig. 3 legends. After 15 min incubation at 30 °C, the reaction was stopped with stop solution. The products and substrate were separated by a 10% denaturing PAGE electrophoresis to assess the substrate binding capacity of the mutants.
Direct enzyme/substrate-binding analysis. This assay was conducted using immobilized substrates on beads and based on a method modified from Gomes and Burgers (39). Briefly, 1 nM Temp-1G with biotin at 5’ end (5’Biotin-GGACTCTGCTCAAGACGGTAGTCAACGTG3’) (IDT Inc., Coralville, IA) was annealed to 1.5 nM Prim-3B (5’CACGTTGACTACCGTC3’) and 1.5 nM Flap-G1 (5’GATGTCAAGCAGTCTCTTTAGGAGAGTCC3’) for flap substrate or Exo-3PT (5’TTGAGGCAGAGTCC3’) for exo-substrate. The annealed flap endo- or exo- nuclease substrate was immobilized to the NeutrAvidin™ beads in buffer A (10 mM Tis-HCl pH 7.5, 1mM EDTA and 1 M NaCl) by incubation for 2–3 hours at room temperature. The unbound substrate was washed off with buffer A. The bead-bound substrate was resuspended in buffer C [30 mM HEPES-NaOH pH 7.5, 125 mM NaCl, 0.2 mg/ml BSA and 1mM dithiothreitol (DTT)]. The binding assay was performed on ice for 20 min in a 16 µl reaction in buffer C with addition of 8 µl substrate-bound beads and the amount of protein as indicated in the Fig. 4 legends. The beads were then washed twice with pre-chilled buffer B (30 mM HEPES-NaOH pH 7.5, 5% glycerol, 0.1 mg/ml BSA, 1mM DTT, 0.01% NP-40, 0.1 mM EDTA, and 125 mM NaCl). 12 µl of 2X protein sample loading buffer was then added. The reaction mixture was boiled for 5 min to release the FEN-1 protein from beads. After 2 min spinning at 14,000 rpm, the supernatant was loaded onto the 10 % SDS-polyacrylamide gel. The proteins were blotted onto a nitrocellulose membrane (Scheilcher & Schuell, Keene, NH) using a Trans-Blot SD semi-dry electrophoretic transfer cell from BioRad (Hercules, California, USA). The blotting was probed with polyclonal antibodies raised in rabbits against hFEN-1 (36). A specific FEN-1 protein was detected using a PIERCE (Rockford, Illinois) superSignal West Pico chemiluminescence kit and a protocol recommended by the manufacturer.
Results

Mutations of R47 or R70 affect hFEN-1 nuclease activities. Since a 3 dimensional crystal structure of human FEN-1 has not been solved, we constructed a molecular model of hFEN-1 using the archaebacterial structures. Based on that model of hFEN-1 (Fig. 1), five residues including R29, R47, R70, R73, and R80 were visualized on the surface of the molecule near the catalytic center. To experimentally test the roles of these amino acid residues in substrate binding and nuclease activity, we created the following non-conservative amino acid substitution mutants: R29A, R47A, R70A, R73A, R80A, R47A/R70A, and R70A/R73A. The mutant proteins were overexpressed and purified to photographic homogeneity as shown in Fig. 2A. These proteins were then assayed for their FEN-1 activities. Fig. 2B and C show that only R47A or R70A among the five single mutants had a significant effect on FEN-1 enzyme activities. Subsequently, R47A, R70A, R73A (as a control since it is so close to R70), R47A/R70A, and R70A/R73A were included for further investigations. The R47A and R70A mutants had a more severe impact on exonuclease rather than on flap endonuclease activity. R70A significantly reduced exonuclease activity while retaining most of its flap endonuclease activity (Table 3). In contrast, the R47A mutation maintains both of activities, but shifted the endonuclease cleavage-site from nucleotide position 19 (Pos19) to 21 (Pos21) on the flap strand and the exonuclease cleavage-site from Pos1, 2, 3 to Pos2 and 3. Double mutants R47A/R70A and R70A/R73A failed to show a synergistic effect on nuclease activities. In fact, the combined mutational effect of R47A and R70A as well as R70A and R73A was additive (Fig. 2B and C).

