Modulation of the 3’→5’ Exonuclease Activity of Human Apurinic Endonuclease (Ape1) by its 5’ Incised Abasic DNA Product

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SUMMARY

The major abasic endonuclease of human cells, Ape1 protein, is a multifunctional enzyme with critical roles in base excision repair (BER) of DNA. In addition to its primary activity as an apurinic/apyrimidinic endonuclease in BER, Ape1 also possesses 3’- phosphodiesterase, 3’-phosphatase, and 3’→5’ exonuclease functions specific for the 3’ termini of internal nicks and gaps in DNA. The exonuclease activity is enhanced at 3’ mismatches, which suggests a possible role in BER for Ape1 as a proofreading activity for the relatively inaccurate DNA polymerase β. To elucidate this role more precisely, we investigated the ability of Ape1 to degrade DNA substrates that mimic BER intermediates. We found that the Ape1 exonuclease is active at both mismatched and correctly matched 3’ termini, with preference for mismatches. In our hands, the exonuclease activity of Ape1 was more active at one-nucleotide gaps than at nicks in DNA, even though the latter should represent the product of repair synthesis by polymerase β. However, the exonuclease activity was inhibited by the presence of nearby 5’ incised abasic residues, which result from the apurinic/apyrimidinidic endonuclease activity of Ape1. The same was true for the recently described exonuclease activity of E. coli endonuclease IV. Exonuclease III, the E. coli homolog of Ape1, did not discriminate among the different substrates. Removal of the 5’ abasic residue by polymerase β alleviated the inhibition of the Ape1 exonuclease activity. These results suggest roles for the Ape1 exonuclease during BER after both DNA repair synthesis and excision of the abasic deoxyribose-5-phosphate by polymerase β.
INTRODUCTION

The formation of apurinic/apyrimidinic (AP) sites in DNA is the most common consequence of exposure of cells to DNA damaging agents of both endogenous and environmental origin (1). AP sites are formed as repair intermediates by DNA glycosylases, which remove certain mismatched bases or base lesions formed by reactive oxygen species, alkylating agents, or other environmental insults. AP sites can also form spontaneously via acid-catalyzed hydrolysis of the N-glycosylic bonds linking the bases to the sugar-phosphate backbone of DNA. Such spontaneous depurination forms an estimated 10,000 AP sites per day in each human cell (2), and the activity of DNA glycosylases would certainly add to this burden. Indeed, the steady-state level of AP lesions is estimated in some studies to be much higher, approaching 50,000 or more per cell depending on its age and tissue source (3,4).

AP sites in mammalian cells are repaired by the DNA base excision repair (BER) pathway. The major human AP endonuclease, Ape1 (also called Apex, HAP1, or Ref-1), initiates BER by hydrolyzing the 5'-phosphodiester bond of the AP site to create a DNA repair intermediate that has a single strand break bracketed by 3'-hydroxyl and 5'-deoxyribose-5-phosphate (dRP) termini. Ape1 interacts with DNA polymerase β (Polβ) during BER to recruit the polymerase to the incised AP site (5), where the polymerase catalyzes both nucleotide insertion and dRP excision. The sequence of these latter two activities is still unclear, but all the combined enzymatic activities of Ape1 and Polβ acting at an AP site would yield a nick that can be sealed by DNA ligase to complete the repair (6,7).
Ape1 is a multifunctional protein with proposed diverse roles in the cell. In addition to its DNA repair activities, Ape1 can activate transcription factors via a redox mechanism (8), form a transcriptional repressor complex for the negative calcium responsive element (9-11), and act as an important target for the granzyme A-mediated cell death pathway (12). In addition to its major role as an AP endonuclease during BER, Ape1 also possesses a weak 3’-phosphatase activity and a 3’-phosphodiesterase activity against abasic residues or fragments (13). These activities are required for the removal of 3’-blocking groups created by ionizing radiation, oxygen free radicals, radiomimetic anti-tumor drugs, and the 3’-AP lyase activities of bifunctional DNA glycosylases (1,14).

Ape1 also has a 3’→5’ exonuclease that excises undamaged DNA nucleotides (see below). Knocking out both alleles coding for Ape1 in mice (the APEX gene) results in early embryonic lethality (15-17), which point to critical roles for Ape1. However, it has not yet been established which Ape1 activities are required for survival. While Ape1 protein levels have been dramatically reduced for short periods in cells in culture using siRNA (12), stable cell lines lacking the protein have not been reported.

The modest 3’→5’ exonuclease activity of the mouse and human Ape1 proteins has been known for some time (18,19), but the biological role of the exonuclease remained obscure. In contrast, the robust AP endonuclease activity of Ape1 is related to the demonstrated roles in BER of exonuclease III in E. coli or Apn1 in yeast (1,13).

