AP Endonuclease 1 Coordinates Flap Endonuclease 1 and DNA Ligase I Activity in Long Patch Base Excision Repair*

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Running Title: APE Stimulates FEN1 and DNA Ligase I
Summary

Base loss is common in cellular DNA, resulting from spontaneous degradation and enzymatic removal of damaged bases. Apurinic/apyrimidinic (AP) endonucleases recognize and cleave abasic (AP) sites during base excision repair (BER). APE1 (REF1, HAP1) is the predominant AP endonuclease in mammalian cells. Here we analyzed the influences of APE1 on the human BER pathway. Specifically, APE1 enhanced the enzymatic activity of both flap endonuclease1 (FEN1) and DNA ligase I. FEN1 was stimulated on all tested substrates, regardless of flap length. Interestingly, we have found that APE1 can also inhibit the activities of both enzymes on substrates with a tetrahydrofuran (THF) residue on the 5’-downstream primer of a nick, simulating a reduced abasic site. However once the THF-residue was displaced at least a single nucleotide, stimulation of FEN1 activity by APE1 resumes. Stimulation of DNA ligase I required the traditional nicked substrate. Furthermore, APE1 was able to enhance overall product formation in reconstitution of BER steps involving FEN1 cleavage followed by ligation. Overall, APE1 both stimulated downstream components of BER and prevented a futile cleavage and ligation cycle, indicating a far-reaching role in BER.

Abbreviations

RPA, replication protein A; BER, base excision repair; AP, apurinic/apyrimidinic; PCNA, proliferating cell nuclear antigen; FEN1, flap endonuclease1; APE1, apurinic/apyrimidinic endonuclease 1; THF, tetrahydrofuran; dRP, deoxyribose phosphate; nt, nucleotide
Introduction

Chromosomal DNA is regularly damaged by reactive oxygen species generated during cellular metabolism and exposure to damaging environmental agents such as ultraviolet light or ionizing radiation. If not efficiently repaired, this damage can disrupt critical cellular processes such as DNA replication or transcription. The pathway responsible for repairing the most frequent types of DNA damage, which cause chemical alteration of nucleotide bases, is base excision repair. Repair is initiated by the action of a damage-specific DNA N-glycosylase that is responsible for the recognition and removal of an altered base through cleavage of the N-glycosylic bond (1,2). Removal generates an apurinic/apyrimidinic (AP) site, a non-coding DNA lesion that is both cytotoxic and mutagenic. The abasic site is the substrate for an AP endonuclease. APE1 (also called HAP1/REF1/APE) is a multifunctional enzyme that hydrolyzes the phosphodiester bond 5’- to an abasic site and is the predominant AP endonuclease in mammalian cells (3-8). Cleavage by APE1 generates a 3’-OH terminus suitable for extension by a DNA polymerase. The resulting 5’-terminus contains a deoxyribose phosphate residue (dRP), which must be removed and replaced in order to complete repair. Following APE1 cleavage, BER can then proceed via two pathways, designated short or long patch repair (2,9-15). In the short patch repair pathway DNA polymerase β adds a single nucleotide and also cleaves the 5’- deoxyribose phosphate residue using an intrinsic deoxyribosephosphate lyase (dRP lyase) activity (16-18). A DNA ligase then seals the nick to complete repair. This generates a repair product wherein only the damaged nucleotide is replaced.

In vivo, a portion of the AP sites become oxidized or reduced prior to repair (13-15), rendering them resistant to cleavage by DNA polymerase β. This necessitates the removal of the damage through long patch BER. It has recently been shown that DNA
polymerase β initiates polymerization in long patch BER by synthesizing a single nucleotide. Polymerase β is proposed then to dissociate and be replaced by DNA polymerase δ/ε for strand displacement synthesis of up to an additional 10 nucleotides, generating a single-stranded flap (18,19). In the absence of polymerase δ/ε, polymerase β is able to rebind and complete synthesis (18). The single strand is removed by flap endonuclease1 (FEN1) to generate a nick. The nick is then sealed by DNA ligase I to complete repair (20,21). The long patch repair pathway results in the removal and replacement of 2-10 nucleotides.

Significantly, many of the protein components involved in long patch BER are also components of the DNA replication machinery, providing a mechanistic link between BER and DNA replication complexes (22-27). The replication proteins proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) were shown to increase the efficiency of BER reconstituted in vitro (28-31). In addition to its role in stimulating DNA polymerase δ, PCNA has been shown to directly interact with both FEN1 and DNA ligase I and stimulate their activities (32-37). The mechanism underlying PCNA directed stimulation of each of these components has been investigated thoroughly, and shown to be mediated through an increase in enzyme-substrate binding (36,37). Moreover, PCNA is believed to provide a DNA targeting function for both replication and repair protein complexes, in which it coordinates sequential protein-protein interactions in order to facilitate enzymatic activity. RPA has been shown to enhance ligation by a unique mechanism involving the direct stimulation of DNA ligase I activity (38).

We have recently shown that the addition of the damage inducible cellular factor p21Cip1,Waf1,Sdi1 prevents PCNA-dependent enhancement of long patch BER in vitro (39). The p21 protein has been shown to bind tightly to PCNA precluding interaction with polymerase δ/ε, FEN1, or DNA ligase I. While this is necessary for complete
inhibition of DNA replication activity during periods of DNA damage, the long patch repair process is needed during such periods. We have proposed that BER can tolerate the loss of PCNA because APE1 can stimulate and coordinate the activities of the BER protein components.

