Interaction between human flap endonuclease-1 (hFEN-1) and proliferating cell nuclear antigen (PCNA) represents a good model for interactions between multiple functional proteins involved in DNA metabolic pathways. A region of 9 conserved amino acid residues (residues Gln-337 through Lys-345) in the C terminus of human FEN-1 (hFEN-1) was shown to be responsible for the interaction with PCNA. Our current study indicates that 4 amino acid residues in hFEN-1 (Leu-340, Asp-341, Phe-343, and Phe-344) are critical for human PCNA (hPCNA) interaction. A conserved PCNA interaction motif in various proteins from assorted species has been defined as Q1(L/I)4X(L/I)4X(F/Y)10, although our results fail to implicate Q1 (Gln-337 in hFEN-1) as a crucial residue. Surprisingly, all hFEN-1 mutants, including L340A, D341A, F343A, and F344A, retained hPCNA-mediated stimulation of both exo- and flap endonuclease activities. Furthermore, our in vitro assay showed that hPCNA failed to bind to the scRad27 (yeast homolog of FEN-1) nuclease. However, its nuclease activities were significantly enhanced in the presence of hPCNA. Four additional Saccharomyces cerevisiae scRad27 mutants, including multiple alanine mutants and a deletion mutant of the entire PCNA binding region, were constructed to confirm this result. All of these mutants retained PCNA-driven nuclease activity stimulation. We therefore conclude that stimulation of eukaryotic hFEN-1 nuclease activities by PCNA is independent of its in vitro interaction via the PCNA binding region.

DNA replication and repair are critical for maintaining genome stability. These processes are in part dependent on the activities of an emerging family of structure-specific endonucleases. These enzymes, typified by eukaryotic flap endonuclease-1 (FEN-1), are substrate structure-specific and multifunctional (1–3). They possess both flap-specific endonuclease and nick-specific exonuclease (ribonuclease) activities and interact with proliferating cell nuclear antigen (PCNA) as well as other proteins (3–5). This unique substrate-specific nature is based on structural elements that have been observed in the three-dimensional structures of prokaryotic FEN-1 homologues (6–10). The N-terminal region and the region located in between the two conserved nuclease motifs three-dimensionally form an arch, thus creating a hole in the middle of the protein. The dimensions of the hole only allow single-stranded DNA, but not double-stranded DNA, to thread through. The presence of positively charged and bulky residues in the helices of the arch region may directly interact with DNA substrate. Other structural features, such as the H3TH motif, dynamically hold the double-stranded portion of the flap substrate (7). Therefore, hFEN-1 nuclease is able to recognize the ends of free 5′ single strand DNA and thread it through the hole, resulting in cleavage at the junction between the single-stranded and double-stranded DNA portions of the substrate (11). At the cellular level, hFEN-1 nuclease is able to localize into nuclei in a cell cycle-dependent and DNA damage-inducible manner (12). To date, nine different proteins involved in DNA metabolic pathways have been reported to interact with hFEN-1 nuclease. They include PCNA, APE-1, DNA2 helicase, RPA, replication factor C (RF-C), pol α, pol β, pol ε, and type II DNA topoisomerase II (13–18). All of these proteins are essentially involved in two different general cellular processes, namely DNA replication and DNA base damage repair, which is consistent with the dual roles of hFEN-1 in DNA replication and repair.