Recently, it was shown that Ape1 acting as an exonuclease can remove therapeutic anti-tumor nucleoside analogs incorporated at the 3’ ends of DNA oligonucleotides, which suggested that Ape1 may contribute to cellular resistance to these drugs (20). This work was further extended to show that the exonuclease activity of Ape1 is enhanced on DNA
mispairs at the 3’ termini of nicks and gaps (21). Since the primary mammalian BER polymerase, Polβ, has relatively low fidelity and lacks an associated proofreading exonuclease (22,23), the Ape1 exonuclease could provide the “missing” proofreading activity during BER (24). To explore a possible role for the exonuclease activity of Ape1 during BER, we investigated the ability of the enzyme to excise nucleotides at the 3’ termini of different BER intermediates.
EXPERIMENTAL PROCEDURES

Reagents and Enzyme. Urea was purchased from American Bioanalytical Corp. (Natick, MA). An acrylamide-bisacrylamide solution (40%, 29:1 ratio) was purchased from Bio-Rad, Inc. (Hercules, CA). E. coli exonuclease III, uracil DNA glycosylase, and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). One unit of exonuclease III is defined by the manufacturer as the amount of enzyme required to release 1 nmol of nucleotides in 30 minutes. E. coli endonuclease IV was purified as previously described (25). Recombinant wild-type human Ape1 and the D283A/D308A mutant form were purified as previously described (26). The R177A mutant form of Ape1 was kindly provided by Drs. Tadahide Izumi and Sankar Mitra of the University of Texas Medical Branch (Galveston, TX). Recombinant wild-type human Polβ was a generous gift from Drs. Rajendra Prasad and Sam Wilson, National Institute of Environmental Health Sciences (Research Triangle Park, NC).

DNA substrates. Oligonucleotides were synthesized and HPLC-purified by Operon Technologies, Inc. (Alameda, CA) or Midland Certified Reagent Company, Inc. (Midland, TX). The sequences of DNA oligonucleotides used in this study are shown in Table I. Upstream strands were 5’ end-labeled by T4 polynucleotide kinase using a molar excess of [γ-32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA). Double-stranded DNA substrates were made by heating the radioactively-labeled oligonucleotides with the appropriate template and downstream oligonucleotides to 90°C and then annealing by slow cooling to room temperature. Unincorporated [γ-32P]ATP was removed using Micro Biospin P-30 columns (Bio-Rad) following manufacturer’s
protocols. DNA substrate purity was monitored by autoradiography following electrophoresis on 8% non-denaturing gels and 12% denaturing polyacrylamide gels containing 7 M urea. The different substrates used in this study are listed in Table II along with the structural components of each substrate. Substrates S2, S4, S6, and S8 were incised with a catalytic amount of Ape1 just prior to use in exonuclease assays. Substrates S22 and S23 were sequentially treated with catalytic amounts of uracil DNA glycosylase and Ape1 to create nicked and gapped DNA with 5’-dRP termini. These substrates were also used immediately following pretreatment due to the labile nature of the 5’-dRP.

Exonuclease assays. Exonuclease reactions were performed in BER buffer (27). Briefly, standard reactions contained 50 mM Hepes-KOH (pH 7.5), 8 mM MgCl₂, 5% (v/v) glycerol, 0.5 mM DTT, 0.1 mg/ml BSA, 10 nM DNA substrate, and enzyme concentrations as indicated in the figure legends. After incubation at 30°C, the reactions were terminated at the indicated times by the addition of formamide loading buffer (90% formamide, 10 mM EDTA, bromophenol blue, and xylene cyanol) and heating at 100 °C for 2 min. The DNA products were then resolved by electrophoresis on acrylamide (14%) gels containing 7 M urea. After drying, the gels were analyzed using a Molecular Imager System (Model GS-525, Bio-Rad), and the results were quantified using Molecular Analyst software (Bio-Rad). The fraction of substrate degraded by the Ape1 exonuclease was calculated as previously described (28). Briefly, the percent exonuclease activity of each reaction was calculated using the following equation:
% exonuclease activity = $N + [(N-1) + 2(N-2) + 3(N-3) \ldots]$ 

where $N$ represents the amount of the 21-mer DNA substrate, $(N-1)$ is the amount of the 20-mer exonuclease product, $(N-2)$ is the amount of the 19-mer exonuclease product, and so on.
RESULTS

Comparison between human Ape1 and E. coli exonuclease III for excision of 3’ mismatches on BER intermediates. Previous reports on the exonuclease activity of Ape1 focused on its activity at 3’ mismatched nucleotides at gaps and nicks in DNA (21,29,30), but the activity of Ape1 on BER intermediates with incised abasic residues was not investigated. We used DNA containing the synthetic AP analog tetrahydrofuran (F) to create a set of DNA substrates that simulate these base excision repair intermediates (Fig. 1A). Tetrahydrofuran residues are resistant to cleavage by β-elimination reactions (31), but are cleaved by Ape1 with the same catalytic efficiency as regular AP sites (19). After 5’ incision by Ape1, F residues serve as stable structural analogs of the 5’-dRP moiety that is produced following 5’ incision of natural AP sites. We found that the Ape1 exonuclease is most active on 3’ A/G mismatches adjacent to a single-nucleotide gap. However, the exonuclease was significantly less active at mismatches positioned at nicks or at gaps bearing 5’-incised F residues (Figs. 1B and 1D).