R47A and R70A have $K_m$ values greater than wild type hFEN-1 protein. To further explore the roles of R47 and R70 in substrate DNA binding, we performed kinetic analysis of the wild type and mutant hFEN-1 enzymes. Conventional steady-state kinetic analyses were carried out employing various concentrations of radioactively labeled substrates, a constant amount of enzyme, and gel electrophoresis. We then measured cleavage activity with both the flap endo- and exo- nuclease DNA substrates. The results were analyzed using Michaelis-Menten kinetics to derive $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values as listed in Table 3. While the $k_{cat}$ values for
flap endonuclease activity were largely not affected, $K_m$ for the R70A, R47AR70A, R70AR73A mutants are doubled. The second order rate constants ($k_{cat}/K_m$) for these three mutants were significantly reduced with the endonuclease activity for 3 folds, suggesting that residue R70 is critical for DNA substrate binding. Interestingly, the R47A/R70A double mutant again revealed an additive effect on kinetic parameters. The effects of these mutations to the exonuclease activity were so severe that their kinetic parameters were not measurable any more. The $K_m$ of the R47A exonuclease activity was slightly increased while the $k_{cat}$ and second order rate constant are both decreased.

*Competition assays indicated that R47A and R70A significantly reduced substrate-binding ability.* Since R47A and R70A of hFEN-1 retained partial flap endonuclease activity, they could not be directly used for the competition assays. However, our previous studies indicated that the D181A hFEN-1 mutant had a wild type or even enhanced binding capability but lost cleavage activity, it could compete for substrates with the wild type enzyme and suppress wild type enzyme activity (38). The competition assay utilized in our current study has been established based on these observations. Knowing biochemical characteristics of the D181A mutant, we created two double mutants D181A/R47A and D181/R70A to confirm the roles of the R47 and R70 residues in DNA binding. Null nuclease activities of these mutants were verified before they were used in competition assays. Circular dichroism spectra of the wild type, single mutants and double mutants were taken and compared to confirm that no significant structural alterations took place due to these mutations (data not shown). Then, if R47 and R70 are involved in DNA binding, the double-mutant proteins, R47A/D181A and R70A/D181A, should demonstrate reduced DNA substrate binding ability in contrast to the D181A mutant. The competition assays were performed based on a constant amount of wild type hFEN-1 protein and increasing amount of mutant proteins (D181A, R47A/D181A or R70A/D181A). The result indicates that the double-mutants did in fact have a significantly reduced ability to suppress the enzyme activity of the wild type hFEN-1 protein (Fig. 3A and B). R47A/D181A has even more severe effects on substrate binding.
Direct DNA-protein binding assays confirm the critical role of R47 and R70 for DNA binding of hFEN-1. To further examine the roles of R47 and R70 in substrate binding, we performed DNA-protein binding assays. This assay was conducted employing biotinylated DNA substrates immobilized onto avidin-coated beads. After FEN-1 proteins were loaded onto the beads with the DNA substrates, the remaining unbound proteins were removed by extensively washing the beads. Bound protein was then dissociated through boiling and subsequently detected by Western blotting using anti-hFEN-1 antibody. The result of this experiment is shown in Fig. 4A and B with the flap DNA and exonuclease substrates. It is clear that R47A and R70A mutations produced significantly lower binding affinity to both of the substrates.

