Lagging Strand DNA Synthesis at the Eukaryotic Replication Fork Involves Binding and Stimulation of FEN-1 by Proliferating Cell Nuclear Antigen*

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The 5' → 3'-exonuclease domain of Escherichia coli DNA polymerase I is required for the completion of lagging strand DNA synthesis, and yet this domain is not present in any of the eukaryotic DNA polymerases. Recently, the gene encoding the functional and evolutionary equivalent of this 5' → 3'-exonuclease domain has been identified. It is called FEN-1 in mouse and human cells and RTH1 in Saccharomyces cerevisiae. This 42-kDa enzyme is required for Okazaki fragment processing. Here we report that FEN-1 physically interacts with proliferating cell nuclear antigen (PCNA), the processivity factor for DNA polymerases δ and ε. Through protein-protein interactions, PCNA focuses FEN-1 on branched DNA substrates (flap structures) and on nicked DNA substrates, thereby stimulating its activity 10- to 50-fold but only if PCNA can functionally assemble as a toroidal trimer around the DNA. This interaction is important in the physical orchestration of lagging strand synthesis and may have implications for how PCNA stimulates other members of the FEN-1 nuclease family in a broad range of DNA metabolic transactions.

In eukaryotic cells, a family of structure-specific endonucleases can be defined based on conserved domains within FEN-1 (flap endonuclease), a 42-kDa enzyme that is both a 5' → 3'-exonuclease and a nick specific 5' → 3'-exonuclease (1). The Saccharomyces cerevisiae analog of FEN-1 is encoded by the RTH1 gene (2, 3). Both human and yeast FEN-1 (yFEN-1) are highly homologous to the human DNA repair gene XP-D and its yeast homologue RAD2 (4). Various DNA metabolic processes are thought to require processing of intermediates by the FEN-1 endonuclease. The enzyme shows the greatest activity as an endonuclease on nicked double-stranded DNA substrates with the 5'-end of the nick expanded into a single-stranded tail (see structure in Fig. 2A), cutting these so-called flap structures at the base of the tail (5). These types of DNA intermediates likely occur during end joining reactions in which DNA ends with limited homology are joined. Mammalian FEN-1 has also been identified as DNase IV, or maturation factor I, a nick-specific 5' → 3'-exonuclease required for nick translation during Okazaki fragment maturation (6–10). Consistent with its corresponding functional activity, mammalian FEN-1 shows sequence homology with the 5' → 3'-exonuclease domain present in Escherichia coli DNA polymerase I (10). The yeast RTH1 gene is dispensable for cell growth, but rth1 deletion mutants are temperature-sensitive for growth and show a terminal phenotype consistent with a defect in DNA replication (2, 3). Genetic studies with yeast rth1 deletion mutants have also shown that FEN-1 functions in the repair of alkylated DNA and in recombination repair. However, yFEN-1 (RTH1) does not function in nucleotide excision repair (2, 3). Rather, in vitro studies have shown that the endonuclease activity of XP-D (the mammalian homologue of RAD2) is required for this repair process (11).

The yeast proliferating cell nuclear antigen (PCNA) is the processivity factor for DNA polymerases δ and ε. It is a homotrimer with a subunit molecular mass of 29 kDa and is highly conserved from yeast to mammalian cells. The crystal structure of yeast PCNA shows that the trimer forms a closed ring with the appropriate dimensions and electrostatic properties to encircle double-stranded DNA and to interact with it using nonspecific contacts (12). Processivity in DNA synthesis is achieved by protein-protein interactions between PCNA and the polymerase, thereby tethering the DNA polymerase at the primer terminus (13). In addition to this structural function during the elongation phase of DNA replication, mammalian PCNA, through its interactions with the cyclin-dependent protein kinase inhibitor p21 (CIP1/WAF1/SDI1), has also been implicated in cell cycle control (14, 15). In this communication we show that PCNA physically interacts with FEN-1 and sequesters it to its site of action, thereby stimulating the activity of FEN-1 10- to 50-fold.