A systematic approach to determining the role of APE1 in the facilitation of BER is to examine its effect upon individual enzymatic steps downstream of the 5'-incision event. Interactions between APE1 and DNA polymerase β have been previously analyzed (17). APE1 was found to facilitate loading of polymerase β onto an incised substrate. Furthermore, during short patch repair APE1 enhanced removal of the dRP-residue by polymerase β without altering polymerization activity. The role of APE1 in long patch BER is still being determined. APE1 was initially shown to directly interact with FEN1 and facilitate FEN1 cleavage in the presence of DNA polymerase β (40), however, the mechanism of stimulation was not determined. We report here that APE1 interacts with both FEN1 and DNA ligase I to stimulate their activities. Furthermore, APE1 coordinates the activities of the nuclease and ligase so that they are most effective on the appropriate substrates for correct stepwise progression through the repair pathway.
Experimental Procedures

Materials—Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Radionucleotides [$\gamma$-32P]ATP (3000 Ci/mmol) and [$\alpha$-32P]dCTP (3000 Ci/mmol) were obtained from Perkin-Elmer Life Sciences. T4 polynucleotide kinase was from Roche Diagnostics, and Sequenase (version 2.0) was from Amersham Biosciences, Inc. Micro Bio-Spin 30 chromatography columns were obtained from Bio-Rad Laboratories. All other reagents were the best available commercial grade.

Preparation of Enzymes/Proteins—Recombinant human DNA ligase I, FEN1, and APE1 were overexpressed and purified as described previously (28,39,41). Purified DNA ligase I was dialyzed into storage buffer (30 mM HEPES (pH 7.5), 10% glycerol, 15% sucrose, 25 mM KCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, and 1 mM EDTA) and stored at -80°C. Purified human APE1 was dialyzed into storage buffer (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, 0.1 mM DTT, 1 mM EDTA, and 10% glycerol) and stored at -80°C.

Oligonucleotide Substrates—Oligomer sequences are listed in Table I, and the primer-template substrates for each individual experiment were constructed as described in the figure legends. In all substrates, the 3’-end regions of the downstream primers share homology with the 5’-ends of their respective templates. The 5’-radioactive end-labeled primers were generated by incubating the oligonucleotide with [$\gamma$-32P]ATP and T4 polynucleotide kinase for 1 hour at 37°C. Unincorporated radionucleotides were removed using Micro Bio-Spin 30 chromatography columns. For substrates that were 3’-labeled, the downstream primer was annealed to the template prior to being radiolabeled using Sequenase (version 2.0) and [$\alpha$-32P] dCTP. The radiolabeled primers
were then purified on a 15% polyacrylamide, 7 M urea denaturing gel. To generate a nick substrate, the respective upstream primer was annealed to the proper template to create a nick between the 3-end of the upstream primer and the 5'-end of the downstream primer. In the case of the flap substrate, the downstream primer creates an unannealed 5'-flap of varying lengths. Substrates were annealed by mixing 1 pmol of the respective downstream primer with 5 pmol of the corresponding template in annealing buffer (10 mM Tris base, 50 mM KCl, and 1 mM EDTA (pH 8.0)) to a final volume of 25 µl. The mixture was heated to 65°C for 10 min and allowed to cool to room temperature. Finally, 10 pmol of the corresponding upstream primer was added and annealed by incubating at 37°C for 1 hour.

**Enzymatic Assays**—Reactions containing the indicated amounts of radiolabeled substrate and enzymes were performed in a buffer consisting of 30 mM HEPES (pH 7.6), 40 mM KCl, 0.01% Nonidet P-40, 0.1 mg/ml bovine serum albumin, and 8 mM MgCl₂. For the DNA ligase assays 0.1 mM ATP was also included. The reaction volume was 20 µl unless otherwise indicated. Reactions were incubated at 37°C, terminated with 15 µl of formamide dye (90% formamide (v/v) with bromophenol blue and xylene cyanol), and heated to 95°C for 5 minutes. All assays were terminated at 3 minutes unless noted in the figure legends. Upon separation on a 15% polyacrylamide, 7 M urea denaturing gel, products were detected by PhosphorImager (Molecular Dynamics) analysis. All assays were performed at least in triplicate.
Results

APE1 enhances both the exo- and endonucleolytic activity of FEN1—Previous studies have demonstrated that APE1 can greatly enhance the overall efficiency of long patch BER reconstituted in vitro, most notably in the absence of PCNA (39). APE1 was proposed to serve as a coordinator for the long patch BER enzymes (39). Additionally, APE1 was found to enhance flap removal by FEN1 in the presence of DNA polymerase β by approximately 3-fold (40). In order to gain a better understanding of the mechanism underlying this latter function of APE1, we have reconstituted cleavage reactions containing only APE1 and FEN1.