Among these FEN-1 interactive proteins, PCNA has been studied intensively. It was originally identified as a processivity factor for DNA polymerase δ (19, 20) and ε (21). PCNA functions as a homotrimer with a subunit molecular mass of 31 kDa for hPCNA (29 kDa for scPCNA) and is highly conserved from yeast to mammalian cells (22). Besides its roles in DNA replication, PCNA has been shown to be a critical regulating factor through its interaction with various important proteins involved in repair and cell cycle control including xeroderma pigmentosum group G (XP) nucleases (23), DNA (cytosine-5) methyltransferase (MCMT) (24), p21 (25), MSH2 (26), and MSH3 (27). In vitro experiments have revealed that PCNA can bind to these proteins and can also stimulate specific enzymatic activities. Under native conditions PCNA exists as a monomer and must homotrimerize in order for it to encircle DNA. PCNA can trimmerize around double-stranded DNA by simple diffusion or trimerize first and slide onto DNA provided there are free ends. However, it is most efficiently loaded as a trimer onto closed circular double-stranded DNA by the accessory protein RF-C in an ATP-dependent process (28, 29). The crystal structure of scPCNA shows that the trimer forms a closed ring with appropriate dimensions and electrostatic properties that en-
The Lieber and Burgers group was the first to report that human and yeast PCNA (hPCNA and scPCNA) interacts with the hFEN-1 and scRad27 nuclease and stimulates their activities, respectively (5, 15). Using the yeast two-hybrid system, Li et al. (15) demonstrated that yeast scRad27 directly binds to scPCNA. This specific interaction was further confirmed by affinity chromatography using scPCNA beads and yeast cell crude extract containing scRad27. Through protein-protein interactions, PCNA focuses hFEN-1 on a branched DNA substrate (flap endonuclease substrate) and on a nicked DNA substrate (exonuclease substrate), thereby stimulating its activity 10–50-fold depending on the salt concentrations used in the reactions (5). The exact mechanism of stimulation remains unclear. Recent in vitro studies suggest that PCNA stimulates nuclease activities by lowering the \( K_m \) value of hFEN-1 for DNA substrates (31). Additionally, there are two regions in PCNA that have been shown to be potential binding sites for hFEN-1. The first of these regions is located in the interdomain connector loop (IDCL) of PCNA (32), and the second sequence is found near the C terminus of PCNA (21, 33). FEN-1 is critical for the removal of oxidative base damage via long patch base excision repair (36–38). The absence of PCNA in reconstituted in vitro base excision repair reactions containing FEN-1 results in an accumulation of uncleaved or unprocessed damaged DNA intermediates (36). A defective hFEN-1/hPCNA complex may result in inefficient removal of certain types of DNA damage or improper processing of Okazaki fragments, which can produce DNA with secondary structure and eventually result in duplication mutations. The accumulation of DNA damage and increased rates of duplication mutations are examples of primary molecular events that may lead to human cancers and heritable genetic diseases such as colorectal cancers, Huntington’s disease, and various ataxias (2, 39, 40).

Via peptide mapping experiments and protein truncation analysis, a conserved region in the C terminus of the hFEN-1 nuclease (337GQRLLDDFFFR345) has been shown to be the PCNA interaction region (23, 32). In this region, deletion or loss of interaction with hPCNA was eliminated (36). In this study, we aimed to elucidate details of the structural and functional relationship of this interaction. Comprehensive alanine mutagenesis was performed in the entire proposed PCNA interaction region. Our results demonstrate that 4 amino acid residues in hFEN-1, Leu-340, Asp-341, Phe-343, and Phe-344, were critical for the in vitro interaction with hPCNA. Individual replacement of each of these 4 amino acid residues with alanines resulted in a loss of interaction with hPCNA in vitro. Unexpectedly, all hFEN-1 mutants, including L340A, D341A, F343A, and F344A, retained hPCNA-mediated stimulation properties of both exonuclease and flap endonuclease activities. Further functional interaction analyses of scRad27 nuclease mutants were also carried out. These mutants include multiple alanine mutations of L340/F343/F344, L340/D341/F343/F344, or 337QQRLLDDFFFR344 or a deletion of these 8 amino acid residues (337–344) in the PCNA-binding region. These alterations resulted in the loss of PCNA binding but retained nuclease activity stimulation. Additionally, human PCNA fails to interact with the scRad27 nuclease, yet it is still able to stimulate the nuclease activities of scRad27. These results suggest that the in vitro interaction of hFEN-1/scRad27 to PCNA and stimula-

| Nucleases | Mutants | Oligos | Oligo sequencea |
|-----------|---------|-------|-----------------|
| hFEN-1    | Q337A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | Q337A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | Q337A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | R339A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | R339A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | R339A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | L340A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | L340A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | L340A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | D341A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | D341A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | D341A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | D342A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | D342A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | D342A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | F343A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | F343A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | F343A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | F344A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | F344A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | F344A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | K345A   | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | K345A-F | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
|           | K345A-R | 5′-CGCAAGGCACGACCggcggGCGCGTTG-3′ |       |
| RAD27     | 3A      | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 3A-F    | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 3A-R    | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 4A      | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 4A-F    | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 4A-R    | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 8A      | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 8A-F    | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | 8A-R    | 5′-GGCTGGATGATTTTCT-3′ |       |
|           | ΔP      | 5′-CATTTCCGATCAGGGCCGCGTGCTGGATTTC-3′ |       |

a Within oligo sequences, lowercase letters represent the triplet codon for alanine, boldface lowercase letters indicate sites of nucleic acid mutagenesis, underlined triplet codons indicate alanine residues when using LFF/AAA DNA as a template, and ▲ represents the location of the deletion of 24 nucleic acids (8 amino acid residues).
ation of nuclease activities are mediated through independent mechanisms.