Changes of cleavage intensities and patterns of FEN-1 proteins with different DNA substrates. The above data allow us to propose that the two amino acids, R47 and R70, are involved in DNA binding of FEN-1 protein. One further question is which parts of the DNA substrate interact with these two amino acids. We designed an experiment using five different substrates. Some of the substrates lack a part of the configuration of the normal double flap substrate. The names and different structural elements of the substrates are indicated in Fig. 5. Analysis of various responses of the wild type protein and R47A and R70A mutants to the different substrates was expected to delineate the specific site of the normal substrate that interacts with these two residues. Fig. 5A shows the cleavage results of three FEN-1 proteins based on normal flap, pseudo Y and 5' overhanging substrates. The result with the normal flap substrate is consistent with those described for Fig. 1B. That is, R70A mutant has a reduction of enzyme cleavage capability, but has no change of cleavage sites in comparison to the wild type; however, mutation R47A changed both cleavage efficiency and sites (a shift from 19nt to 21nt and larger products). With pseudo Y substrate missing the upstream primer, the enzyme activities of the wild type and the R47A, R70A mutants were reduced, particularly at the 21nt cleavage site. With a 5’ overhanging substrate, the enzyme activities of the three FEN-1 proteins were all further reduced and the cleavage products were shifted up to 21, 22 and 23nt without any 19nt product. The wild type had new cutting sites on the single-stranded flap. R70A seemed to have an equivalent or more enzyme activity compared to the wild type when the
products of the wilt type enzyme were added up; whereas R47A had a weaker enzyme activity than that of R70A or wild type FEN-1.

In addition to the above findings, we further tested if R47 and R70 could be responsible for the interaction with the 3’ end overlapping nucleotide of the upstream primer in the double flap substrate. It has recently been reported that FEN-1 enzyme activity can be significantly stimulated by the addition of an extra nucleotide (cytosine) at the 3’ end of the upstream primer of the normal flap substrate (40). We expect to see the loss or significant reduction of the activity stimulation in the two mutants, R70A and R47A, if the two amino acids are important for the interaction with the 3’ overlapping nucleotide of the upstream primer of the double flap substrate. Obviously, this study gave an opposite result (Fig. 5B). Taken together, we may propose that R47 and R70 residues interact with the upstream duplex of the flap substrate.

Protonation of R47 or R70 resulted in a single cleavage site at Pos21 resembling R47A mutation. The endonucleolytic activity of hFEN-1 produces two major products. The cleavage sites of these two products are at nucleotide 21 (Pos21) and 19 (Pos19), counting from the 5’ end of the flap DNA strand. When the positively charged amino acid residue R47 was replaced by a neutral alanine, the cleavage site preferentially shifts from Pos19 to Pos21. We show here that the cleavage sites of hFEN-1 proteins were altered with the change of pH value for both the wild type and mutants except R47A (Fig. 6). Interestingly, under basic conditions beyond pH 7.2, the Pos19 product appeared more than the Pos21, whereas the Pos21 accumulated when pH is more acidic. This phenomenon indicates that protonation of R47 may shift the area or position of substrate/protein interactions, resulting in a single cleavage site as seen in the R47A mutant (Fig. 6A). Similarly, the cleavage sites in the exo- substrate has shifted to Pos2 and Pos3 when the proteins were protonated (Fig. 6B).

Mutations of R47 and R70 produce an enzyme that is less tolerant of Mg++ competition with substrate binding. A high concentration of metal ions interferes with enzyme activity. To test if decreased activity is due to
competition between the metal ions and positively charged amino acid residues with DNA substrate, we assayed the Mg$^{2+}$ dependence of the five mutants indicated previously. Our results show that the mutants have slightly lower maximal activities at optimal Mg$^{2+}$ concentration for both flap endonuclease and exonuclease activities (Fig. 7 and the data not shown). Moreover, the metal concentration ranges for the activities have significantly reduced toward the lower [Mg$^{2+}$] end in R47 A, R70A mutants, and their derivatives, compared to the wild type enzyme. However, R73A had a very similar profile as the wild type enzyme. This result may indicate that mutations of substrate binding residues R47 and R70 lead to decreased tolerance of Mg$^{2+}$ competition with substrate binding.
Discussion