Fig. 1 shows a titration of APE1 into FEN1 cleavage assays using either a nick (lanes 1-6) or nick-1nt flap substrate (lanes 7-12). In the absence of FEN1, APE1 had no effect on these substrates (data not shown). The reactions were performed in substrate excess so that the amount of product formation is indicative of the initial rate of reaction. FEN1 cleavage of the nick substrate generates a 1-nt product and FEN1 cleavage of the nick-1-nt flap substrate generates both 2-nt and 1-nt products. Differences in product mobility are the result of two different nucleotides being released. Upon addition of APE1 to the FEN1 assay, an enhancement of product formation was observed (lanes 2-6 and 8-12). For both the nick and 1-nt flap substrate, there was a consistent increase in product formation and thus fold-stimulation, with increasing concentrations of APE1. This indicates that APE1 is able to stimulate both FEN1 exonucleolytic and endonucleolytic activities. APE1-mediated stimulation of FEN1 cleavage was maximally 10-fold as compared to reactions lacking APE1. Interestingly, the addition of APE1 did not alter the cleavage specificity of FEN1 on these substrates, merely increased the amount of product formed, implying that APE1 is not able to significantly change substrate positioning within the FEN1 active site.
APE1 does not appear to enhance FEN1 cleavage by altering substrate structure—We have recently shown that the preferred cellular substrate for FEN1 involves the formation of a double-flap at the cleavage junction rather than a nick-flap (42). The double flap is created when one nucleotide of the downstream flap reanneals and displaces the 3'-terminal nucleotide of the upstream primer. Interaction of APE1 with damaged DNA is proposed to induce a bend of approximately 35° (43). We considered that APE1 might stimulate cleavage by melting the substrate into a configuration with short flaps resembling the natural double flap that serve as better FEN1 substrates. To examine this possibility we compared APE1 stimulation of FEN1 activity on a series of substrates containing adjacent bound primers (Fig. 2A). The primer lengths were the same but they differed in that the terminal nucleotides of one or both primers adjacent to the nick were unannealed. If APE1 created one of these substrate configurations, then APE1 stimulation would be eliminated or significantly decreased on substrates already possessing the preferred structures. FEN1 alone was generally more active on the substrates with flaps (compare lane 3 with lanes 10, 17, and 24). Titration of APE1 into each of these reactions resulted in a similar degree of stimulation of product formation over the levels produced by FEN1 alone.

Fig. 2B shows time courses of FEN1 cleavage using the same substrates as utilized in Fig. 2A. For these experiments, APE1 and FEN1 concentrations remained fixed, and substrate was provided in excess in order to allow multiple reaction cycles. The presence of APE1 in the assays (lanes 5-8, 13-16, 21-24, and 29-32) continuously stimulated cleavage throughout the time course. Quantitation of the data indicates that FEN1 stimulation ranged between 5-10 fold. These results show that the stimulation is not dependent on a specific substrate structure and occurs over multiple reaction cycles.

APE1 stimulates FEN1 activity on increasing flap lengths—Strand displacement
synthesis by DNA polymerase δ can create different length flaps during long patch BER. These can then reconfigure into the preferred double flap structure. We measured FEN1 cleavage and APE1 stimulation on substrates with this flap configuration and different flap lengths. Substrates were created with either a nick or a one, two, or six nucleotide flap. They were prepared using a fixed length downstream primer and one of four upstream primers having an increasing length of overlap with the downstream primer. The overlapping flap substrates can equilibrate into a variety of flap configurations, but we have previously shown that only the one with the single nucleotide 3′-flap is the primary FEN1 substrate. We determined FEN1 cleavage and the effects of increasing concentrations of APE1 on each of the four substrates (Fig. 3A). FEN1 activity alone was generally higher on double flap substrates as compared to the nick substrates (best seen by disappearance of starting substrate), as we had found earlier (42). Increasing concentrations of APE1 progressively stimulated the flap cleavage reaction (lanes 4-7, 11-14, 18-21, 25-28).

Notably, APE1 did not change the cleavage specificity. In each case, FEN1 captured the equilibration intermediate with a one nucleotide 3′-flap and cleaved one nucleotide into the annealed region of the downstream primer. Since the label is at the 5′-end of the downstream primer, this should and did yield labeled products one, two, or six nucleotides in length for the different flap substrates. The patterns of products increased in intensity with progressive addition of APE1 but did not change in size.

**APE1 alters FEN1 activity differently on abasic substrates** —The previous experiments were carried out utilizing substrates containing a 5′-terminal phosphate residue. We also were interested in whether there would be a difference in APE1 stimulation when using substrates containing a 5′-terminal abasic residue. We chose to address this question by using a tetrahydrofuran (THF) residue, which has been shown to...
simulate a naturally reduced abasic site. Such a site would be a typical intermediate of long patch base excision repair \textit{in vivo}. APE1 is able to recognize and cleave 5’- to a THF residue embedded within a DNA oligonucleotide (44). It has also previously been shown that FEN1 cleavage is not dependent upon strict recognition of the 5’-terminal nucleotides, as other similarly sized structures do not inhibit FEN1 cleavage (45).