Exonuclease III is the E. coli homolog of Ape1 and shares many of the same DNA repair activities (13). In addition to being an AP endonuclease, exonuclease III also possesses potent 3’-phosphodiesterase and 3’-phosphatase activities, and a well-characterized and robust 3’→5’ exonuclease (32,33). Exonuclease III shares structural homology with Ape1 reflected in 28% amino acid sequence identity, including highly conserved catalytic residues (29,34). To determine if exonuclease III activity was inhibited similarly to the Ape1 exonuclease by the presence of 5’-incised F residues at nicks and gaps, we assayed the ability of exonuclease III to degrade these same four
DNA substrates across a range of enzyme concentrations. Exonuclease III exhibited only a small difference in activity between gaps and nicks (Fig. 1C, panels 1 and 3). The bacterial enzyme was not inhibited at all by the presence of the incised dRP analog (Fig. 1C, panels 2 and 4). Thus, the inhibition of Ape1’s 3’→5’ exonuclease activity is a feature specific to the mammalian enzyme.

*Ape1 exonuclease activity for correctly matched 3’ termini is inhibited by the presence of incised abasic residue.* Previous experiments indicated that Ape1 is generally inefficient at excising non-mismatched nucleotides (19,21,35), but that the enzyme may also be more active at excising some properly paired nucleotides than certain mismatched pairs (29). The difference in these reports may depend on the overall sequence context of the DNA substrate, which can affect the exonuclease activity by up to two orders of magnitude (29). Our experiments utilized a well-characterized 51-mer oligonucleotide substrate that has long been used by several groups for routine BER assays (26,36). In the sequences used here (Fig. 2A), kinetic analysis showed that Ape1 was able to digest a properly matched A/T pair (Fig. 2B), albeit at a reduced efficiency compared to an A/G mismatch. On the 3’ mismatch, the same enzyme concentration resulted in digestion of most of the mismatched substrate even at the earliest time point (Fig. 2C). A comparison of estimated initial rates from exonuclease assays using a reduced amount of Ape1 indicated that the exonuclease activity is at least 3-fold more active on an A/G mismatch than on an A/T pair in this sequence context. Whereas previous studies found no difference in the exonuclease activity at nicks or gaps (21,29), we found that Ape1 was at least 3-fold more active at gaps than at nicks in the sequence context used here, whether
or not a 3’ mismatch was present (Fig. 2). Furthermore, these assays showed that Ape1 was inhibited at least 3-fold by the presence of incised F residues at both nicks and single-nucleotide gaps, consistent with the data shown in Figure 1.

**Separate inhibitory effects of an abasic residue and a 5’ phosphate on Ape1 exonuclease activity.** We next wanted to explore in greater detail the role of different structural elements at the 5’ end of nicks and gaps that might affect the Ape1 3’→5’ exonuclease activity. Panel A and the tops of each box of panel C in Figure 3 illustrate schematically the various DNA structures that were analyzed. The presence of a 5’-phosphate at a single-nucleotide gap did not affect the exonuclease activity (Fig. 3B). However, the presence of a 5’-phosphate at a nick inhibited the exonuclease activity 8-fold compared to a 5’-hydroxyl (Fig. 3B).

We also assayed the Ape1 exonuclease activity on substrates with termini bearing 5’-unphosphorylated deoxyribose moieties or 5’-mismatched nucleotides, as well as substrates lacking a downstream strand to determine the relative inhibitory contribution made by the structural elements at the 5’ terminus of each substrate (Fig. 3C). In the sequence context of our DNA substrate, differences between single-nucleotide gaps and nicks were observed only when the 5’ phosphates were present. Consistent with data shown in Figures 1 and 2, the Ape1 exonuclease was relatively inefficient at both nicks and single-nucleotide gaps when an abasic residue containing an adjacent phosphate group was present at the 5’ terminus (Fig. 3C, panels a, e). The Ape1 exonuclease activity increased only slightly when the 5’ phosphate was removed to yield a 5’ terminal abasic residue (Fig. 3C, panels b, f). However, substitution of the abasic residue with a
guanine nucleotide to create a 5' G/G mismatch increased the 3'→5' exonuclease activity of Ape1 (Fig. 3C, panels c, g). DNA lacking a downstream double-stranded structure was a very poor substrate for the Ape1 exonuclease activity (Fig. 3C, panel d). This observation is consistent with structural studies, DNase footprinting analysis, and enzymatic data demonstrating that Ape1 requires at least three base pairs of duplex DNA both upstream and downstream of its active site for maximal AP endonuclease activity (37-39).