MATERIALS AND METHODS

Site-directed Mutagenesis of FEN-1 and RAD27—All mutant proteins created for the current study were prepared using the QuickChange® site-directed mutagenesis kit from Stratagene (La Jolla, CA). A pair of mutagenic primers for each mutant was synthesized at the City of Hope DNA/RNA/peptide synthesis core facility. All together, 12 mutants of human FEN-1 and yeast Rad27 were made for this study. Mutations and corresponding oligo sequences are listed in Table I for clarity. Mutagenesis reactions contain 50 ng of template pET28 derived plasmid harboring the wild type FEN-1 (41), RAD27 (34), or the 3A recD27 mutant (this study) gene sequence, 125 ng of each primer, a 10 mM dNTP mix, and 1 μl of Taq DNA polymerase mix, and 1 μl of Phy tag enzyme (Stratagene, La Jolla, CA). The column was then washed with 40 mM imidazole over a total volume of 300 μl. Fractions were collected in 2-ml aliquots for a total of 25 fractions, and appropriate fractions were analyzed using SDS-PAGE to determine which ones contained purified protein. Fractions containing the desired protein were pooled and then exchanged into a buffer of 20 mM Tris-Cl, pH 7.9, 150 mM NaCl using a desalting column (Amersham Pharmacia Biotech). Protein concentration was determined using the Bio-Rad (Bradford) protein assay reagents, and then an equal volume of glycerol was added to each protein sample for storage at −20 °C.

FEN-1/PCNA Binding Assay—A binding assay protocol was designed based on the published methods developed by Gary et al. (36) and Stucki et al. (42). Briefly, cells harboring the wild type or mutant His-tagged human FEN-1 or yeast Rad27 protein, and non-histidine-tagged human or yeast PCNAs were grown, induced, and harvested as described previously (34, 42). Binding reactions contain 150 μl of crude cell extracts of the appropriate FEN-1 or Rad27 protein and the appropriate PCNA protein, 150 μl of a 50% slurry of beads charged with 50 mM NiSO₄ and 450 μl of Tris-buffered sample (50 mM Tris-Cl, pH 7.4, 150 mM NaCl). To facilitate the protein-protein interaction, samples were gently shaken at 4 °C for 90 min. Beads were collected by gravity and then washed four times with wash buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 60 mM imidazole). Following the final wash, beads were resuspended in 75 μl of a 50% slurry of beads charged with 50 mM NiSO₄ and 450 μl of Tris-buffered sample (50 mM Tris-Cl, pH 7.4, 150 mM NaCl). To facilitate the protein-protein interaction, samples were gently shaken at 4 °C for 90 min. Beads were collected by gravity and then washed four times with wash buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 60 mM imidazole). Following the final wash, beads were resuspended in 75 μl of 2% protein sample loading buffer (100 mM Tris, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol), boiled, the supernatant was collected, followed by analysis using SDS-PAGE. The gel was stained with Coomassie Blue R250 for visualization of proteins that were bound to the beads. A FEN-1/PCNA complex was confirmed by visualizing the presence of both a protein band for the desired protein band for human FEN-1 (−42 kDa) or scRad27 (−42 kDa) and a band for hFEN-1 (−38.5 kDa) or Yeast Rad27 (−33.5 kDa). The FEN-1/PCNA complex was confirmed by visualizing the presence of both a protein band for the desired human or yeast PCNA (−31 and 29 kDa, respectively) on the gel.