Fig. 3B compares FEN1 cleavage activity on THF substrates with either a nick or a one, two, or six residue 5’-flap. These have the same basic structure as the previous set of equilibrating substrates, except that a THF is the 5’-terminal residue of the downstream primer. They would represent structures made by a reduced substrate after cleavage by APE1 and then extension of the upstream primer by zero, one, two, or six nucleotides. FEN1 alone was able to cleave each of the substrates (lanes 3, 10, 17, 24). FEN1 cleavage of the nick-THF residue occurred at a position 2 residues in from the 5’-end (lanes 2-7). Since FEN1 is unable to cleave immediately adjacent to an abasic residue (22), it cleaves at the next available site. Interestingly, upon addition of APE1 to these assays, this cleavage product was greatly reduced rather than stimulated (lanes 4-7). Here, APE1 interaction, presumably with the substrate, prevents rather than stimulates FEN1 activity. For substrates with one, two, and six residue flaps, addition of APE1 produced levels of stimulation similar to those seen with assays done using substrates containing 5’-terminal phosphate residues. Another difference from the unmodified substrate is in the size of cleavage products generated by FEN1. Cleavage of the THF-containing substrate generated predominantly a 2-residue cleavage product for the nick, 1nt-flap, and 2nt-flap structures, while cleavage of the 5’-terminal phosphate containing substrates generated a 1-nucleotide product for the nick and 1nt-flap and a 2-nucleotide product for the 2nt-flap. Both sets of substrates primarily generated a 6-nucleotide cleavage product for the 6nt-flaps. Differences in cleavage specificity are likely due to a substrate-dependent shift in the positioning of the active site of the FEN1 enzyme.
Specificity did not appear to be influenced by the addition of APE1 to the reactions. Overall, these results show that the structures of the THF substrates influence the cleavage specificity of FEN1 and the way in which APE1 influences FEN1 activity.

Fig. 3C shows time courses of reactions directly comparing the reaction rates for the 1-nt, 2-nt, and 6-nt flaps containing either a 5'-terminal phosphate or 5'-terminal THF residue in the absence or presence of APE1. In each case, the presence of a THF residue did not significantly alter the reaction kinetics for FEN1 cleavage, nor did it affect the amount of APE1 stimulation. Interestingly, at the earliest time points (0.5, 1, and 2 min) of the cleavage reaction using the 1-nt flap, there was a slightly reduced stimulation from APE1 on the THF-containing substrate. However, by the later time points the fold-stimulation was comparable to that seen using either the 2-nt or 6-nt flap substrates. Also in comparing the effects of increasing APE1 concentration at three minutes (Fig. 3B, lanes 10-14), the stimulation of FEN1 on the 1-nt THF flap substrate appears slightly less than that seen for the 2-nt and 6-nt THF flap substrates. Very likely APE1 binding to the nick or one nucleotide flap substrates interferes with the FEN1 reaction. This would promote FEN1 cleavage of longer over shorter flaps. In general, regardless of the presence of the THF-residue, APE1 stimulation of these cleavage reactions was 3-4 fold.

APE1 enhances DNA ligase I activity —We next measured the effect of APE1 on ligation, the final step of BER. We have previously shown that both PCNA and RPA (38) stimulate DNA ligase I activity through independent mechanisms (38,39). We reconstituted ligation reactions containing human DNA ligase I in the absence or presence of APE1 on a nicked substrate (Fig. 4). In the absence of DNA ligase I, APE1 had no effect on the nicked radiolabeled substrate (lane 2). Ligase I alone at a concentration of 0.06 nM joined a small amount of downstream primer (18-mer) to the
upstream primer (25-mer) to generate the 43-nt ligation product (lane 3). These reactions were done in substrate excess so that the amount of product generated would be indicative of the initial rate of the reaction. Upon titration of APE1 into the reaction, product formation was stimulated maximally 10-fold (lanes 4-8). Again, previous experiments have shown that other proteins such as single-stranded DNA binding protein (SSB) from Escherichia coli do not have stimulatory effects upon DNA ligase I. Gel mobility shift experiments using DNA ligase I and APE1 have not generated definitive results as to the effect of APE1 on ligase I binding to nicked DNA (data not shown). However, it is attractive to hypothesize that the mechanism for APE1 stimulation of both DNA ligase I and FEN1 are similar.

APE1 inhibits DNA ligase I activity on THF-nick substrates—The presence of a THF residue on the 5'-terminus of the downstream primer of a nicked substrate was shown to inhibit FEN1 cleavage activity (Fig. 3B). Additionally, gel shift assays indicate that APE1 preferentially binds to a nick-THF substrate preventing FEN1 binding and cleavage (data not shown). We sought to determine what effect APE1 had upon DNA ligase I activity on a nick-THF substrate. Ligation of a THF-terminal downstream primer by DNA ligase I during repair would be a deleterious, futile process that reverses the APE1 cleavage reaction. Fig. 5 shows a titration of APE1 into ligation reactions containing DNA ligase I (1.0 nM) with two different substrates, one containing a 5'-terminal phosphate on the downstream primer and the other containing a 5'-terminal THF on the downstream primer. These substrates are identical in sequence with the exception of the THF residue. In the absence of APE1, DNA ligase I is able to join the 28-mer downstream primers with the 25-mer upstream primers to generate a 53-mer product (lanes 1 and 7). Increasing the concentration of APE1 in the assays containing the substrate with the 5'-terminal phosphate resulted in an enhancement in ligation
product formation (lanes 2-6). The THF substrate could be ligated, but at a lower efficiency than the unmodified substrate. However, on the substrate containing a 5’-terminal THF residue, progressive addition of APE1 to the reaction increasingly inhibited and ultimately prevented ligation (lanes 8-12). Very likely APE1 limits access to the THF-containing substrate by blocking the binding of DNA ligase I. This tight interaction appears to protect abasic sites within the DNA from undesired ligation prior to their removal.