_Ape1 and E. coli endonuclease IV are both inhibited by 5'-incised abasic DNA at nicks and gaps_. To verify that the inhibitory effect of 5'-dRP on the 3'→5' exonuclease activity of Ape1 is a general effect rather than a sequence-specific feature of our DNA substrates, we assayed the Ape1 exonuclease activity on substrates whose sequences resemble some other previously characterized substrates (tops of each panel in Fig. 4A; (29)). In this sequence context, which contained a 3'-A/C mismatch at nicks and gaps, Ape1 was slightly more active at the nicks than at the single-nucleotide gaps (Fig. 4A, compare panels a-c with d-f; Fig. 4B compare panel a to b). Ape1 continued to be inhibited at nicks and gaps bearing 5'-incised F residues (Fig. 4A, panels c,f; Fig. 4B, panels a,b), although to a slightly lesser extent than previously observed (3-fold instead of 4-fold as shown in Fig. 2). Consistent with experiments shown in Fig. 3, we observed that Ape1 exonuclease was more active at nicked DNA when the 5' phosphate was removed (Fig. 4B, panel b). However, we also observed the same increase in exonuclease activity at gapped substrates lacking the 5'-phosphate (Fig. 4B, panel a).
A second AP endonuclease from *E. coli*, endonuclease IV, was also recently demonstrated to possess an intrinsic 3’→5’ exonuclease activity (40). Although structurally unrelated to Ape1, endonuclease IV also has AP endonuclease, 3’-phosphodiesterase, and 3’-phosphatase activities. Endonuclease IV is inducible as part of the oxidative stress-activated SoxRS regulon of *E. coli* (41), and the enzyme plays a specialized role in repairing oxidatively damaged DNA (42). To determine whether endonuclease IV is also inhibited by the presence of a 5’ abasic residue, which can arise from oxidatively damaged AP sites (1), we assessed the ability of the endonuclease IV exonuclease to degrade these same substrates bearing 5’ phosphates or F residues.

Endonuclease IV was nearly 2-fold more active than Ape1 as an exonuclease on gapped substrates lacking a 5’ phosphate or bearing 5’ F (Fig. 4A, compare filled wedges representing endonuclease IV to open wedges representing Ape1; Fig. 4B compare panels a,b to c,d). Furthermore, endonuclease IV was also inhibited at nicks and gaps bearing 5’ F residues, relative to the same substrates lacking F. This effect was smaller than that observed for Ape1. Interestingly, endonuclease IV was somewhat more active on nicks and gaps when the 5’ phosphate was present than when it was removed (Fig. 4B, panels c and d), the reverse of the effect seen for Ape1.

**Excision of 5’-dRP by Polβ activates Ape1 exonuclease at an incised natural AP site.**

Using substrates with the same DNA sequence as those characterized in Figure 4, we next wanted to assess the Ape1 exonuclease activity at a hydrolytic AP site following incision by Ape1, and to determine whether the addition of Polβ could stimulate the exonuclease activity. Substrates bearing a single-nucleotide gap or a nick, each
containing a 5'-dRP adjacent to a 3' A/C mismatch, were generated by treatment of DNA containing a single dUMP residue with uracil-DNA glycosylase to create an AP site. This treatment was followed by incision with a catalytic amount of Ape1 (Ape1:DNA ratio 1:40). Addition of Polβ to the Ape1 exonuclease reactions resulted in stimulation of the exonuclease activity on nicks with 5’ dRP, but not at single-nucleotide gaps with 5’-dRP (Fig. 5). Since dRP excision by Polβ has been proposed to be the rate-limiting step of BER (43), dNMP misincorporation by Polβ prior to dRP excision would result in a mismatch adjacent to a nick, displacing the dRP by one nucleotide. The stimulation of the Ape1 exonuclease activity is likely due to the dRP lyase activity of Polβ and not protein-protein interactions, since Polβ was unable to stimulate Ape1 on substrates containing preincised F residues, which are resistant to β-elimination by Polβ (data not shown). The excision of 5’-dRP by Polβ on DNA containing mismatches has not been reported, but our results suggest that Polβ favors excision of the displaced 5’-dRP when a 3’-mismatch is present.
DISCUSSION

The 3'→5' exonuclease activity of the murine (18) and human (19) Ape1 proteins was identified some time ago, but only recently characterized in detail. The apparent specificity of this exonuclease activity for 3'-mismatched DNA at internal nicks and gaps suggested a role for Ape1 as a proofreading exonuclease for DNA polymerase β (21) which lacks its own intrinsic 3'→5' exonuclease activity (23). In E. coli, the primary repair polymerase is DNA polymerase I, which possesses its own 3'→5' exonuclease activity and thus does not need to rely on other proteins for proofreading of polymerization errors. Although other proteins with 3'→5' exonuclease activity are present in mammalian cells (44), Ape1’s role in initiating the repair of abasic sites, demonstrated physical interaction with Polβ, and ability to stimulate 5’-dRP excision by Polβ (5) strongly implicate Ape1 in proofreading for Polβ during BER.

