In eukaryotic flap endonuclease 1, the C terminus is essential for substrate binding

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Abstract

Flap endonuclease 1 (Fen1) is a structure-specific metallonuclease with important functions in DNA replication and DNA repair. It interacts like many other proteins involved in DNA metabolic events with proliferating cell nuclear antigen (PCNA), and its enzymatic activity is stimulated by PCNA in vitro. The PCNA interaction site is located close to the C terminus of Fen1 and is flanked by a conserved basic region of 35-38 amino acids in eukaryotic species but not in archaea. We have constructed two deletion mutants of human Fen1 that lack either the PCNA interaction motif or a part of its adjacent C-terminal region and analyzed them in a variety of assays. Remarkably, deletion of the basic C-terminal region did not affect PCNA interaction but resulted in a protein with significantly reduced enzymatic activity. Electrophoretic mobility shift analysis revealed that this mutant displayed a severe defect in substrate binding. Our results suggest that the C terminus of eukaryotic Fen1 consists of two functionally distinct regions that together might form an important regulatory domain.
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Fen1 (5′-exonuclease-1 or flap endonuclease-1) is a multi-
functional structure-specific metallonuclease that is impor-
tant for DNA metabolic events such as replication and repair. Its
main function in replication is proposed to be the removal of the
displaced RNA-DNA primers synthesized by DNA polymerase
α-primase during discontinuous lagging strand replication (re-
viewed in Ref. 1). In DNA repair, Fen1 appears to be required
for nonhomologous end joining of double-strand DNA breaks
(2) and for the removal of DNA base damage and single-strand
breaks, likely through participation in the excision and resyn-
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excision repair (reviewed in Ref. 3). The relevance of these
observations is underscored by \textit{in vivo} data using yeast Fen1-
null mutant strains that display severely impaired phenotypes
such as temperature sensitivity for growth with a terminal
phenotype consistent with a defect in replication, sensitivity for
DNA damaging agents such as UV radiation, and alkylating
agents and defects in telomere maintenance (4–7). Because
does not, or a part of its adjacent C-terminal region and analyzed these
mutants in a variety of assays. Consistent with results of a very

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recent study (21), we found that the PCNA interaction motif is indispensable for stimulation of Fen1 activity by PCNA. Moreover, our data reveal that the basic C-terminal tail is not important for PCNA interaction. However, this part of the protein is required for substrate binding. Our results indicate two functionally distinct regions within the C-terminal part of human Fen1 that might together form an important regulatory domain.

**EXPERIMENTAL PROCEDURES**

**Nucleic Acid Substrates—Oligonucleotides** for preparing the substrates for the Fen1 assays were purchased from Microsynth GmbH (Balgach, Switzerland). F12_01 is a 39-mer composed of 19 nucleotides complementary to nucleotides 698–717 of the plasmid pBlueScript SK- (pBS) and a 20-nucleotide noncomplementary tail. F12_05 is a 19-mer of the same sequence without the noncomplementary tail, and F12_02 is a 30-mer complementary to nucleotides 669–698 of single-stranded pBS. Oligonucleotides F12_01 and F12_05 were labeled at the 5’ end using [γ-32P]ATP and T4 polynucleotide kinase. Free ATP was removed on MicroSpin™ G-25 columns. To generate the substrates for the endo- and exonuclease assays, the appropriate primers were mixed with single-stranded pBS DNA in 1:1 molar ratio in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, heated to 75 °C, and slowly cooled to room temperature. The templates for the mobility shift assay were created by hybridizing 5’-labeled F12_01 or F12_05 and unlabeled F12_02 to a complementary 49mer oligonucleotide, corresponding to nucleotides 669–717 of pBS.