Comparison of inhibition by APE1 of FEN1 cleavage and DNA ligase I activity on THF-nick substrates—Figure 6 shows a graphical representation comparing FEN1 cleavage and DNA ligase I joining reactions on nick-THF substrates using increasing amounts of APE1. Unlike the previous assays that contained very limited levels of enzyme relative to substrate, we increased the quantity of enzyme used in order to determine the efficiency of APE1 inhibition of the cleavage or ligation reactions. In the absence of APE1, 0.5 nM FEN1 and 0.2 nM DNA ligase I were able to generate 59% and 45% conversion of substrate to product, respectively. As APE1 was titrated into these reactions, DNA ligase I activity was inhibited 2-fold at the lowest concentration of APE1 to over 40-fold at the highest concentration of APE1. FEN1 activity, on the other hand, only decreased from 59% product formation to 49% product formation at the lowest concentration of APE1. This difference in inhibition appeared to be less significant as the amount of APE1 used in the assay was increased. At the highest levels of APE1, FEN1 was further inhibited to 4% cleavage product formed, an approximate 10-fold inhibition of cleavage.

APE1 stimulates sequential cleavage and ligation—The final steps of long patch BER involve sequential reactions by FEN1 and DNA ligase I. The above results indicate
that APE1 can individually enhance the enzymatic activities of both FEN1 and DNA ligase I. We next reconstituted the final steps of BER in the presence and absence of APE1 on a model oxidized substrate to determine whether APE1 can enhance overall repair product formation. Fig. 7 compares FEN1 cleavage and DNA ligase I joining of a 6-nt flap substrate containing a 5’-terminal THF residue in the absence or presence of APE1. Ligation of this substrate requires removal of the THF-containing flap, necessitating two steps of the repair pathway. This substrate was radiolabeled at the 3’-terminus in order to monitor both the cleavage and subsequent ligation reactions. In the absence of APE1, FEN1 cleavage of the 29-nt downstream primer generated a 23-nt cleavage product (lane 7). In the presence of APE1, production of the 23-nt cleavage product was enhanced (lane 14). This resulted in an approximate 4.5-fold stimulation of product formation. Ligation of the cleavage product (23-nt) with the upstream primer generates a 54-nt repair product. In the absence of APE1, approximately 8% of the total starting material was converted to repair product (lane 21) at the 30 min time point. In the presence of APE1, 35% of the starting material was converted to repair product (lane 28). This generates approximately a 4-fold stimulation of product formation. Together these data suggest that APE1 can promote the sequential actions of two critical BER components, FEN1 and DNA ligase I, both individually and as part of the DNA repair complex in order to facilitate the efficient production of repaired DNA.
Discussion

Long patch base excision repair provides cells with the means to remove and replace damaged nucleotide bases. A characteristic of this repair pathway is that it employs many of the same proteins as lagging strand DNA replication (22-27). In mammals, these include DNA polymerase δ/ε, RPA, FEN1, and DNA ligase I. Maximum efficiency of long patch base excision repair reactions carried out both in cell extracts and using purified proteins requires the participation of the PCNA toroid. PCNA is a homotrimer that encircles double-stranded DNA (46). It was first characterized as a sliding clamp for the DNA polymerase δ/ε complex to allow for processive DNA synthesis by tethering the polymerase to DNA. Subsequent studies of interactions with other replication proteins have suggested that PCNA tethers DNA replication and repair proteins to the DNA substrate in order to increase the efficiency of the reaction (36,37).

In mammals, the cellular response to DNA damage includes the expression of p21, which binds to and inactivates PCNA. Addition of p21 was also shown to inhibit long patch BER reconstituted using purified proteins (39). The presence of the BER component APE1 was shown to stimulate steps in long patch BER occurring after APE1 incision of the abasic site. APE1 allowed an efficient completion of repair even in the absence of PCNA. This result led us to propose that aside from its role as a nuclease, APE1 has evolved to facilitate the steps of BER in a way that can partially compensate for the lack of PCNA (39).

A more complex role for APE1 is suggested from other results. Aside from its primary role in the recognition and 5’-cleavage of abasic sites, APE1 has been shown to actively displace the DNA glycosylase from a damage site (47-49). Additionally, APE1 is able to facilitate the loading of DNA polymerase β onto the incised substrate and to
enhance polymerase β dRPase activity (17). It has also recently been shown that APE1 is able to interact with PCNA (40). This interaction, which has no functional consequence on APE1 activity, may be a way to directly recruit the long patch BER components to sites of APE1 bound to damaged DNA (40). The significance of APE1 is also highlighted by results showing that a knock-out of APE1 in mice is lethal (50).

Here we have explored the influence of APE1 on the final two steps of long patch BER, flap cleavage by FEN1 and joining of the strands by DNA ligase I. Our results show that both reactions are stimulated by the presence of APE1. FEN1 has also been previously shown to directly interact with APE1 in vitro (40).

Examining the FEN1 cleavage reaction first, we found that APE1 displayed a similar level of stimulation for both the endo- and exonucleolytic activities of FEN1. Also, the sequence of the substrate did not influence the stimulation. Since APE1 binding of the substrate induces a bend, we considered that it may have effected stimulation by altering the nick substrate structure to create a double-flap. However, when such a structure was preformed on the substrate, stimulation was still observed. Natural substrates have equilibrating double flaps. APE1 also stimulated cleavage on these structures. Overall these results indicate that the stimulatory process is not dependent on either the flap configuration or length.

Cleavage of a double-flap structure, the natural substrate of FEN1, produces a nick that is a substrate for DNA ligase. DNA ligase I, thought to conduct the final ligation step in long patch BER, was also stimulated by the presence of APE1. DNA ligase I has a high affinity for a nick site, making it unlikely that APE1 would be able to effectively compete with ligase for that binding site. It is therefore unlikely that DNA ligase I was held on at the nick by APE1. We hypothesize that the stimulation was a result of a direct interaction of APE1 with the ligase.