The specific activity of the Ape1 exonuclease varies rather widely among different studies (21,29,35). Some variation is expected due to differences in the activity or purity of recombinant enzymes prepared by each group. The Ape1 exonuclease activity can also differ by as much as two orders of magnitude depending on the sequence of the DNA substrate (21,29). However, exonuclease III is expressed at high levels in E. coli and may constitute a highly active exonuclease contaminant that co-purifies with Ape1. To verify that the 3’→5’ exonuclease activity is intrinsic to Ape1 and not due to the contaminating activity of another nuclease, we assayed two independently purified preparations of histidine-tagged Ape1 (12,45) in addition to the untagged wild-type Ape1 used in this study. The two histidine-tagged Ape1 proteins were found to possess similar levels of exonuclease activity as the wild-type enzyme (data not shown), and like the
wild-type Ape1, the exonuclease activities of both histidine-tagged Ape1 proteins were also inhibited on DNA containing a 5’-incised abasic residue (data not shown). This inhibition of Ape1 exonuclease contrasts with the lack of such inhibition for purified exonuclease III. Furthermore, the AP endonuclease-defective D283A/D308A mutant form of Ape1, which was purified in the same manner as the wild-type Ape1 used in this study (26), did not possess measurable exonuclease activity (data not shown), a further indication that contaminating bacterial exonucleases are not responsible for the observed activity. Finally, the pattern of exonuclease products formed by Ape1 in our assays is distinct from that produced by exonuclease III. Exonuclease III is much more active than Ape1 as a 3’→5’ exonuclease and even small concentrations of exonuclease III can sequentially degrade DNA to produce multiple products (Figure 1C). Consistent with results published by Hadi et al. (29), the Ape1 used in this study excised primarily one or two nucleotides. Digestion of DNA with high concentrations of Ape1 or over long periods of incubation did not result in the excision of more than two nucleotides from the original single-nucleotide gap or nick substrates.

The relationship between the exonuclease function of Ape1 and the product of its AP endonuclease activity, 5’-dRP, is intriguing in view of the high affinity of Ape1 for incised AP sites (26). Mammalian DNA BER is coordinated by a series of protein-protein and protein-DNA interactions that facilitate hand-off of DNA repair intermediates from one protein to the next (46). Tight binding of BER proteins to their products may protect the repair intermediates from the cellular milieu to limit DNA degradation or the activation of deleterious recombination pathways.
We demonstrate here that the exonuclease activity of Ape1 is inhibited by 5'-dRP at nicks and gaps and enhanced when the 5'-dRP is removed by Polβ. While this paper was in preparation, an in press report has appeared that independently demonstrates the inhibitory effect of a 5'-incised tetrahydrofuran residue (58). This suggests a possible regulatory mechanism whereby excision of 5'-dRP coordinates Ape1’s exonuclease activity during BER. Inhibition of Ape1’s exonuclease activity by the 5'-dRP residues (that result from incision at AP sites) would protect the undamaged DNA 5’ of the AP site from degradation prior to nucleotide incorporation by Polβ. This protective mechanism may be biologically significant given that the cellular levels of Ape1 can far surpass the concentration of Polβ (47,48). The report that the majority of AP sites detected in cells is already 5’ incised supports this concept (4). Furthermore, if dRP excision is rate-limiting during BER (43), then dNMP incorporation by Polβ would usually precede dRP excision. Temporally placing the Ape1 exonuclease activity after dRP excision may then ensure that the proofreading exonuclease is activated only after DNA repair synthesis has occurred. The intrinsic preference of the exonuclease for mismatches would further limit the role of Ape1 to error correction.

Crystal structures of Ape1 in complex with duplex DNA containing the abasic site analog tetrahydrofuran indicate a contact between the side chain of the arginine-177 (R177) and the 5’-phosphate at the abasic incision site (38). R177 is absent from exonuclease III and appears to be conserved only in the Ape1 homologs of higher eukaryotes. Since the Ape1 exonuclease, but not exonuclease III, exhibited specificity for different DNA substrates, we hypothesized that a protein-DNA contact involving R177 might be responsible for the observed inhibition of the Ape1 exonuclease on DNA
containing 5’-incised AP sites. We already observed that disruption of this putative contact by removal of the 5’-phosphate at nicks increased the exonuclease activity. Thus, elimination of this putative contact at the protein level, by an alanine substitution at R177 in Ape1, might increase the exonuclease activity on nicked DNA containing 5’-phosphates. Instead, we found the R177A mutant of Ape1 to exhibit an overall 60-80% reduction in exonuclease activity, with no change in its substrate specificity (data not shown). Noting that both 5’-phosphorylated and 5’-unphosphorylated F residues inhibit Ape1 (Fig. 3), we conclude that contacts to the 5’-phosphate specifically do not constitute a major determinant of the exonuclease inhibition. Compared to the wild-type protein, the R177A mutant also exhibited a similar reduction in its ability to excise 3’-phosphoglycolate esters (data now shown). Because the R177A protein exhibited wild-type AP endonuclease activity, some protein residues clearly modulate the 3’-phosphodiesterase and 3’→5’ exonuclease activities selectively, even though all of Ape1’s nuclease activities probably share a single active site carrying out hydrolysis (38).