**Enzymes and Proteins—Human Fen1 cDNA (gift of A. Dutta) was cloned into the pET234d vector (Novagen).** Deletion mutagenesis was performed with the QuickChange™ site-directed mutagenesis kit from Stratagene according to the instruction manual. The following primers were used for the Fen1(ΔP) mutant (amino acids 337–344): 5’-GACGCGCCAGGGCAACAAAGGTACGCCGGCTCACTCT-3’ and 5’-AGGGTACGCCGTCAGCTTGGTCTGCTGGCGGCCTT-3’. For the Fen1(ΔC) mutant (aa 380–380), oligos 5’-GCGAAGGGAGGAGAAGAGAACCTGTGCCGCCGC3’ and 5’-CGGCCGAATGTTCTCCCTCTTGCTGCTGGCGGC-3’ were used, respectively. Wild type and mutant Fen1 were overexpressed in Escherichia coli strain BL21(DE3)PlysE as histidine-tagged proteins and purified to homogeneity via Nickel charged metal chelating resin (HiTrap; Amersham Pharmacia Biotech) and fast protein liquid chromatography MonoS chromatography. Human PCNA was produced in E. coli using the plasmid pT7pcDNA (gift from B. Stillman) and purified to homogeneity as described (22). Replication factor C (RF-C) was purified from nuclear extract of 60 g of harvested HeLa cells as described (23). Human replication protein A (RF-A) was overexpressed in E. coli strain BL21(DE3) harboring the expression plasmid p11d-trp-A and purified according to Ref. 24.

**Native Gradient PAGE—** The electrophoresis was performed in 8–25% polyacrylamide gels using the Phast System (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The gels were stained with Coomassie Blue.

**Affinity Interaction Binding Assay—** Interaction was measured as described (25). Briefly, E. coli strain BL21(DE3)PlysE harboring wild type or mutant Fen1 and PCNA expression plasmids was grown at 37 °C, and expression was induced. After harvesting by centrifugation, cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and briefly sonicated. The cell lysates were clarified by centrifugation. Binding assay mixtures (500 μl) contained 50 μl of 50% nickel-nitrilotriacetic acid-Sepharose resin (Invitrogen), 150 μl of each cell lysate, and 50 μM Tris-HCl, pH 7.5, 150 mM NaCl to increase mixing efficiency. These mixtures were incubated for 90 min at 4 °C with constant gentle agitation and washed six times with 0.8 ml of buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 60 mM imidazole), and bound protein complexes were subsequently visualized on Coomassie-stained 12% SDS-polyacrylamide gels.

**Fen1 Assay—** The assays were performed in a final volume of 12.5 μl containing 40 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 5 mM dithiophreitol, 200 μg/ml bovine serum albumin, 50 mM of DNA substrate, 50 ng of RF-A, and indicated amounts of NaCl, ATP, PCNA, and RF-C. After addition of wild type or mutant Fen1, the reactions were incubated at 30 °C for 15 min and stopped with 2.5× stop buffer (95% formamide, 20 mM EDTA, 0.05% each bromphenol blue, and Xylene cyanol). Products were separated on 15% denaturing polyacrylamid gel, visualized by autoradiography and quantified on a PhosphorImage using the ImageQuant software (Molecular Dynamics).

**Mobility Shift Assay for Fen1—** Binding reactions were carried out as described (26). Briefly, a total volume of 20 μl contained 50 mM Tris-HCl, pH 8, 10 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 50 μg/ml bovine serum albumin, 50 fmol of labeled oligonucleotide template, and indicated amounts of wild type or mutant Fen1. After incubation at 20 °C for 10 min, reactions were loaded on 7% polyacrylamide gels containing 0.5× TBE and run at 50 V for 3 h. The gels were dried and exposed to x-ray films.

**RESULTS**

**A Conserved Basic C-terminal Tail Flanks the PCNA-binding Motif of Eukaryotic Fen1—** The structures of archaea Fen1 reveal a saddle-shaped, single-domain α/β protein with a deep positively charged cleft along one face and an unusual “helical clamp” through which a single-stranded flap strand is likely threaded. The C-terminal nine residues extend away from the body of the enzyme (Fig. 1A and Refs. 12 and 13). These residues are homologous to critical residues of eukaryotic proteins known to interact with PCNA (15), and, based on these data, a model of how Fen1 could be hooked to PCNA during DNA replication was proposed (12). However, Fen1 from eukaryotic species have additional 35–38 residues at the C terminus that are not present in the archaea orthologue. B, sequence alignment of the Fen1 C termini from various eukaryotic species. The PCNA-binding motif and the basic C-terminal tail are shaded and the conserved residues are printed in bold. C, SDS-PAGE analysis of purified wild type and mutant Fen1. 1.5 μg of each sample were separated on a 12% SDS polyacrylamid gel and stained with Coomassie Blue.