The interplay of proteins becomes more complex on THF substrates that represent
repair intermediates expected \textit{in vivo}. A nick substrate with a THF at the 5’-side of the nick represents a natural intermediate of repair formed just after APE1 cleavage of an abasic site. THF represents a nucleotide that has been stripped of its base and reduced. The THF structure is not recognizable to FEN1 as a nucleotide. Although the THF does not prevent FEN1 cleavage from occurring, it does alter the way in which FEN1 deals with the substrate. FEN1 cannot cleave between the THF and the adjacent nucleotide residue and must instead cleave between the next recognizable nucleotides (22). Inability to cleave off the THF does not present a problem in long patch BER since polymerization displaces the THF into a flap. The flap then reconfigures to a double flap. FEN1 cleaves at the base of the 5’-flap to produce a nick, which is subsequently ligated.

The presence of APE1, which is normally stimulatory, was strongly inhibitory to both FEN1 and DNA ligase I on the nick-THF substrate. Since it is unnecessary for FEN1 to cleave this substrate, the inhibitory effect would not influence BER. Significantly, DNA ligase I is capable of resealing the THF nick, reversing the initial APE1 cleavage. This highly undesirable reaction creates a futile cleavage and ligation cycle that would short circuit the repair process. APE1-directed inhibition of the ligation reaction coordinates steps in the repair process to ensure that the THF lesion is removed before ligation can occur. Once the THF is displaced into a flap, APE1 stimulation of FEN1 activity is restored. FEN1 can then create a lesion-free nick for ligation.

The final experiment examined the effect of APE1 on the last two steps of long patch BER beginning with a THF-terminated flap. With just FEN1, an enhancement of cleavage was seen. When the reaction contained both FEN1 and DNA ligase I, both cleavage and ligation were enhanced. Increase in final product formation was not as great as would be expected from the amounts of stimulation of the FEN1 and the DNA ligase I reactions when measured alone. This may be because the mechanism of FEN1 stimulation involves higher affinity binding of the nuclease to the substrate. Thus, FEN1
binds the substrate in addition to the nick product, better in the presence of APE1. Because of the enhanced binding of FEN1 to the nick product, it may not cycle off quickly to allow DNA ligase binding. However, the overall effect of APE1 is to stimulate the rate of final repaired DNA product formation.

In summary, APE1 does not act merely as a component of the long patch BER pathway, but also as a facilitator and coordinator of most of the steps. It was previously found to displace the DNA glycosylase from the abasic site prior to cleavage (47-49) and to facilitate loading of DNA polymerase β (17). We show here that it blocks re-ligation of a damaged site but promotes later flap cleavage and ligation of an unmodified nick. The exact mechanisms by which APE1 stimulates both FEN1 and DNA ligase I are still under investigation.

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Figure Legends

Fig. 1. **Enhancement of FEN1 exonucleolytic and endonucleolytic activity by APE1.**
Reactions of 20 µl containing 10 fmol of DNA substrate and 0.5 fmol of FEN1 were performed as described under Experimental Procedures. The substrate is comprised of either D1:T1:U1 (*lanes 7-12*) or D5:T1:U1 (*lanes 1-6*) (see Table I) containing a $\gamma^32P$ radiolabel at the 5-end of the upstream primer (as indicated by the asterisks). *Lanes 1 and 7* contain FEN1 and substrate. *Lanes 2-6 and 8-12* contain APE1 in addition to FEN1 and substrate. The following amounts of APE1 were added (as indicated by the triangles): 1.0, 2.0, 5.0, 10, and 50 fmol. The reactions were incubated at 37°C for 3 min. Substrate and product sizes are as indicated. Schematic representations of the substrates are located above the figure. FEN1, flap endonuclease 1; APE1, AP endonuclease 1.

Fig. 2. **APE1 stimulation of FEN1 activity is not mediated through alterations of substrate structure.** A, reactions of 20 µl containing 5 fmol of DNA substrate and 0.5 fmol of FEN1 (*where indicated*) were performed as described under Experimental Procedures. *Lanes 1-7* contain a nick substrate comprised of D2:U2:T2. *Lanes 8-14* contain a gap-1nt 5-flap substrate comprised of D4:U2:T2. *Lanes 15-21* contain a gap-1nt 3-flap substrate comprised of D2:U6:T2. *Lanes 22-28* contain a gap-1nt 5-and 3-flap substrate comprised of D4:U6:T2. Each substrate contains a $\gamma^32P$ radiolabel at the 5-end of the downstream primer (as indicated by the asterisks). *Lanes 1, 8, 15, and 22* contain only substrate. *Lanes 2, 9, 16, and 23* contain both substrate and 25 fmol of APE1. *Lanes 3, 10, 17, and 24* contain substrate and 0.5 fmol of FEN1. *Lanes 4-7, 11-14, 18-21, and 25-28* contain substrate, FEN1, and increasing amounts of APE1 (1.0, 5.0, 10, and 25 fmol) as indicated by the triangles. Reactions were performed at 37°C for 3 min. Substrate and product sizes are as indicated on the gel. Schematic representations
of the substrates are located above the figure. B, time-course assays (0.5, 1.0, 3.0 and 5.0 min as indicated by the triangles) were performed using 25 fmol of DNA substrate and 2.5 fmol of FEN1 in 100 µl reactions in the absence or presence of APE1 (25 fmol) as described in Experimental Procedures. Reactions were incubated at 37°C, and aliquots of 20 µl were removed at the times listed above. Lanes 1-8 contain substrate comprised of U2:D2:T2. Lanes 9-16 contain substrate comprised of U2:D4:T2. Lanes 17-24 contain substrate comprised of U6:D2:T2. Lanes 25-32 contain substrate comprised of U6:D4:T2. Substrate and reaction product sizes are as indicated on the gel. Schematic representations of the substrates are located above the figure. FEN1, flap endonuclease 1; APE1, AP endonuclease 1.