The finding that the exonuclease activities of both Ape1 and E. coli endonuclease IV are inhibited by the presence of 5’-incised abasic residues at nicks and gaps suggests an evolutionarily defined role for this mode of inhibition. Ape1 and endonuclease IV are unrelated at the amino acid sequence and protein structural levels. Endonuclease IV utilizes three tightly bound zinc metal ions for catalysis (49) while Ape1 uses magnesium (38). Furthermore, the mammalian and bacterial proteins bind DNA in very different ways. Ape1 makes contact mostly with the strand containing the flipped out abasic residue and induces a 35° bend in the DNA (38). Endonuclease IV makes significant contacts with both the damaged and undamaged strands to flip out both the abasic DNA
and the unpaired nucleotide, which produces an 85° kink in the DNA (49). Despite these differences, both proteins have combined AP endonuclease, 3’-phosphodiesterase, and 3’→5’ exonuclease, and 3’-phosphatase activities, albeit at different relative levels (50). The biological roles in BER of the 3’→5’ exonuclease activities of both endonuclease IV and exonuclease III from *E. coli* are still unclear. However, the modulation of the endonuclease IV exonuclease activity by the 5’-incised abasic DNA suggests a specific regulatory mechanism in BER for endonuclease IV but not for exonuclease III.

How Ape1’s exonuclease activity is coordinated during BER remains an intriguing question. As demonstrated by DNAse footprinting and structural data, Ape1 occludes at least 3 base pairs of double-stranded DNA upstream and downstream of AP sites when bound as an AP endonuclease (37-39). However, it is unclear whether Ape1 remains at the AP site while Polβ inserts a nucleotide and excises the 5’ dRP residue. It is possible that recruitment of Polβ to the repair site results in displacement of Ape1, which would have to be recruited back to the repair site following DNA synthesis. Yet another possibility is that Ape1 and Polβ are both part of a multi-protein repair complex that coordinates BER. Although a protein complex containing enzymatic activities proficient for the complete repair of uracil has been isolated from bovine testis nuclear extracts, only Polβ and DNA ligase I were identified as individual components of this complex (51). Both Ape1 and Polβ interact physically with Xrcc1 (52-54), p53 (55), and PCNA (56,57), but no stable complex containing all these proteins has yet been identified. It seems likely that the dynamic processes of BER require transient interactions to allow the processing of large numbers of lesions on a daily basis in mammalian cells.
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REFERENCES


FOOTNOTES

1 The abbreviations used are: AP, apurinic/apyrimidinic; Ape1, AP endonuclease 1; Polβ, DNA polymerase β; dRP, deoxyribose-5-phosphate; BER, base excision repair
FIGURE LEGENDS

Figure 1. **Substrate specificity of Ape1 exonuclease compared to *E. coli* exonuclease**

**III. A,** Schematic diagram of oligonucleotide substrates (see Table II for detailed
domains). Asterisks and carets denote the locations of the $^{32}$P label and internal 3’-
OH termini, respectively.  **B,** Ape1 exonuclease activity. Standard enzyme reactions
contained 10 nM DNA with increasing concentrations of Ape1 denoted by the wedges
(no enzyme, 0.5, 1.5, 5, 15, or 50 nM); The enzyme incubations were 10 min. The intact
DNA substrates are indicated by an arrow.  **C.** Exonuclease III activity on various
structures. The reactions contained increasing amounts of exonuclease III, as denoted by
the wedges (no enzyme, 0.0001, 0.001, 0.01, 0.1, or 1 unit). The arrow indicates the
intact DNA substrates.  **D,** Quantification of Ape1 exonuclease activity. Experiments as
shown in panel B were quantitated in a phosphorimager. Bars indicate the average
fraction of each DNA substrate degraded in 10 min. Error bars indicate the standard error
of the mean (n=3).

Figure 2. **Inhibition of Ape1 exonuclease activity by a 5’-terminal abasic site analog,**
tetrahydrofuran.  **A,** Schematic diagrams of fully base-paired (S5, S6, S7, and S8) and
3’-A/G mismatched (S1, S2, S3, and S4) substrates (see Table II for details). Asterisks
and carets denote the locations of the $^{32}$P label and internal 3’-OH termini, respectively.
**B,** Exonuclease activity of Ape1 on fully base-paired DNA substrates. The reactions
contained 10 nM DNA and 25 nM Ape1. The fraction of total substrate converted to
product at each time point is shown. Error bars indicate the standard error of the mean


for each data point (n=3). C, Ape1 exonuclease activity on substrates with internal 3’
A/G mismatches. The reactions were performed and analyzed as described for panel B,
except that some reactions with substrate S1 contained only 5 nM Ape1 (open squares).

Figure 3. Inhibition of Ape1 exonuclease by 5’-phosphate at nicked DNA. A,
Schematic diagram of oligonucleotide substrates (see Table II for details). Asterisks and
carets denote the locations of [32P] label and internal 3’-OH termini, respectively. The p
denotes the location of an unlabeled 5’-phosphate. B, Effect of downstream 5’-phosphate
on Ape1 exonuclease activity at single-nucleotide gaps and nicks. The reactions
contained 10 nM DNA and 25 nM Ape1. Error bars indicate the standard error of the
mean for each data point (n=3). C, Ape1 exonuclease activity on nicked and single-
nucleotide-gapped substrates. A diagram of each substrate is shown above its respective
gel image (see Table II for details). Asterisks and carets denote the locations of the [32P]
label and internal 3’-OH termini, respectively. The p denotes the location of an unlabeled
5’-phosphate. Arrows indicate the migration of the intact DNA substrate. The reactions
were performed as described for panels B and C. The wedges indicate a time course (0,
5, 10, or 15 minutes).