**EXPERIMENTAL PROCEDURES**

Materials—The yeast strains used were PY26 (Matα, ura3–52, leu2–3, 112, trp1Δ1, prb1–1122, prc1–407, pap4–3, snu1–1:CEN LEU2) and its Jrr1 derivative PY59 (as PY26, but Jrr1::HIS3; which was made by insertional activation of the RTH1 gene using plasmid pR2.10 (3). Strains were grown and extracts prepared and concentrated with 0.35 g/ml ammonium sulfate as described (16). E. coli single-stranded DNA binding protein, yeast FEN-1, and yeast PCNA were overproduced in E. coli and purified as described (1, 17). Yeast replication factor C (RF-C) was purified as described (18). PCNA or bovine serum albumin was coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer’s protocol. Beads contained 3 mg of PCNA/ml of beads or 4 mg of BSA/ml of beads.

Two-hybrid Analysis—The entire POL30 gene or pol 30–52 gene (containing the S115P mutation) was fused to the bacterial LexA DNA binding domain in vector pCh435 (17). Screening of a library of yeast cDNAs fused to the GAL4 activation domain using the lea-pol30–52 construct as bait was carried out essentially as described (19). In the one positive RTH1 isolate obtained, fusion with the activation domain of GAL4 occurred at amino acid 81. The strength of the interaction was 2.5 units of β-galactosidase activity for lea-POL30 and 2.2 units for lea-pol30–52, whereas negative controls were 0.5–0.8 units of β-galactosidase activity.

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from the PCNA beads was accomplished at 0.6 M NaCl, indicating that salt bridges contribute substantially to the PCNA-
yFEN-1 interaction. Subsequent treatment with ionic deter-
gents did not release additional yFEN-1 (Fig. 1A). The most straightforward conclusion of these experiments is that PCNA must encircle the double-stranded DNA in order to stimulate yFEN-1, and the mutant hybrid method, an excess of the latter mutant protein also blocked the binding of yFEN-1 to PCNA beads. Finally, we showed that the beads can bind yFEN-1 from crude yeast extracts. That the species detected on the Western blot is yFEN-1 follows from its comigration with purified yFEN-1 and the absence of a signal with Δthr1 extracts (Fig. 1C). Although yFEN-1 is not the major polypeptide species from crude extracts bound to PCNA beads, it can be detected as a distinct band on silver stained gels, which is absent when extracts are used from an isogenic Δthr1 strain (data not shown).

The functional interaction between yFEN-1 and PCNA was probed with DNA substrates that are probable intermediates in DNA end joining (flap structures, Fig. 2A) and in DNA replication (nicked duplexes). On a model flap structure, PCNA stimulated the activity of yFEN-1 about 10-fold (Fig. 2B) based on PhosphorImager quantitation. It is important to note that, in contrast to assays with nicked substrates described below, only PCNA and yFEN-1 were added in this assay. Therefore, stimulation of yFEN-1 activity can be directly attributed to its interaction with PCNA rather than with other accessory factors. Stimulation requires that PCNA is a trimer. The pcna-52 mutant exists as a monomer in solution (17). Despite the fact that this mutant protein exhibits a similar affinity for yFEN-1 as wild-type PCNA, it failed to stimulate yFEN-1 activity (Fig. 2C; see also Fig. 1B). The measured interaction signals between POL30-lexA or pol30-52-lexA and RTH1 fused to the GAL4 activation domain were identical and weak (see “Experimental Procedures”). As the two-hybrid method may detect both direct and indirect interactions, we turned to biochemical methods to investigate a possible interaction between yFEN-1 and PCNA.

The existence of a specific protein-protein interaction between yFEN-1 and PCNA was confirmed by affinity chroma-
tography on PCNA beads. Yeast FEN-1 bound specifically to PCNA beads but not to control BSA beads (Fig. 1A). Elution from the PCNA beads was accomplished at 0.6 M NaCl, indicating that salt bridges contribute substantially to the PCNA-FEN-1 interaction. Subsequent treatment with ionic deter-
gents did not release additional yFEN-1 (Fig. 1A). The specificity of the interaction was demonstrated by the observa-
tion that an excess of PCNA blocked binding of yFEN-1 to the beads (Fig. 1B). As expected from the observed in vivo interaction between yFEN-1 and the mutant pcna-52 by the two-
form is unable to do so. Because this mode of PCNA loading occurs by nonspecific diffusion onto DNA ends, a large excess is required to observe substantial stimulation (21). Thus, at the lowest concentration of PCNA tested (0.02 μg), which represents a 20-fold molar excess over DNA substrate, very little stimulation was observed (Fig. 2C). RF-C is required for the efficient loading of PCNA at primer termini in an ATP-dependent manner (18, 22–24). If RF-C would efficiently and appropriately load PCNA at flap structures, one would expect to observe stimulation of yFEN-1 activity at PCNA levels stoichiometric with DNA substrate. However, inclusion of RF-C and ATP in the nuclease assay did not give a significant further stimulation of yFEN-1 beyond that observed by PCNA alone, either at high concentrations of PCNA (Fig. 2B) or at low concentrations of PCNA, which show only minimal stimulation (data not shown). Possibly, RF-C fails to recognize the flap structure as a docking site for PCNA. Or, alternatively, PCNA loaded by RF-C is on the wrong double-stranded side of the flap structure necessary for yFEN-1 stimulation. These observations with the yeast enzymes were extended to the human system. Human PCNA stimulated the endonucleolytic activity of human FEN-1 on the flap structure substrate to a similar degree (data not shown).