To explain the structure-specific nature of FEN-1, Bambara and his colleagues have proposed a thread-through model based on their elegant biochemical data from mammalian enzymes (41, 42). They hypothesized that there exists a hole in the FEN-1 protein that is large enough for single stranded DNA to thread through but not for double-stranded DNA. Circumstantial evidence supporting this hypothesis has emerged. The structure of a viral homologue of FEN-1, T5 exonuclease, has revealed an arch structure formed by an N-terminal helix and a helical region located between the two conserved nuclease motifs. Several positively charged and bulky amino acid residues exist on the inner side of this archway, which may be involved in interactions with ssDNA substrate (31). Recently, Xu et al. (43) experimentally tested the DNA binding role of the corresponding residues in the 5' nuclease of Escherichia coli DNA polymerase I. They found that two highly conserved basic residues, K78 and R81, reside close to the phosphodiester bond immediately 5' to the cleavage site, while the third highly conserved residue, R20, may interact with the phosphodiester 3' to the cleavage site. Another lysine residue (K83) has been identified in T5 5'-3' exonuclease as a determinant for cleavage sites in the flap DNA substrate (44). Unfortunately, these residues are not conserved in eukaryotic homologues. Therefore, information revealed from experimentation with the prokaryotic homologues may not be directly applicable to the eukaryotic enzymes. The crystal structures of two archae FEN-1 proteins have indicated the presence of an unusual helical arch motif, which forms a hole in the FEN-1 protein (32, 33). It was proposed that the active-site groove, the arch region, and the metal ion environments constrain possible duplex and flap strand substrate positions and support induced conformational changes of FEN-1 proteins upon DNA binding and catalysis (32).

In addition, two structural motifs possibly mediate binding of the double-stranded and single-stranded regions of the flap DNA substrate. They include the H3TH motif which forms a platform that links nonspecific dsDNA to the active site and the helical clamp motif which enables passage of the 5’ flap strand and caps the active site.
to facilitate phosphodiester bond cleavage (32). While the importance of these structural elements needs to be tested, we found that two arginine residues from other structural elements are also critical for binding DNA substrates in this report.

Arginine 47 and 70 appear to be involved in substrate binding via a variety of experiments in this report. The nuclease assays indicated that the R70A mutation more severely impairs flap endo- and exo-nuclease activities as compared to R47A. Direct binding assays and kinetic analysis further verified this observation. R47 is a major determinant of the cleavage sites. However, when the R47A or R70A mutation was combined with D181A mutation and the double mutants were employed to carry out the suppression assay with the wild type enzyme, the R47A/D181A mutant was not able to suppress wild type enzyme activity as efficiently (Fig. 3). This result indicates that R47A/D181A has more severely impaired binding ability to both flap and nicked double stranded DNA substrates while its conformation is largely uncharged as verified by CD spectra. The impaired binding is probably due to a synergistic effect of the R47A and D181A mutations on the single protein.

It is reasonable to hypothesize that there exist various structural elements of the FEN-1 molecule that interact with different portions of the flap DNA substrate. The enzymatic activities respond to heterogenous substrate configurations, ranging from the double flap (Fig. 5B), 5’ single flap, pseudo Y (missing the upstream primer), 5’ overhang (Fig. 5A), nicked double stranded duplex (Fig. 2B) to a simple duplex configuration (34). The results might indicate that R70 of human FEN-1 protein is mainly involved in the interaction with upstream primer. We have quantitatively compared product intensities of the wild type and R70A enzymes with normal flap and pseudo Y substrates and found that the overall enzyme activity reduction between wild type and R70A proteins is similar. There is no further activity reduction due to additional loss of the upstream substrate configurations (the loss of primer). Mutation of R47 leads to reduction of cleavage efficiency at 19nt site. This is probably due to the interaction between R47 and the upstream template strand.
Loss of the upstream primer did not affect the cleavage efficiency at 19nt of the R47A while loss of the whole upstream duplex completely abolished production of 19nt with all three FEN-1 proteins (Fig. 5A). This is consistent with our other observation that protonation of R47, mimicking the alanine substitution for R47, also shifted the cleavage sites from Pos19 and 21 to the single site of Pos21 based on flap substrate and from Pos 1, 2 and 3 to Pos 2 and 3 based on exo-substrate. A shortened single-stranded flap (6 nucleotides, Fig. 5B) also resulted in a single cleavage site, possibly due to restriction of enzyme movement on the flap substrate.