Fig. 3. APE1 stimulates FEN1 activity on flaps of increasing length. A, reactions of 20 µl containing 5 fmol of DNA substrate and 0.5 fmol of FEN1 (where indicated) were performed as described in Experimental Procedures. Lanes 1-7 contain substrate comprised of U2:D2:T2. Lanes 8-14 contain substrate comprised of U3:D2:T2. Lanes 15-21 contain substrate comprised of U4:D2:T2. Lanes 22-28 contain substrate comprised of U5:D2:T2. In each case, flaps are formed by increasing the length of the upstream primer with sequences complimentary to the template. The 5′-terminus of the downstream primers is radiolabeled with γ\(^{32}\)P. Lanes 1, 8, 15, and 22 contain only substrate. Lanes 2, 9, 16, and 23 contain substrate and APE1 (25 fmol). Lanes 3, 10, 17, and 24 contain substrate and FEN1 (0.5 fmol). Lanes 4-7, 11-14, 18-21, and 25-28 contain substrate, FEN1 (0.5 fmol) and increasing amounts of APE1 (1.0, 5.0, 10 and 25 fmol) as indicated by the triangles. Reactions were performed at 37°C for 3 min. Substrate and reaction product sizes are as indicated on the gel. B, reactions of 20 µl containing 5 fmol of DNA substrate and 0.5 fmol of FEN1 (where indicated) were performed as described in “Experimental Procedures”. Substrates in this assay contained
identical sequences as those in Fig. 3A with the exception that the 5'--terminal residue of the downstream primers all contain a THF in place of the nucleotide representing an oxidized abasic residue. Lanes 1-7 contain substrate comprised of U2:D3:T2. Lanes 8-14 contain substrate comprised of U3:D3:T2. Lanes 15-21 contain substrate comprised of U4:D3:T2. Lanes 22-28 contain substrate comprised of U5:D5:T2. Reactions were performed at 37°C for 3 min. Lanes 1, 8, 15, and 22 contain only substrate. Lanes 2, 9, 16, and 23 contain substrate and APE1 (25 fmol). Lanes 3, 10, 17, and 24 contain substrate and FEN1 (0.5 fmol). Lanes 4-7, 11-14, 18-21, and 25-28 contain substrate, FEN1, and increasing amounts of APE1 (1.0, 5.0, 10, and 25 fmol) as indicated by the triangles. Substrate and reaction product sizes are indicated on the gel. C, reactions of 180 µl containing 45 fmol of DNA substrate and 4.5 fmol of FEN1 in the absence or presence of APE1 (90 fmol) were performed as described in Experimental Procedures. Reactions were performed at 37°C and 20 µl aliquots were removed at 0, 0.5, 1.0, 2.0, 3.0, 5.0, 10, and 15 min. Substrates compared were 1nt-THF (U3:D3:T2) to 1nt-REG (U3:D2:T2), 2nt-THF (U4:D3:T2) to 2nt-REG (U4:D2:T2), and 6nt-THF (U5:D3:T2) to 6nt (U5:D2:T2). The 3-end of the downstream primer was radiolabeled with α↑32P. Graphical analysis is presented of the conversion of substrate to cleavage product (% cleaved) as a function of time (min) for FEN1 alone on regular substrates (triangles), FEN1 alone on THF substrates (diamonds), FEN1 and APE1 on regular substrates (crosses or circles), and FEN1 and APE1 on THF substrates (squares). The amount of substrate cleaved to product was determined by quantitating the amount of substrate and product on denaturing polyacrylamide gels using PhosphorImager analysis. FEN1, flap endonuclease 1; APE1, AP endonuclease 1; THF, tetrahydrofuran.

Fig. 4. APE1 stimulates DNA ligase I activity. Reactions of 20 µl containing 5 fmol of nicked DNA substrate and 1.2 fmol of DNA ligase I were performed as described in
Experimental Procedures. The substrate is comprised of \textit{U}1:D1:T1 containing a $\gamma^{\text{32P}}$ on the downstream primer as indicated by the asterisk. \textit{Lane 1} contains only substrate. \textit{Lane 2} contains substrate and APE1 (25 fmol). \textit{Lane 3} contains substrate and DNA ligase I (1.2 fmol). \textit{Lanes 4-8} contain DNA substrate, DNA ligase I (1.2 fmol), and increasing amounts of APE1 (1.0, 2.0, 5.0, 10, and 25 fmol) as indicated by the triangle. Reactions were performed at 37°C for 2 min. Substrate and ligation product sizes are as indicated on the gel. Schematic representation of the substrate is located above the figure. APE1, AP endonuclease 1.