**Table II.**

| Proteins | PCNA binding motif sequences | Organisms | GenBank™ no. |
|----------|-----------------------------|-----------|-------------|
| hFEN-1   | 334GSTQGRDLDDDFKVT          | Homo sapiens | NP004102    |
| RnFEN-1  | 334GSTQGRDLDDDFKVT          | Rattus norvegicus | AF281018    |
| MmFEN-1  | 334GSTQGRDLDDDFKVT          | Mus musculus | NP032025    |
| xiFEN-1b | 334GSTQGRDLDDDFKVT          | Xenopus laevis | AAP88707    |
| xFEN-1   | 334GSTQGRDLDDDFKVT          | X. laevis | AAP88707    |
| dmFEN-1  | 334ACQTVRLDDDFKVL           | Drosophila melanogaster | T13692    |
| CeFEN-1  | 334SGTQGRDLDFKGN            | Caenorhabditis elegans | AAFA6553    |
| osFEN-1  | 334KGQGRLDDDFKPV            | Oryza sativa | BAA36171    |
| Rad27p   | 334STQGRDLDDDFKV            | Schizosaccharomyces pombe | P95750    |
| SpRad2   | 334TIPQGRDLDDDFKV           | Plasmmodium falciparum | AA01445    |
| PheF1    | 347VTQYRDLNDFFAC           | Pyrococcus horkoshii | A71015    |
| PhF1     | 328ARGQSTELSDFKVK           | Pyrococcus abyssi | E75117    |
| PoF1     | 328AVQGRSLDFKRR            | Pyrococcus furiosus | T46993    |
| FEN-1    | 318LTQGRLSDFK               | M. thermotolerans | C69985    |
| afF1     | 326KTSQATLSDFK              | Archaeoglobus fulgidus | NP069102    |
| apF1     | 390GRQGRSLDFMG             | Aeropyrum pernix | H72765    |
| myF1     | 315KTRQTLSDWF              | Methanococcus jannaschii | C64480    |
| taF1     | 326QTRQPGSLDFK             | Thermoplasma acidophilum | CAC12164    |
| hBF1     | 317AAQTQGRDLDFW            | Halobacterium sp. NRC-1 | AA0146980    |
| Other representative proteins | | | |
| XPG      | 98TQQLRDLDDDFKVK           | Homo sapiens | P28715    |
| DNA ligase I | 001NQRQLESQFRRR     | Homo sapiens | M36067    |
| MCM7     | 158STRQTTISSLPAK            | Homo sapiens | X63682    |
| Tigger Cds2 | 86AQLQGQTSLSLFK            | Homo sapiens | U89973    |
| Cdc27    | 359KPCQGQISFGFK             | Homo sapiens | M74062    |
| P57      | 266KLGPQISDFATR            | Homo sapiens | U84889    |
| P21 pep2 | 142KQRQTSDFHK              | Homo sapiens | P35936    |
| UDG2     | 018KQRQTSDFHK              | Homo sapiens | K04470    |
| hMYH     | 509QRQGQISDFHK             | Homo sapiens | T03629    |
| hMSh3    | 018PARQAVQSLDFQ            | Homo sapiens | J04810    |
| hMSh6    | 001MSRQKSLDFHK             | Homo sapiens | P52701    |
| Pol32    | 335LKKQGQLESQFRRK          | Homo sapiens | Z49543    |

Protein Overexpression and Purification—Protocols for protein overexpression and purification have been described in our previous work (34, 42). After transformed E. coli BL21(DE3) (5) cells were amplified, protein expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were harvested, soluble FEN-1 and Rad27 wild type and mutant proteins were extracted in a Tris-based buffer (20 mM Tris, pH 7.9, 150 mM NaCl) and were bound to a 5-ml Ni⁺⁺ chelating column, which is facilitated by a fast protein liquid chromatography system (Amersham Pharmacia Biotech). The column was then washed with 40 mM imidazole, and specifically bound protein was eluted over a gradient spanning a concentration range from 40 to 350 mM imidazole over a total volume of 50 ml. Fractions were collected in 2-ml aliquots for a total of 25 fractions, and appropriate fractions were analyzed using SDS-PAGE to determine which ones contained purified protein. Fractions containing the desired protein were pooled and then exchanged into a buffer of 20 mM Tris-Cl, pH 7.9, 150 mM NaCl using a desalting column (Amersham Pharmacia Biotech). Protein concentration was determined using the Bio-Rad (Bradford) protein assay reagents, and then an equal volume of glycerol was added to each protein sample for storage at −20 °C.

FEN-1 and PCNA Interaction
for 60 min. Polynucleotide kinase enzyme was inactivated by heating the sample at 72 °C for 10 min. 80 pmol each of the Temp-3B and Prim-1G oligos were then added to the labeled oligos, respectively. The samples were incubated at 70 °C for 5 min, followed by slow cooling to 25 °C to allow the oligos to slowly anneal and form the flap endonuclease and exonuclease substrates as shown in Fig. 2 (A and B). Substrates were precipitated at −20 °C overnight after adding 20 μl of 3 M NaOAc and 1 ml of 100% ethanol. Substrates were collected by centrifugation, washed once with 70% ethanol, and resuspended in sterile water.