Although these results allowed us to believe that R47 and R70 could be involved in the interaction with upstream duplex of the normal flap substrate, it did not exclude a possibility that the residues also interact with the single-stranded flap strand. As revealed previously, the mutation of R70 or R47 into alanine has a significant effect on the exonuclease activity of FEN-1 with a substrate missing the flap strand. Particularly, the R70A mutation led to the loss of exonuclease activity (Fig. 2, Table 3). This result indicates that R47 and R70 are not involved in the interaction with the flap strand.

The FEN-1 enzyme activity requires divalent metal ions such as Mg$^{2+}$ and Mn$^{2+}$. These metals are critical for the stimulation of FEN-1 protein by stabilizing protein structure and participating in the catalysis reaction. On the other hand, higher concentrations of the metal may affect DNA-protein interaction or complex stability. Certainly, this hypothesis requires further investigation. Nevertheless, it may explain why nucleases have an optimal metal concentration for their activities, beyond which enzyme activity is inhibited. For the enzyme activity of wild type hFEN-1, the optimal Mg$^{2+}$ concentration was shown to be around 5 mM. If a high concentration of Mg$^{2+}$ disrupts protein-DNA interactions as we hypothesized, we would expect that neutralization of the positively charged amino acid residues involved in protein-DNA interaction could shift the optimal Mg$^{2+}$ concentration to a lower position. To test this hypothesis, we assayed the Mg$^{2+}$ dependence of the five mutants. Our results show that the Mg$^{2+}$ concentration range for the nuclease activities shifted to the lower
side for all the mutants except for R73A (Fig. 7), indicating that mutations of R47 and R70 decrease the enzyme’s tolerance to Mg$^{++}$ competition with substrate binding.

In summary, this report represents an attempt to identify eukaryotic FEN-1 residues involved in substrate binding. The interactions of the residues R47 and R70 have mainly or partly been mapped to the upstream double stranded portion of a flap or a nicked duplex DNA substrate. Protonation of the residues and high concentrations of metal ions interferes with such key protein-substrate interactions. Further work will be needed to achieve a more comprehensive understanding of the structural and functional relationship of this unique family of structure-specific nucleases, particularly a more completed definition of key structural elements for substrate binding.
Acknowledgement

Authors thank Li Zheng and other members in the Shen’s laboratory for their technical assistance and stimulating discussions during the course of this study and Tim O’Connor for his critical comments on the manuscript. We are grateful for the opportunity to collaborate with J. A. Tainer laboratory at the Scripps Research Institute in solving the crystal structure of *Pyrococcus furiosus* FEN-1 nuclease, which was used as a template for molecular modeling in this study (CA81967). This work was supported by NIH grant CA73764 to B.H.S.
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Figure Legends

Fig. 1. Three dimensional view of the human FEN-1 amino acid residues, R47, R70 and R73. The human FEN-1 structure was modeled based on the crystal structures of *Methanococcus jannaschii* and *Pyrococcus furiosus* FEN-1 proteins. The coordinates of these two proteins were downloaded into the molecular modeling program Insight II. The sequence of the representative FEN-1 proteins was then aligned with the archaeal proteins, respectively, and structural information was used to modify the final alignment of all the FEN-1 sequences. The residues emphasized in this study are indicated in red.

Fig. 2. Enzyme activities of FEN-1 mutant proteins. Site-directed mutagenesis, protein overexpression and purification, DNA substrate preparation and FEN-1 nuclease assays were described in Experimental Procedures section. Reactions were carried out with FEN-1 protein and 44.4 nM flap endo- or exo- nuclease substrate in a 15 µl reaction volume containing 50 mM Tris (pH 8.0) and 10 mM MgCl₂. 34.9, 69.8, 139.5, or 279 nM of hFEN-1 enzyme was used in each reaction. All reactions were incubated at 30 °C for 15 min and terminated by adding an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). An aliquot of each reaction was then run on a 12% denaturing PAGE and the reaction products were visualized by autoradiography. A. Purified FEN-1 proteins. Lane 1), molecular weight marks; 2) wild type FEN-1 protein; 3) R47A; 4) R70A; 5) R73A; 6) R47A/R70A; and 7) R70A/R73A. B. Flap endonuclease activities of FEN-1 proteins. C. Exonuclease activities of FEN-1 proteins.