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**FIG. 1. Mutations at the C terminus of human Fen1.** A, structure of Methanococcus jannaschii Fen1 (13). The region of homology to the PCNA-binding motif in human Fen1 is indicated (P). The basic C-terminal tail is not present in the archaea orthologue. B, sequence alignment of the Fen1 C termini from various eukaryotic species. The PCNA-binding motif and the basic C-terminal tail are shaded and the conserved residues are printed in bold. C, SDS-PAGE analysis of purified wild type and mutant Fen1. 1.5 μg of each sample were separated on a 12% SDS polyacrylamid gel and stained with Coomassie Blue.
failed to interact with PCNA in several assays, although it still contained an intact PCNA interaction motif. Thus, these C-terminal residues could be involved in PCNA interaction, or they could play another as yet unknown function. In a quest for Fen1 mutants that would differ from wild type Fen1 in their affinities for PCNA, we selectively deleted two regions at the C terminus of human Fen1. The first deletion comprised the entire PCNA interaction motif (amino acids 337–343) and was called Fen1(ΔP). The second deletion mutation, called Fen1(ΔC), enclosed the last 21 C-terminal amino acids (amino acids 360–380). Wild type and mutant Fen1 were overexpressed in E. coli as polyhistidine (His<sub>6</sub>)-tagged proteins and purified to homogeneity (Fig. 1C).

The Basic C Terminus of Human Fen1 Is Not Required for PCNA Binding—First, we tested whether the C-terminal tail of human Fen1 is involved in PCNA interaction. We have previously shown that the complex formed by PCNA and Fen1 is stable enough to survive electrophoresis on native gels (15). The complexes appear as shifted bands on Coomassie-stained gels (Fig. 2A). PCNA alone runs at about 67 kDa (lane 1), despite its actual mass of 86.3 kDa, reflecting the compactness of the trimer and the high overall negative charge of the protein. Fen1(WT) alone with an estimated pI of 8.78 does not enter the gel because of its net positive charge at pH 8.44 of the gel buffer, and the same is true for Fen1(ΔC) but not Fen1(ΔP) (Fig. 2A). Fen1(WT) and Fen1(ΔC), a complex with a molecular mass of 263 kDa was apparent (lane 5), whereas the activity of Fen1(ΔP) cleaved 80 and 90% of the input template (50 fmol) in 15 min, respectively, the same amount of Fen1(ΔC) cleaved only about 8% of the template. Thus, at nonsaturating enzyme concentrations, Fen1(ΔC) is about 10 times less active than Fen1(ΔP) and Fen1(ΔC) was clearly impaired (lane 8–10). Although 2.5 ng (60 fmol) of Fen1(WT) and Fen1(ΔP) cleaved 80 and 90% of the input template (50 fmol) in 15 min, respectively, the same amount of Fen1(ΔC) cleaved only about 8% of the template. Thus, at nonsaturating enzyme concentrations, Fen1(ΔC) is about 10 times less active than Fen1(WT) and Fen1(ΔP). Fen1(ΔC) displayed a reduced enzymatic activity even at highly saturating enzyme concentrations (25 ng; 0.6 pmol).

Next, we tested whether the exonucleolytic activity of the mutant Fen1(ΔC) was impaired as well. Purified wild type and mutant Fen1 were examined on a circular partial duplex DNA molecule containing a nick (Fig. 3B). Fen1 digests the upstream oligonucleotide exonucleolytically in the 5′ to 3′ direction, thus releasing a labeled 5′ mononucleotide, which could be resolved and quantitated on 15% urea polyacrylamid gels. As expected, Fen1(ΔC) exonucleolytic activity was also severely
reduced (lanes 18–20). Again, the activities of Fen1(WT) and Fen1(DP) were almost identical (Fig. 3B, lanes 12–14 and 15–17). Although 5 ng of (120 fmol) of Fen1(WT) and Fen1(DP) cleaved 84 and 81% of the input template (50 fmol) in 15 min, respectively, no detectable cleavage product could be observed when 50 fmol of the template was incubated with 5 ng of Fen1(DC). When the same amount of template was incubated together with 50 ng of Fen1(DC), only 24% were cleaved in 15 min. At these concentrations, Fen1(WT) and Fen1(DP) cleaved almost 100%.