For PCNA-independent assays, reactions were carried out with 120 fmol of hFEN-1 or scRad27 and 500 fmol of flap or exonuclease sub- strate in 15 μl of Tris buffer (pH 8.0), 10 mM MgCl2, and 100 μg/ml BSA. For PCNA-dependent flap endonuclease activity stimulation assays, 120 fmol of the appropriate FEN-1 or Rad27 was mixed with 2.6 μl of 5X reaction buffer with high salt concentration (250 mM Tris-Cl, pH 8.0, 50 mM MgCl2, 375 mM NaCl), 500 fmol of flap substrate, and 3,000 fmol of human or yeast PCNA. Each reaction was then brought to a total volume of 13 μl with water. As for the PCNA-dependent exonuclease activity stimulation assays, 240 fmol of FEN-1 or Rad27 protein, 3,000 fmol of human or yeast PCNA, and 500 fmol of exonuclease substrate were mixed with 2.6 μl of 5X reaction buffer with low salt concentration (250 mM Tris-Cl, pH 8.0, 50 mM MgCl2, and 125 mM NaCl). Each reaction was then brought to a total volume of 13 μl with water. 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% bromphenol blue, 0.05% xylene cyanol). An aliquot of each reaction was then run on a 15% denaturing PAGE at 1900 V for 1 h. The gel was dried at 70 °C for 50 min, and then visualized by autoradiography.

RESULTS

Identification of hFEN-1 Residues That Are Crucial for hPCNA Binding—Previous studies have determined some of the amino acid residues in the C-terminal region of hFEN-1 that are crucial for its ability to bind hPCNA (23, 32). Our current study aimed to comprehensively analyze each amino acid residue in the consensus PCNA binding region of hFEN-1 to determine which residues were necessary for binding. Based on previous findings, we chose to focus on the region of hFEN-1 that includes residues Gln-337 through Lys-345 (3, 12, 32). This region resembles the consensus conserved PCNA binding motif, QXGXXQ(L/D)XXGFP(Y/F)8. The residues in bold are conserved in many FEN-1 nucleosome homologs and some other PCNA interacting proteins, as shown in Table II, and serve as excellent candidates to begin mutagenic analysis.

We individually mutated 8 of the 9 amino acids in this region to alanine in order to study the PCNA binding role of each residue. The ninth residue, glycine, was excluded in the individual mutational analysis, but was later included in a mutant complementation assay (Fig. 1). Surprisingly, Gln-337 was not important even though this glutamine residue is highly conserved in the XPG family is actually shorter than proposed and only resembles the consensus PCNA binding motif. This result may allow hFEN-1 to overcome this inhibition. Each of the eight mutant hFEN-1 proteins created in this study was highly purified in order to analyze in vitro stimulation of endonuclease and exonuclease activities by hPCNA. Logically, one would expect that all mutants that retained binding ability would also lose their ability to be stimulated. To perform PCNA-dependent nuclease activity assays, we chose to use linearized DNA oligo substrates as shown in Fig. 2 (A and B), which are identical to the substrates used in the original report by Lieber and Burgers (15). Endonuclease and exonuclease activities of each mutant were first tested in the absence of NaCl to confirm that each protein was functional as described under “Materials and Methods” (data not shown). Following this confirmation, we individually assayed the stimulation of both endonuclease and exonuclease cleavage for the eight mutant hFEN-1 proteins as well as the wild type protein. Interestingly, we observed that endonuclease activity of all eight mutants was still stimulated by hPCNA (Fig. 2A). In agreement with these results, we also observed that exonuclease activity of all eight mutants was also stimulated by hPCNA (Fig. 2B). These results were quite unexpected. One observation of importance is that previously created PCNA binding-deficient hFEN-1 and scRad27 mutants have never been assayed for stimulation (e.g. Refs. 36 and 44). Our analysis of the hPCNA-mediated stimulation of hFEN-1 mutant nuclease activity clearly demonstrates that amino acid residues, which were shown to be necessary for binding, were not required for stimulation.

hFEN-1 or Rad27 Binding and Activity Stimulation by Human and Yeast PCNA—When the Lieber and Burgers’ group first demonstrated the interspecies interaction and stimulation between hFEN-1/scRad27 and its respective PCNA, they also reported that the interspecies stimulation of hFEN-1 by scPCNA was poor (5). This observation was then utilized to explain genetic data obtained in rad27 mutant complementation experiments by hFEN-1 (44, 45). Our current work was designed to determine if the human and S. cerevisiae PCNA proteins (hPCNA and scPCNA, respectively) were able to bind to hFEN-1 or scRad27 and stimulate the nuclease activities intra- and interspecifically. First, we tested the ability of both PCNAs to bind to hFEN-1 or scRad27. Fig. 3 shows the interspecific binding properties of hFEN-1 and scRad27 to human and yeast PCNA. The data indicate that hFEN-1 binds to hPCNA and scPCNA with no apparent qualitative differences. scRad27 binds only to scPCNA under the same reaction conditions, whereas it failed to bind to hPCNA. This indicates that the regions of hPCNA and scPCNA where hFEN-1 binds are similar enough that hFEN-1 is unable to discriminate between the two sites. On the other hand, the results in Fig. 3 also show that scRad27 is able to bind scPCNA, but not hPCNA. This may
illustrate subtle differences of hFEN-1 and scRad27 binding to PCNA from different species.