Figure 4. Comparison of Ape1 and E. coli endonuclease IV exonuclease activities at
A/C mismatches in different DNA structures. A, Schematic diagrams of the substrates
used in this experiment are shown in each panel (see Table II for details). Asterisks and
carets denote the locations of the [32P] label and internal 3’-OH termini, respectively.
The reactions contained 10 nM DNA substrate and either 0.2, 1, or 5 nM Ape1 (open
wedges) or 0.2, 1, or 5 nM *E. coli* endonuclease IV (filled wedges). Control lanes without added enzyme are indicated (—). **B,** Quantification of enzyme titrations shown in panel B. Panel a: activity of Ape1 on gapped substrates. Panel b: activity of Ape1 on nick substrates. Panel c: activity of endonuclease IV on gap substrates. Panel d: activity of endonuclease IV on nick substrates.

Figure 5. **Activation of Ape1 exonuclease activity by Polβ dRP lyase.** Each 25-µl reaction contained 20 nM DNA substrate, 5 nM Ape1 and where indicated, 10 nM Polβ. The fraction of total substrate converted to product was plotted for each time point. Error bars indicate the standard error of the mean for each data point (n=3).
### Table I
DNA oligonucleotides used for exonuclease assays

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>DNA sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upstream Strands</strong></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>*GCTTGATGCCTGCAGGTCG</td>
</tr>
<tr>
<td>A2</td>
<td>*GCTTGATGCCTGCAGGTCGGAATCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td>A3</td>
<td>*TAGAGGATCCCCGCTAGCGG</td>
</tr>
<tr>
<td>A4</td>
<td>*TAGAGGATCCCCGCTAGCGGGAATCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td><strong>Downstream Strands</strong></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>pCTCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td>B2</td>
<td>CTCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td>B3</td>
<td>pCTCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td>B4</td>
<td>TCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td>B5</td>
<td>TCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td>B6</td>
<td>GTCTAGAGGATCCCCGGGTACCGAGCTGA</td>
</tr>
<tr>
<td><strong>Template Strands</strong></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGATCGAAGC</td>
</tr>
<tr>
<td>C2</td>
<td>TCGAGCTCGGTACCCGGGGATCCTCTAGAGGCACCTGAGGCAAGCCAGGCAAGC</td>
</tr>
<tr>
<td>C3</td>
<td>TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGATCGAAGC</td>
</tr>
<tr>
<td>C4</td>
<td>TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGATCGAAGC</td>
</tr>
<tr>
<td>D0</td>
<td>TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGATCGAAGC</td>
</tr>
<tr>
<td>D1</td>
<td>TCGAGCTCGGTACCCGGGGATCCTCTAGACCGGGGATCGAAGC</td>
</tr>
</tbody>
</table>

The nucleotide targets for excision are indicated in boldface.

* indicates the locations of the $[^{32}P]$ label.

F and U denote tetrahydrofuranyl and uridine residues, respectively.

The p marks the location of a chemically synthesized 5’-phosphate group.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Gap or Nick</th>
<th>Nucleotides at 3' termini</th>
<th>Structures at 5' termini</th>
<th>Oligonucleotides annealed</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>gap</td>
<td>A/G mismatch</td>
<td>phosphate</td>
<td>A1:B3:C2</td>
</tr>
<tr>
<td>S2*</td>
<td>gap</td>
<td>A/G mismatch</td>
<td>pF</td>
<td>A2:C2</td>
</tr>
<tr>
<td>S3</td>
<td>nick</td>
<td>A/G mismatch</td>
<td>phosphate</td>
<td>A1:B3:C4</td>
</tr>
<tr>
<td>S4*</td>
<td>nick</td>
<td>A/G mismatch</td>
<td>pF</td>
<td>A2:C4</td>
</tr>
<tr>
<td>S5</td>
<td>gap</td>
<td>A/T pair</td>
<td>phosphate</td>
<td>A1:B3:C1</td>
</tr>
<tr>
<td>S6*</td>
<td>gap</td>
<td>A/T pair</td>
<td>pF</td>
<td>A2:C1</td>
</tr>
<tr>
<td>S7</td>
<td>nick</td>
<td>A/T pair</td>
<td>phosphate</td>
<td>A1:B1:C1</td>
</tr>
<tr>
<td>S8*</td>
<td>nick</td>
<td>A/T pair</td>
<td>pF</td>
<td>A2:C3</td>
</tr>
<tr>
<td>S9</td>
<td>gap</td>
<td>A/T pair</td>
<td>hydroxyl</td>
<td>A1:B4:C1</td>
</tr>
<tr>
<td>S10</td>
<td>nick</td>
<td>A/T pair</td>
<td>hydroxyl</td>
<td>A1:B4:C3</td>
</tr>
<tr>
<td>S11</td>
<td>gap</td>
<td>A/T pair</td>
<td>F</td>
<td>A1:B5:C1</td>
</tr>
<tr>
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<td>gap</td>
<td>A/T pair</td>
<td>G/G mismatch</td>
<td>A1:B6:C1</td>
</tr>
<tr>
<td>S13</td>
<td></td>
<td>A/T pair</td>
<td></td>
<td>A1:C1</td>
</tr>
<tr>
<td>S14</td>
<td>nick</td>
<td>A/T pair</td>
<td>F</td>
<td>A1:B5:C3</td>
</tr>
<tr>
<td>S15</td>
<td>nick</td>
<td>A/T pair</td>
<td>G/G mismatch</td>
<td>A1:B6:C3</td>
</tr>
<tr>
<td>S16</td>
<td>gap</td>
<td>A/C mismatch</td>
<td>hydroxyl</td>
<td>A3:B4:D0</td>
</tr>
<tr>
<td>S17</td>
<td>gap</td>
<td>A/C mismatch</td>
<td>phosphate</td>
<td>A3:B3:D0</td>
</tr>
<tr>
<td>S18</td>
<td>gap</td>
<td>A/C mismatch</td>
<td>pF</td>
<td>A3:B5:D0</td>
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<tr>
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<td>nick</td>
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<td>A3:B3:D1</td>
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<td>nick</td>
<td>A/C mismatch</td>
<td>pF</td>
<td>A3:B3:D1</td>
</tr>
<tr>
<td>S22</td>
<td>gap</td>
<td>A/C mismatch</td>
<td>dRP</td>
<td>A4:D0</td>
</tr>
<tr>
<td>S23</td>
<td>nick</td>
<td>A/C mismatch</td>
<td>dRP</td>
<td>A4:D1</td>
</tr>
</tbody>
</table>