In summary, these data are in agreement with a recent study by Gomes and Burgers (21) showing that a mutation within the PCNA-binding motif does not affect the nuclease activity of yeast Fen1 at low salt concentration. In addition, our data reveal that the PCNA independent endo- and exonucleolytic activities of a Fen1 mutant lacking the basic C-terminal tail are severely defective when compared with wild type activity.

Both the PCNA-binding Motif and the C-terminal Tail of Human Fen1 Are Important for PCNA-dependent Endo- and Exonuclease Activity—Endo- and exonucleolytic activities of Fen1 are inversely proportional to monovalent salt concentrations in the physiological range (27). However, it has been shown that PCNA can stimulate Fen1 activity up to 50-fold under physiological salt conditions (16, 18). Kinetic analysis revealed that PCNA enhances Fen1 binding stability, thus increasing the cleavage efficiency (28). To efficiently stimulate Fen1 activity, the PCNA trimer must encircle the DNA and must be located ‘below’ the flap (15). In our study, PCNA-dependent Fen1 activity was determined at 100 mM NaCl. Under these conditions, Fen1 alone is inactive (Fig. 4A). For stimulation, the ATP-dependent clamp loader RF-C must be present to load PCNA at the flap junction and at the position of the nick, respectively, because PCNA cannot spontaneously load onto circular DNA molecules in the absence of RF-C. The human single-stranded DNA binding protein RP-A was also present in this assay to prevent nonproductive association of RFC to single-stranded DNA (29). First, wild type and mutant Fen1 nuclease activities were determined in the presence of 100 mM NaCl (Fig. 4B). Fen1(DP) was inactive, even at the highest concentration tested (lanes 5–7). This result is again in agreement with a recently characterized similar yeast Fen1 mutant (21), although this mutant carrying two point mutations within the PCNA-binding motif shows weak activity at long incubation times. Fen1(DC) displayed some activity at higher concentrations, but this activity was also severely impaired compared with Fen1(WT) activity; in the presence of PCNA, as little as 0.05 ng (1.2 fmol) Fen1(WT) cleaved 85% of the input template in 15 min (lane 2), whereas the same amount of Fen1(DC) cleaved only 8.5% (lane 8). Remarkably, the difference between Fen1(WT) and Fen1(DC) activity seems to be the same as in the...
absence of PCNA, although in the PCNA independent assay, the enzyme concentration was 50-fold higher. When the PCNA-dependent exonucleolytic activity of wild type and mutant Fen1 was tested, Fen1(ΔP) was, as expected, completely inactive (lanes 15–17), and Fen1(ΔC) was about 10 times less active than Fen1(WT) (lanes 12–14 and 18–20).

In summary, Fen1(ΔP) is completely defective under PCNA-dependent conditions. Because this mutant is unable to form a complex with Fen1 and PCNA is absolutely required for Fen1 stimulation. Moreover, PCNA-dependent endo- and exonucleolytic activity of Fen1(ΔC) is as much reduced as the PCNA-independent activity, which suggests that the C-terminal tail has no influence on PCNA interaction and stimulation.