Additionally, using hPCNA and scPCNA, we assayed the stimulation of flap endonuclease and exonuclease activities of hFEN-1 and scRad27, and we clearly demonstrated that nuclease activities are stimulated in the presence of hPCNA or scPCNA (Fig. 4). Even though the interspecific binding abilities of hFEN-1 and scRad27 differ, it is clear that both proteins are stimulated by the corresponding interspecific PCNA to a similar degree. Again, these data further confirm the fact that deficiency of our hFEN-1 mutants in hPCNA binding does not necessarily correlate with stimulation of nuclelease activities.

Multiple Mutations of Conserved Residues in the PCNA Interaction Motif of Rad27—Our study has identified the residues critical for hFEN-1 binding to hPCNA, whereas additional data have detailed which residues are important for PCNA binding by other proteins such as ligase I, p21, and MCMT (Table II) (23, 24, 44, 46). Our experiments have also demonstrated that the nuclelease activities of single amino acid mutants can still be stimulated by hPCNA. One of the possible explanations is that the presence of other unidentified conserved residues in the PCNA binding region are able to serve as a back-up for stimulation by compensating for single residue mutations. Three residues (LXXFF) in this motif are highly conserved and are essential for binding to PCNA by ligase I, p21, MCMT, hFEN-1, and scRad27 (23, 24, 32, 44, 46). These residues are equivalent to the Leu-340, Phe-343, and Phe-344 residues in hFEN-1 and the Leu-343, Phe-346, and Phe-347 residues in scRad27. We mutagenized these residues in scRad27 to alanine (L343A/F346A/F347A, termed the 3A mutant hereafter). We opted to create this mutant in scRad27 so that it could potentially be used for future in vivo studies in S. cerevisiae. Characterization of the 3A mutant was performed with the single mutant hFEN-1 proteins. Binding assays of the 3A mutant revealed that this mutant lost its ability to bind to scPCNA (Fig. 5). scPCNA-dependent endonuclease and exonuclease activity assays were also performed. Clearly, the endonuclease and exonuclease activities of the 3A mutant were significantly enhanced by scPCNA (Fig. 6). However, there is an additional conserved amino acid residue, Gln-337 in hFEN-1, which is highly conserved in PCNA-binding proteins, but has been experimentally proven to not be critical for hFEN-1 binding to hPCNA. In order to clearly demonstrate that the glutamine residue plays no role in binding or stimulation, we constructed a quadruple mutant in scRad27 (Q340A/L343A/F346A/F347A, termed the 4A mutant hereafter). The binding (Fig. 5) and stimulation assays (Fig. 6) of the 4A mutant resulted in data similar to that for the 3A mutant and...
to the single mutants of hFEN-1. These data indicate that multiple mutations of conserved residues in the putative PCNA binding region do not abolish in vitro PCNA-mediated stimulation of nuclelease activities. These properties led us to believe that more comprehensive group mutations, or a deletion of the entire region, may be necessary to create a mutant that cannot have its nuclelease activities stimulated.

Recently, Stucki et al. (43) constructed a deletion mutant of hFEN-1 (FEN-1ΔP) in order to eliminate 8 residues (Gln-337 through Phe-344) in the putative PCNA binding motif. Results showed that FEN-1ΔP completely lacked nuclelease activity stimulation in hPCNA-dependent assays. The authors concluded that the physical interaction between hFEN-1 and hPCNA is absolutely required for hFEN-1 stimulation. To further elucidate the meaning of our results as well as the conclusion made by Stucki et al. (43), we constructed two additional mutants in scRad27, in which we either changed all 8 residues (Gln-340 through Phe-344) in the PCNA binding motif to alanines, or in which we deleted all 8 residues (termed the ΔP mutant hereafter) in this region.

As expected, both the 8A and ΔP mutants were deficient in their ability to bind to scPCNA (Fig. 5). In contrast to what Stucki et al. (43) has shown, our results revealed that both the 8A and ΔP scRad27 mutants could have their endonuclease and exonuclease activities stimulated in the presence of scPCNA (Fig. 6). This phenomenon may explain the lack of observable phenotypes of S. cerevisiae cells containing a Rad27 gene harboring the F346A/F347A double mutation (44, 45). The F346A/F347A scRad27 mutant was unable to produce significant phenotypic differences when incorporated in the S. cerevisiae chromosome, probably due to that this double mutant’s stimulation properties are similar to that of wild type.