Fig. 3. The suppression of wild type FEN-1 nuclease activities by the mutant D181A was significantly reduced by additional mutations at R47 or R70. The reaction was performed based on 93 nM of wild-type FEN-1 and various concentrations of mutant protein as indicated. To start the assay, the wild-type protein was mixed with a mutant first, and then the mixture was added to reaction buffer with flap endo- or exo- nuclease substrate. A. Competition based on flap DNA substrate. B. Competition based on exo- substrate.
Fig. 4. Substrate binding assay of hFEN-1 proteins. The assay was described in the Experimental Procedures section. 97.1, 194.2, 388.4, or 680.2 nM of wild type and mutant proteins was added to a 20 μl reaction volume. Panel A shows the result of binding assay based on a flap DNA substrate and panel B is the assay results based on an exo- substrate. For both panels A and B, the top shows Western blotting results while the bottom shows the quantification of the bound proteins.

Fig. 5. Enzymatic activities of FEN-1 with different DNA substrates. The reaction condition is the same as that described in Fig. 2. 69.8, 139.5, or 279 nM of hFEN-1 protein was added to each reaction. A. Enzymatic activities of FEN-1 proteins with a flap, a pseudo Y, and a 5’ overhang substrate. B. Enzymatic activities of FEN-1 proteins with a single flap substrate (SFS) or a double flap substrate (DFS).

Fig. 6. pH effects on enzymatic activities and cleavage sites of hFEN-1 proteins. Reactions were carried out with 139.5 nM FEN-1 protein and 44.4 nM flap- or exo- DNA substrate in a 15 μl reaction volume containing 50 mM Tris and 10 mM MgCl₂ with different pH values. The pH value of the Tris buffer was adjusted in a 5x stock solution (250 mM Tris and 50 mM MgCl₂) to be 5, 6.3, 7.2, 8, 8.8, 9.6, and 10.4, respectively. The reaction products were visualized by auto-radiography and the intensity of main products was measured using IP Lab Gel program. Pos1, Pos2, Pos19 and Pos21 represent the cleavage products with length of 1, 2, 19 and 21 nucleotides, respectively. Except for R47A and R47A/R70A, all other proteins have a cleavage site shift on flap DNA substrate (A) and exo- DNA substrate (B) with the change of pH values.

Fig. 7. Mg²⁺ dependence of endonuclease and exonuclease activities of hFEN-1 proteins. Reactions were carried out with 139.5 nM FEN-1 protein and 44.4 nM of flap- or exo- DNA substrate in a 15 μl reaction volume containing 50 mM Tris pH 8.0 and different concentrations of MgCl₂. The final concentrations of MgCl₂ in the reactions were 0.1, 0.3, 1, 3, 6, 10, 15, 20, 25, and 30 mM, respectively. After reaction, products
were visualized by auto-radiography and the intensity of products was measured using IP Lab Gel program, the percentage of products was calculated based on the ratio of product intensity over the sum of intensities of product and substrate left-over.
Table 1. Critical amino acid residues identified in human FEN-1 via site-directed mutagenesis and biochemical analysis

| Designated regions | Biochemical functions      | Residues                  | References |
|--------------------|----------------------------|---------------------------|------------|
| N and I            | Catalytic center           | D34, D86, E158, E160, E178, D179, D 181, D233 | 34, 38     |
|                    | Substrate binding          | R47, R70                  | This study |
| III                | PCNA interaction           | L340, D341, F343, F344    | 25         |
| IV                 | Nuclear localization       | K354, R355, K356, K365, K366, K367 | 15         |
Table 2. DNA oligos for construction of DNA substrates and creation of FEN-1 mutants