**DISCUSSION**

In this study, we have characterized two deletion mutants of human Fen1 lacking residues at the C terminus that have been proposed to be critical for interaction with PCNA. These mutants were compared with wild type Fen1 in a variety of assays. The mutant Fen1(ΔC) lacking 7 amino acids (amino acids 337–343) near the C terminus behaved very similarly to mutants recently characterized by other groups (21, 25). These studies and the data presented here provide a consistent body of evidence that the bimolecular interaction between these two proteins is mediated by a consensus PCNA-binding motif (QXXM/ILXXF/Y), which is present in several other proteins known to interact with PCNA including p21, XP-G and DNA ligase I. On the other hand, at least two regions within PCNA seem to mediate the interaction with Fen1, and the protein-protein contacts between Fen1 and PCNA are slightly different in solution from the contacts that occur when the proteins are complexed with DNA (15, 21). The relevance of the PCNA/Fen1 interaction via the interaction motif is underscored by *in vivo*
data using yeast strains with mutations within this conserved region (21, 30). In another report, it was suggested that the basic C-terminal tail of Fen1 was also important for the interaction with PCNA, because a deletion mutant lacking 17 amino acids at the C terminus failed to interact with PCNA, although this mutant still contained an intact PCNA interaction motif (17). We have constructed a similar mutant, Fen1(ΔC), lacking 21 amino acids at the very C terminus and tested it for PCNA interaction in a native PAGE-based assay and in an affinity interaction binding assay. In our hands, no difference in the ability to interact with PCNA could be detected between this mutant and wild type Fen1, whereas the Fen1(ΔP) mutant was completely unable to interact with PCNA. This discrepancy may partially result from differences in the experimental design or alternatively the additional C-terminal residues on the Fen1 mutant of Chen et al. (17) may affect the folding of the PCNA-binding motif so that it no longer can interact with PCNA. Our data, however, show that the short PCNA-binding motif of Fen1 is responsible for the bulk of the PCNA interactions of the mutant Fen1(ΔC) to a relatively equal extent under physiological salt conditions.

On the other hand, both the endo- and exonucleolytic activities of the mutant Fen1(ΔC) were significantly reduced compared with the wild type enzyme. This defect could be observed in a PCNA-dependent background, as well as under conditions where Fen1 displays activity without PCNA. We therefore conclude that this defect is not due to the inability of Fen1(ΔC) to interact with PCNA. Moreover, the ability of this mutant to associate with an oligo flap template and a short nicked double-stranded DNA was severely reduced. Our results clearly demonstrate the importance of the C-terminal basic tail for the binding portion of the nucleolytic reaction. Because the active site of the enzyme is composed of residues of the N-terminal and intermediate conserved regions (31, 32), it is rather unlikely that the C terminus is also involved in the cleavage portion of the enzymatic reaction. The simplest explanation for the defect in substrate binding of Fen1(ΔC) would be the lack of several positively charged residues (8 lysines and 1 arginine) that may specifically or unspecifically contact the negatively charged phosphate groups of the DNA backbone and contribute to the stability of the enzyme substrate complex. If this is true, then the question arises why such an “anchor” is not present in the archaea Fen1 orthologues, which have structure-specific mechanisms for DNA substrate binding and catalysis resembling the human enzyme (33). Another even more interesting possibility would be that the C-terminal tail loops back toward the enzymatic center, thus providing structural elements that might modulate the stability of the enzyme substrate complex. We could recently show that Fen1 is an acetylated protein in vivo. This Fen1 acetylation is significantly increased after UV treatment of the cells and is most probably mediated by the histone acetyl transferase domain of the transcriptional coactivator p300. There is a detectable interaction between these two proteins both in vitro and in vivo, which is mediated by the C-terminal tail of Fen1. Moreover, we showed that the acetylated Fen1 possessed a reduced enzymatic activity, thus resembling our Fen1(ΔC) mutant. Possibly, one or several lysine residues at the C-terminal tail are “neutralized” upon acetylation, which could lead to a destabilized enzyme-substrate complex, as shown here for the Fen1(ΔC) mutant. This model suggests that the C-terminal tail of eukaryotic Fen1 contains regulatory regions that are modified upon treatment of the cells with damaging agents. This modification might regulate the enzymatic activity itself or the subnuclear localization by modulating the DNA binding stability and/or specificity. In summary, our results suggest that the C terminus of eukaryotic Fen1 consists of two functionally distinct regions: one that mediates PCNA interaction, which is important for stimulation of enzymatic activity and/or targeting Fen1 to sites where its action is required, and a second region that functions in recognition of the DNA substrate. This second region is not present in archaea orthologues of Fen1. It therefore has been suggested that it might be required for nuclear localization of the enzyme in eukaryotes (20, 34), although the importance of such a putative nuclear targeting signal for Fen1 has not been shown. Our data suggest that this basic C-terminal tail of eukaryotic Fen1 most likely has an additional function.

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REFERENCES
C Terminus of Eukaryotic Fen1