**DISCUSSION**

Employing a strategy of individually mutating each of the 8 amino acid residues to alanine residues in the C-terminal putative hPCNA binding motif of hFEN-1 (Table II) allowed us to systematically analyze hFEN-1’s ability to bind to hPCNA as well as to determine the effect on the stimulation of its nuclelease activities by hPCNA. Our intentions were to precisely determine which residues in hFEN-1 are crucial for binding to hPCNA and then correlate the binding results of individual mutants with their stimulation properties. Our results revealed interesting and novel findings that should expand our overall knowledge of hFEN-1’s in vitro binding and stimulation characteristics and may help to further our understanding of previously published in vivo results.

Previous biochemical analysis of PCNA and its interaction with various proteins revealed a conserved sequence in these proteins that is necessary for their ability to bind to PCNA. An 8-amino acid residue consensus PCNA binding motif was derived and is represented by the following sequence: QXXLX(T/I)LXXF(Y/F). Traditionally, this motif is located at, or near, either the N or C terminus of PCNA-binding proteins. In Table II, boldface letters in the putative PCNA binding motifs of various proteins indicate that residues are highly conserved. Mutational analysis of this motif in hFEN-1 was performed in order to determine the identity of the residues essential for binding to PCNA. Previous analysis of five proteins has partially determined the residues that were essential for PCNA binding by the scRad27, hFEN-1, p21, ligase I, and MCMT proteins (23–25, 32, 44, 46, 47). Residues equivalent to (L/I)4, Phe-347, and (F/Y)α of the PCNA consensus binding motif are essential for PCNA binding by all five proteins. In our current study, point mutations in the consensus PCNA binding motif of hFEN-1 were created and characterized. Binding analysis of each mutant using crude cell extracts unambiguously showed which residues were essential for hFEN-1 to bind to hPCNA (Fig. 1). In the C-terminal PCNA binding region of hFEN-1, we
determined that residues Leu-340, Asp-341, Phe-343, and Phe-344 (equivalent to Leu-343, Asp-344, Phe-346, and Phe-347 in scRad27) were essential for binding. Our experiments further confirmed that the glutamine residue is not essential for the hFEN-1-hPCNA interaction and may not actually be a part of the PCNA binding motif even though it is highly conserved. Instead, aspartate 341, which is conserved in the eukaryotic FEN-1/XPG nuclease family, is a crucial residue in the consensus motif (Table II). Our results suggest that the consensus PCNA binding motif for the FEN-1/XPG family could be better represented as (L/I)X(D/E)XX(F/W)X(D/E)X.

The most interesting and unexpected results obtained in this study involved the analysis of the hPCNA-mediated stimulation of the nuclease activities of hFEN-1 and scRad27 proteins that contained single or multiple mutations in the C-terminal PCNA interaction domain. In order to correlate the experimentally observed binding abilities of mutants with their stimulation properties, all mutant hFEN-1s were analyzed for their hPCNA-mediated flap endonuclease and exonuclease stimulation using in vitro assays (5, 43). We predicted that all mutants that lost binding ability to hPCNA would also lose their ability to be stimulated, and, likewise, mutants that retained binding ability would also retain hPCNA-mediated stimulation of their endo/exonuclease activities. However, all mutants created still retained hPCNA-mediated stimulation of flap endonuclease (Fig. 2A) and exonuclease (Fig. 2B) activities. It was also considered that the presence of PCNA could produce an excluded volume effect similar to the mechanism in which the presence of excess BSA is able to enhance the activity of some restriction enzymes. This possibility was tested using BSA and polyethylene glycol 4000 and observed that this was not the case (data not shown). Alternatively, we also considered the possibility that an undetectable amount of residual binding to hPCNA may exist in any (or all) of the hPCNA binding deficient single mutants because our binding assay may not be sensitive enough to detect residual binding levels. Therefore, we addressed this possibility by mutating multiple conserved residues in combination. The following four mutants of scRad27 were also constructed for this experiment: 1) a triple mutant by changing Leu-343, Phe-346, and Phe-347 all to alanines (3A mutant), 2) a quadruple mutant by changing Gln-340, Leu-343, Phe-346, and Phe-347 all to alanines (4A mutant), 3) a mutant in which residues Gln-340 through Phe-347 were all changed to alanines (8A mutant), and 4) a mutant with a deletion of these 8 residues (∆P mutant). We assayed binding of these mutants to scPCNA and flag endonuclease and exonuclease activity stimulation by scPCNA. As expected, all of these mutants lost their ability to bind to scPCNA (Fig. 5). All of the scRad27 proteins containing multiple mutations (3A, 4A, and 8A) or a deletion (∆P) also retained normal nuclease activity stimulation mediated by scPCNA (Fig. 6).