| Oligo name | Oligo sequence | Used to construct |
|------------|----------------|-------------------|
| Flap-G1    | 5’GATGTCAGCAGTCTAACTTTGAGGAGTCACTCC3’ | Flap/pseudo-Y/5’ overhanging substrate |
| Temp-1G    | 5’GGACTCTGCCTCAAGACGTTAGTCAACGTG3’ | Flap/pseudo-Y/exo-substrate |
| Prim-3B    | 5’CACGGTCTACCCGTC3’ | Flap/exo-substrate |
| Exo-3PT    | 5’TGTAGGAGAGAGTCC3’ | Exo-substrate |
| 5’ overhanging   | 5’GGACTCTGCCTCAAGACGTTAGTCAACGTG3’ | Flap/exo-substrate |
| Prim-3B    | 5’CACGGTCTACCCGTC3’ | Flap/exo-substrate |
| Exo-3PT    | 5’TGTAGGAGAGAGTCC3’ | Exo-substrate |
| Prim-N     | 5’ACGAGCAGCTGTC3’ | Single flap substrate (SFS) |
| Prim-D     | 5’ACGAGCAGCTGTC3’ | Double flap substrate (DFS) |
| Flap-ND    | 5’AAAAAACACGCTGTCAGCTC3’ | SFS/DFS |
| Temp-ND    | 5’ACAAGACGAGCAGCAGCTC3’ | SFS/DFS |

R29A-F  5’GAGCTACTTTTACACGTCAAAAGTGGCCATTG3’  R29A
R29A-R  5’CAATGGCCACATTAGCGCCCAAGGTAGCTC3’  R29A
R47A-F  5’CCTGATTGCTGTGCCCAGGGTGGGATG3’  R47A
R47A-R  5’CATCCACCCCACCTGGGGCAACAGCAATCAGG3’  R47A
R70A-F  5’GGGCATGTTTCTACCGCAGCATGTCAT3’  R70A
R70A-R  5’CATGCGAATGCTGCGTACGTGGAACATGCCC3’  R70A
R73A-F  5’CTACCGCAACATTACGCATGATGATGATAACGGC3’  R73A
R73A-R  5’GCCGTTATGCACTCAGCGCCATCAGCAGCTC3’  R73A
K80A-F  5’GGATACGGGCATCAGGCCCTGATGTATGCTTTTG3’  K80A
K80A-R  5’CAAGACCATACACCGGAGCGAGCCGATGCCTATTCC3’  K80A
R70R73AA-F  5’CTACGGCAGCATGCGATGATGATAACGGC3’  R70A/R73A
R70R73AA-R  5’GGATACGGGCATCAGCGCCCTGATGTATGCTTTTG3’  R70A/R73A
Table 3. Kinetic parameters of wild type and mutant FEN-1 proteins

| Proteins         | Enzyme activity | \( K_m \) (nM) | \( k_{cat} \) (1/min) \( \times 10^{-2} \) | \( \frac{k_{cat}}{K_m} \) (1/nM•min) \( \times 10^{-4} \) |
|------------------|-----------------|----------------|---------------------------------------------|-----------------------------------------------------|
| wtFEN-1          |                 | 45.2           | 3.5                                         | 7.7                                                 |
| R47A             |                 | 76.3           | 3.1                                         | 4.1                                                 |
| R70A             |                 | 101.2          | 2.6                                         | 2.6                                                 |
| R73A             |                 | 47.5           | 3.6                                         | 7.6                                                 |
| R47A/R70A        |                 | 111.8          | 2.5                                         | 2.2                                                 |
| R70A/R73A        |                 | 106.7          | 2.7                                         | 2.5                                                 |
| wtFEN-1          | Endo-           | 56.2           | 2.5                                         | 4.4                                                 |
| R47A             |                 | 74.7           | 1.6                                         | 2.1                                                 |
| R70A             |                 | nd*            | nd                                          | nd                                                  |
| R73A             |                 | 55.6           | 2.6                                         | 4.7                                                 |
| R47A/R70A        |                 | nd             | nd                                          | nd                                                  |
| R70A/R73A        |                 | nd             | nd                                          | nd                                                  |

*nd: The relevant parameters were unmeasurable due to the impaired enzyme activity.
A.

Flap endonuclease activity (\%)

\[ \text{[FEN-1 mutants] (\mu M)} \]

D181A
D181A/R47A
D181A/R70A

B.