* indicates substrates that were created by treatment with a catalytic amount of Ape1 following annealing.

pF denotes a phosphorylated tetrahydrofuran residue.

F denotes an unmodified tetrahydrofuran residue.

a, see Table I for oligonucleotide sequences.

b, substrate was a primer-template pair lacking a downstream primer.

c, to create this substrate, oligonucleotide B5 was phosphorylated by T4 polynucleotide kinase prior to annealing.
A

\begin{align*}
S5 &: A^T \_ T \_ G \_ A \\
S6 &: A^T \_ p^T \_ T \_ G \_ A \\
S7 &: A^p \_ T \_ T \_ A \\
S8 &: A^p \_ p^F \_ T \_ A \\
S1 &: A^A \_ G \_ G \_ A \\
S2 &: A^A \_ p^T \_ G \_ A \\
S3 &: A^p \_ T \_ G \_ A \\
S4 &: A^p \_ p^F \_ G \_ A \\
\end{align*}

B

\begin{align*}
\text{Time (min)} & & 0 & 5 & 10 & 15 \\
\% \text{ exonuclease activity} & & 0\% & 10\% & 30\% & 50\% \\
S5 & & \text{△} & \text{△} & \text{△} & \text{△} \\
S6 & & \text{□} & \text{□} & \text{□} & \text{□} \\
S7 & & \text{●} & \text{●} & \text{●} & \text{●} \\
S8 & & \text{●} & \text{●} & \text{●} & \text{●} \\
\end{align*}

C

\begin{align*}
\text{Time (min)} & & 0 & 5 & 10 & 15 \\
\% \text{ exonuclease activity} & & 0\% & 20\% & 60\% & 80\% \\
S1 & & \text{△} & \text{△} & \text{△} & \text{△} \\
S2 & & \text{□} & \text{□} & \text{□} & \text{□} \\
S3 & & \text{●} & \text{●} & \text{●} & \text{●} \\
S4 & & \text{●} & \text{●} & \text{●} & \text{●} \\
\end{align*}

Wong et al., Fig. 2
Wong et al., Fig. 3
**Fig. 4**

**A**

S16 \( \text{A}^\text{A} \text{CGA} \)

S17 \( \text{A}^\text{A} \text{pT} \text{CGA} \)

S18 \( \text{A}^\text{A} \text{pT} \text{CGA} \)

S19 \( \text{A}^\text{A} \text{pC} \text{GA} \)

S20 \( \text{A}^\text{A} \text{pC} \text{GA} \)

S21 \( \text{A}^\text{A} \text{pC} \text{GA} \)

**B**

**Gaps**

- S16
- S17
- S18

% exonuclease activity vs. nM Ape1

**Nicks**

- S19
- S20
- S21

% exonuclease activity vs. nM Ape1

**C**

**Gaps**

- S16
- S17
- S18

% exonuclease activity vs. nM endonuclease IV

**D**

**Nicks**

- S19
- S20
- S21

% exonuclease activity vs. nM endonuclease IV

Wong et al., Fig. 4
A  

S22  

C  

G  

A  

S23  

C  

A  

B  

% exonuclease activity  

S22  

S22 + PolB  

S23  

S23 + PolB  

Time (min)
Modulation of the 3'-to-5' exonuclease activity of human apProduct
Donny Wong, Michael S. DeMott and Bruce Demple

J. Biol. Chem. published online July 10, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306065200

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