This finding was supported by our further observation that hPCNA was able to stimulate nuclease activities of scRad27 even though it can not bind to scRad27. The original purpose for performing the experiment on interspecific binding and stimulation of hFEN-1 and scRad27 proteins by human or yeast PCNAs was to verify the previous data that scPCNA could not stimulate hFEN-1 nuclease activities (5). Our recent observation shows that hFEN-1 could fully complement scRad27 functions when it was used to rescue rad27 knockout strains (26). This result led us to hypothesize that the scPCNA should be able to bind to hFEN-1 and stimulate its nuclease activities or vice versa. As expected, our present study indicated that the scPCNA could indeed bind to hFEN-1 protein (Fig. 3) and stimulate its nuclease activities (Fig. 4) in vitro. Unexpectedly, we also revealed that hPCNA could stimulate the nuclease activities of scRad27, but was not able to bind to the protein, which support our hypothesis that the binding and stimulation of FEN-1 proteins by PCNA are mediated via independent mechanisms.

The above results led us to consider the possible existence of additional unidentified motif(s) and/or structural elements in RAD27 (or hFEN-1) that could be responsible for the stimulation properties of the wild type and various mutant proteins by PCNA. In a recent report, Gomes and Burgers (48) described that the ability of scRad27 to bind to two distinct regions in scPCNA (the IDCL and C terminus) was regulated by DNA. A yeast IDCL mutant, pcna-79 (IL126, 128AA), failed to interact with scRad27 in solution, but surprisingly, was still very active in stimulating scRad27 nuclease activity. In contrast, a C-terminal mutant, pcna-90 (PK252, 253AA), exhibited wild type binding to scRad27 in solution, yet poorly stimulated the nuclease activities of scRad27. When proteins were individually loaded onto a DNA substrate (resembling our exonuclease substrate) that was coupled to magnetic beads, wild type scPCNA and pcna-79, but not pcna-90, formed a complex with scRad27 and the DNA substrate. These results indicated that the presence of an appropriate DNA substrate dictated the region of PCNA to which Rad27 binds.

The data presented here are contradictory to what Stucki et al. (43) have published recently. Stucki et al. constructed a deletion mutant of hFEN-1 lacking the PCNA interaction motif, which is equivalent to our ∆P scRad27 mutant described here. The sequences of these regions in the two proteins are identical except for 1 residue. They have carried out PCNA interaction and stimulation assays with their mutant and concluded that the PCNA interaction motif is indispensable for stimulation of hFEN-1 activities by hPCNA. Our results using proteins containing single mutant, multiple mutants, or a deletion mutation in the C-terminal region required for in vitro PCNA interaction did not result in significant loss of stimulation. This discrepancy may have resulted from origins of proteins, differences in experimental design, how the mutant proteins were handled, or the removal of an additional amino acid residue, Phe-347, in the yeast enzyme (deletion of 8 residues in scRad27 versus 7 residues in hFEN-1). Among these possibilities, the deletion mutant protein that Stucki et al. and the one that we have constructed may have different folding properties, solubility, and consequently different biochemical functions. We have found that the recombinant scRad27 proteins with multiple point mutations or deletions are not as stable as wild type scRad27 nuclease. The most deleterious case was the 8A mutant, where 8 amino acid residues (337–344) were converted into a large cluster of alanines. Despite this, we were able to purify the mutant proteins and perform the nuclease activity assays. All of the mutants included in this study have a wild type level of nuclease activities in PCNA-independent assays. Their nuclease activities could be stimulated by PCNA to a level similar to that of the wild type nuclease. Therefore, the stimulation that we observed with these mutants is most likely specific and real. Several mutants, including 3A and 4A, were introduced into rad27 null mutant cells using a yeast expression plasmid (pDB20) and failed to show the significant phenotype observed in the rad27 null mutants, which is consistent with previous observations (42, 44, 45). This indicates that the activity stimulation instead of the in vitro hFEN-1/hPCNA interaction is responsible for the functional deficiency observed in vivo.

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crystal structure of Pyrococcus furiosus FEN-1 nuclease, which is used as a template for molecular modeling in this study.

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Stimulation of Eukaryotic Flap Endonuclease-1 Activities by Proliferating Cell Nuclear Antigen (PCNA) Is Independent of Its in Vitro Interaction via a Consensus PCNA Binding Region
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