Fig. 5. \textbf{APE1 inhibits DNA ligase I activity on a THF-nick substrate.} Reactions of 20 µl containing 10 fmol of DNA substrate and 2 fmol of DNA ligase I were performed as described in Experimental Procedures. \textit{Lanes 1-6} contain substrate comprised of \textit{U}2:D2:T2. \textit{Lanes 7-12} contain substrate comprised of \textit{U}2:D3:T2. The 5-terminus of the downstream primers was radiolabeled with $\gamma^{\text{32P}}$. Both substrates are identical in sequence with the exception that the 5-terminal nucleotide of D3 is replaced with a THF residue to represent an oxidized abasic site. \textit{Lanes 1 and 7} contain DNA substrate and DNA ligase I (2 fmol). \textit{Lanes 2-6} and \textit{8-12} contain DNA substrate, DNA ligase I (2 fmol), and increasing amounts of APE1 (1.0, 2.0, 5.0, 10, and 50 fmol) as indicated by the triangles. Reactions were performed at 37°C for 3 min. Substrate and ligation product sizes are as indicated on the gel. Schematic representations of the substrates are located above the figure. Lig I, DNA ligase I; APE1, AP endonuclease 1; THF, tetrahydrofuran.

Fig. 6. \textbf{Comparison of APE1 inhibition of DNA ligase I and FEN1 activity on a THF-nick substrate.} Graphical representation is presented of the conversion of substrate to cleavage or ligation product (percentage) as a function of APE1 concentration (nM) for
reactions of 20 µl containing 10 fmol of DNA substrate and either 5.0 fmol of FEN1 or 4.0 fmol of DNA ligase I that were performed as described in Experimental Procedures. Reactions were incubated at 37°C for 4 min. Substrate is comprised of U₂:D₃:T₂ containing a γ³²P label on the 5-end of the downstream primer. APE1 amounts used in the assays are: 1.0, 2.0, 5.0, 10, and 25 fmol. The dark grey bars represent FEN1 cleavage and the light grey bars represent DNA ligase I ligation. The % of substrate ligated or cleaved is represented on the y axis, and the concentration of APE1 (nM) is on the x axis. The amount of substrate converted to product was determined by quantitating the substrate and product on denaturing polyacrylamide gels by PhosphorImager analysis. APE1, AP endonuclease 1; FEN1, flap endonuclease 1; Lig, DNA ligase I.

Fig. 7. APE1 stimulation of cleavage followed by ligation on a 6nt-THF flap substrate.
Reactions of 140 µl containing 70 fmol of DNA substrate and 5 fmol of FEN1 (lanes 1-28), 5 fmol of DNA ligase I (lanes 15-28) were performed in the absence (lanes 1-7 and lanes 15-21) or presence (lanes 8-15 and lanes 22-28) of 35 fmol of APE1 as described in Experimental Procedures. Reactions were performed at 37°C and 20 µl aliquots were removed at 1.0, 2.0, 3.0, 5.0, 10, 15, and 30 min as indicated by the triangles. Substrate is comprised of U₅:D₃:T₂ containing an α³²P radiolabel at the 3-end of the downstream primer. Substrate and reaction product sizes are as indicated on the gel. Cleavage of the 29nt substrate produces the 23nt product and ligation with the 31nt upstream primer generates the 54nt band. Schematic representation of the substrate is depicted above the figure. APE1, AP endonuclease 1; FEN1, flap endonuclease 1, LigI, DNA ligase I, nt, nucleotide.
# TABLE I. Oligonucleotide sequences (5’-3’)

| Downstream Primers |                  |                   |
|--------------------|------------------|-------------------|
| D₁ (18-mer)        | GTAAAACGACGGCCAGT |
| D₂ (28-mer)        | CCACCGTGCCACCGACG |
| D₃ (28-mer)        | FCCCCGTCCACCGACG |
| D₄ (28-mer)        | TCACCGTGCCACCGACG |
| D₅ (19-mer)        | AGTAAAACGACGGCCAG |

| Upstream Primers   |                  |                   |
|--------------------|------------------|-------------------|
| U₁ (25-mer)        | CGCCAGGGTTTTCAGTCACG |
| U₂ (25-mer)        | CGACTGAGCCAGCCTAATTCT |
| U₃ (26-mer)        | CGACCGTGCCAGCCTAATTCT |
| U₄ (27-mer)        | CGACCGTGCCAGCCTAATTCT |
| U₅ (31-mer)        | CGACCGTGCCAGCCTAATTCT |
| U₆ (25-mer)        | CGACCGTGCCAGCCTAATTCT |

| Templates          |                  |                   |
|--------------------|------------------|-------------------|
| T₁ (44-mer)        | GCACTGGCGGCGTCGTTTACGTCGACTGGAGGACCAACCCTG |
| T₂ (54-mer)        | GCACTGGCGGCGTCGTTTACGTCGACTGGAGGACCAACCCTG |

F-denotes tetrahydrofuran residue
|    | Nick   | 1nt | 2nt | 6nt |
|----|--------|-----|-----|-----|
| APE1 | - + -  | - + - | - + - | - + - |
| FEN1 | - - + + + + + - + + + + + + - + + + + + + + + + + |

- 28
- 6
- 2
- 1

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
|       | Nick-THF | 1nt-THF | 2nt-THF | 6nt-THF |
|-------|----------|---------|---------|---------|
| APE1  | - + -    | - + -   | - + -   | - + -   |
| FEN1  | - - + + + + - - + + + + - - + + + + - - + + + + - - + + + + |
AP endonuclease 1 coordinates flap endonuclease 1 and DNA ligase I activity in long patch base excision repair
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