In contrast to these results with flap substrates, we observed no obvious stimulation of yFEN-1 activity by PCNA on model oligonucleotides containing a nick (data not shown).² PCNA is known to slide rapidly across linear DNA molecules (25). As the more rapid sliding of PCNA across these small linear double-stranded DNA substrates, in comparison to the sterically hindered flap substrates, might not provide a significant mean residence time for PCNA in order to interact productively with yFEN-1, we turned to circular DNA substrates from which PCNA, once loaded, would not be able to dissociate (Fig. 3). RF-C and ATP are absolutely required to load PCNA at primer termini of circular substrates (21). In agreement with these known properties of PCNA, we observed stimulation of yFEN-1 activity by PCNA but only if RF-C and ATP were also present. Interestingly, the inclusion of salt (75 mM NaCl) in the assay revealed the functional interaction between PCNA and yFEN-1 most profoundly. As PCNA is efficiently loaded by RF-C onto the DNA at physiological salt levels, it in turn is capable of loading yFEN-1 and hence stimulating its activity (Fig. 3). In the absence of PCNA, yFEN-1 fails to interact with the DNA at these salt concentrations, and a modification of the assay to very low salt and magnesium concentrations is essential to detect yFEN-1 activity at nicks (5). No stimulation was observed with the monomeric mutant pcna-52 (data not shown).

The interaction between FEN-1 and PCNA may have bearing on the structure of the lagging strand DNA replication complex. Because FEN-1 does not negatively affect DNA synthesis by DNA polymerase δ or ε holoenzyme (i.e., does not compete with these polymerases for binding to PCNA),³ it may form an integral part of this complex and mediate coupled synthesis and maturation of Okazaki fragments (Fig. 4). In the presence of DNA polymerase δ or ε, the complex would carry out nick translation until DNA ligase I seals the nick (9). In mammalian cells, an RNase H is also required for in vitro Okazaki fragment maturation (7, 26). However, it is not known at this time whether this enzyme forms an integral component of this maturation complex.

In addition to replication, the interaction between FEN-1 and PCNA may have broader implications in DNA metabolism as well. In nucleotide excision repair, for example, PCNA has been shown to play an important role (27, 28). Although FEN-1 likely is not involved in this reaction, a highly homologous structure-specific nuclease, XP-G or RAD2, is absolutely required. Based on the involvement of PCNA in nucleotide excision repair and the presence of homology between FEN-1 and RAD2, it is possible that PCNA may also interact with RAD2 to facilitate its loading and thereby excision of damaged nucleotides. Thus, there may be a common theme in various aspects of DNA metabolism, in addition to DNA replication, in which a processivity factor stimulates a structure-specific nuclease in processing nicked and branched DNA intermediates.

Based on the studies here, we conclude that the functional interaction between PCNA and FEN-1 is important in the orchestration of lagging strand processing at the eukaryotic DNA replication fork. The interaction of PCNA with other FEN-1 family members may be generally important to a wide variety of transactions involving branched DNA intermediates.

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² PCNA stimulation assays were carried out at various salt levels, either with or without RF-C and ATP. The maximum observed stimulation was about 1.5-fold.

³ DNA polymerase δ holoenzyme assays were carried out as described in Ref. 18 with subsaturating levels of PCNA (3 ng, 35 fmol). Addition of a 20-fold molar excess of FEN-1 (30 ng, 700 fmol) inhibited the reaction by about 10%.
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