Exonuclease activity (\%)

\[ \text{[FEN-1 mutants] (\mu M)} \]
A.

\[
\begin{array}{cccccccc}
100 & 75 & 50 & 37 & kDa & 1 & 2 & 3 & 4 & 5 & 6 & 7 \\
\end{array}
\]

B.

\[
A 3' GTGCAACTGATGGCAGAACTCCGTCTCAGG \\
TGTAGGCAAGGTCC \\
\]

C.

\[
\begin{array}{cccccccc}
16nt \quad 3nt \quad 2nt \quad 1nt \\
\end{array}
\]

\[
5' TGTAGGCAAGGTCC \\
3' GTGCAACTGATGGCAGAACTCCGTCTCAGG \\
5' CACGTTGACTACCGTC \\
\]

\[
\begin{array}{cccccccc}
C & wt & R47A & R70A & R73A & R47A/R70A & R70A/R73A \\
\end{array}
\]

\[
\begin{array}{cccccccc}
34nt \quad 21nt \quad 19nt \\
\end{array}
\]
### A.

| Substrate Protein | Normal flap | Pseudo Y flap | 5' overhanging flap |
|-------------------|-------------|---------------|---------------------|
| None              | wt          | None          | wt                  |
| None              | R70A        | None          | wt                  |
| None              | R47A        | None          | wt                  |

**Flap strand**
- Normal flap: 5'...GATGTCAAGCAGTCCTAAGT...
- Pseudo Y flap: 5'...GATGTCAAGCAGTCCTAAGT...
- 5' overhanging flap: 5'...GATGTCAAGCAGTCCTAAGT...

**Template**
- Normal flap: 3' GTGCAACTGATGGGAGAGACCTTCCTCAGG
- Pseudo Y flap: 3' GTGACACTGATGGGAGAGACCTTCCTCAGG
- 5' overhanging flap: 3' GTGACACTGATGGGAGAGACCTTCCTCAGG

**Upstream primer**
- Normal flap: 5'CACGTTGACTACCGTC
- Pseudo Y flap: 5'CACGTTGACTACCGTC
- 5' overhanging flap: 5'CACGTTGACTACCGTC

**Downstream duplex**
- Normal flap: 3' GTGCAACTGATGGGAGAGACCTTCCTCAGG
- Pseudo Y flap: 3' GTGCAACTGATGGGAGAGACCTTCCTCAGG
- 5' overhanging flap: 3' GTGCAACTGATGGGAGAGACCTTCCTCAGG

**Protein**
- Normal flap: wt, wt, wt
- Pseudo Y flap: wt, wt, wt
- 5' overhanging flap: wt, wt, wt

**Flap Strand Lengths**
- Normal flap: 34nt, 21nt, 19nt
- Pseudo Y flap: 34nt, 21nt, 19nt
- 5' overhanging flap: 34nt, 21nt, 19nt

**Flap Strand Numbers**
- Normal flap: 19nt, 21nt
- Pseudo Y flap: 19nt
- 5' overhanging flap: 19nt
B.

### Normal flap (NF)

- Protein: None
- Substrate: NF
- 21nt
- 10nt
- 9nt

### Double flap (DF)

- Protein: None
- Substrate: DF
- 21nt
- 10nt
- 9nt

**Table:**

| Protein | None | None | wt | R70A | R47A |
|---------|------|------|----|------|------|
| Substrate | NF | DF | NF | DF | NF | DF |

**Sequences:**

- Normal flap (NF): 5' CGCTGTCTCGCT 3'<br>3' CGTTCGCAGACAGCGACAGAGCGA<br>5' ACGAGCGTCTGT
- Double flap (DF): 5' AAAAAAAAAACGCTGTCTCGCT 3'<br>3' CGTTCGCAGACAGCGACAGAGCGA<br>5' ACGAGCGTCTGT
Arginine residues 47 and 70 of human flap endonuclease-1 are involved in DNA substrate interactions and cleavage site determination
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J. Biol. Chem. published online May 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111941200

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