Nucleotide Sequence and DNA Secondary Structure, as Well as Replication Protein A, Modulate the Single-stranded Abasic Endonuclease Activity of APE1

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A major role of the multifunctional human Ape1 protein is to incise at apurinic/apyrimidinic (AP) sites in DNA via site-specific endonuclease activity. This nuclease function has been well characterized on double-stranded (ds) DNA substrates, where the complementary strand provides a template for subsequent base excision repair events. Recently, Ape1 was found to incise efficiently at AP sites positioned within the single-stranded (ss) regions of various biologically relevant DNA configurations. The studies within indicated that the ss endonuclease activity of Ape1 is poorly active on ss AP site-containing polyadenine or polythymine oligonucleotides, suggesting a requirement for some form of DNA secondary structure for efficient cleavage. Computational, footprinting, and biochemical analyses indicated that the nature of the secondary structure and the proximity of the AP site influence Ape1 incision efficiency significantly. Replication protein A (RPA), the major ssDNA-binding protein in mammalian cells, was found to bind ss AP-DNA with similar affinity as unmodified ssDNA and ds AP-DNA with lower affinity. Consistent with their known relative DNA binding affinities, RPA blocks/inhibits the ss, but not ds, AP endonuclease function of Ape1. Moreover, RPA inactivates Ape1 incision activity at an AP site within the ss region of a fork duplex, but not a transcription-like bubble intermediate. The data herein suggested a model whereby RPA selectively suppresses the nontemplated ss cleavage activity of Ape1 in vivo, particularly at sites of ongoing replication/recombination, by coating the ssDNA.

Apurinic/apyrimidinic (AP) endonuclease 1 (Ape1) is the major mammalian repair protein for abasic sites in DNA (1). This enzyme catalyzes incision of the phosphodiester backbone immediately 5′ to an AP lesion, initiating a cascade of events that involves components of the base excision repair (BER) pathway and aims to remove the abasic residue and re-establish genetic integrity (2). Abasic sites are common products in DNA, arising either spontaneously (at a rate of 10,000 events per mammalian genome per day), via accelerated base release because of chemical modification, or through enzyme (glycosylase)-catalyzed removal of a damaged base (3, 4). If un repaired, AP lesions present mutagenic and cytotoxic challenges to the cell (5). Deficiencies in Ape1 activity in mice have been associated with increased spontaneous mutation frequencies in both somatic and germ line cells (6), as well as increased cancer susceptibility and reduced survival in the face of exogenous oxidizing agents (7). It is noteworthy that, although uncommon, human Ape1 protein variants with reduced function have been identified (8).

It is generally accepted that Ape1 is the predominant AP site incision enzyme in mammals, accounting for >95% (if not all) of the total cellular AP endonuclease activity (9). In fact, recent evidence argues that its AP endonuclease function is essential for cell viability (10, 11). For many years, this activity had been characterized on double-stranded (ds) AP-DNA substrates (12–14), as it was presumed that a successful BER event would take place exclusively on a template-containing (instructional) duplex DNA molecule. Recently, however, it was shown that Ape1 exhibits a robust endonuclease activity at AP sites in single-stranded (ss) oligonucleotides, in some instances greater than in dsDNA, as well as in several complex and biologically relevant ss structures, such as primer-template duplexes, bubble conformations, and fork-like arrangements (15, 16). Presently, little is known about how these more “exotic” activities of Ape1 are modulated or how the resulting incision products are handled by the cell.

Replication protein A (RPA) is the most abundant ssDNA-binding protein in mammalian cells, present at roughly 100,000 molecules per cell (17). This heterotrimeric complex was originally identified as an essential component of simian virus (SV40) DNA replication in vitro (18–20). More recent studies have demonstrated that RPA operates in many processes of eukaryotic DNA metabolism, including repair and recombination (21, 22). Its primary role is presumed to be in modulating or coordinating these various DNA transactions, via both its well-characterized DNA binding activity and its assorted interactions with other proteins (17). In this study, we aimed to identify the DNA structural elements that influence Ape1 ss AP site incision activity and to determine the effect of RPA on Ape1 ss and ds AP endonuclease functions. Our results herein reveal that Ape1 requires some form of DNA secondary conformation for proficient ss AP site incision, that the type and location of the secondary structure with respect to the AP lesion can have a significant impact on Ape1 efficiency, and that RPA is likely a key negative regulator of Ape1 ss cleavage activity in vivo.

EXPERIMENTAL PROCEDURES

Proteins and Oligonucleotide Substrates—Recombinant human Ape1 protein was purified as described previously (23). Recombinant human RPA was purified as detailed by Henricksen et al. (24) with some modification. Briefly, Rosetta(DE3)pLyS5 (Novagen) strains were transformed with p11dtRPA, and the RPA complex was expressed at 37 °C...
**Ape1 ss Incision Activity**

TABLE 1  
Deoxyribose oligonucleotides

| Name          | Sequence (5′ to 3′)                  |
|---------------|-------------------------------------|
| 19 poly(A)-F  | AAAAAAAAAAFAAAAAAAAAA               |
| 19 poly(T)-F  | TTGGGTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| 26 poly(T)-F  | TTGGGTTTTTTTTTTTTTTTTTTTTTTTTTTTT |
| 34 poly(F)-D  | AATTGCCCGTGGCAGCTACTAGAATTC        |
| 26FDA         | AATTGCCCGTGGCAGCTACTAGAATTC        |
| 26FDD         | AATTGCCCGTGGCAGCTACTAGAATTC        |
| 26FDA1        | AATTGCCCGTGGCAGCTACTAGAATTC        |
| 26FDD1        | AATTGCCCGTGGCAGCTACTAGAATTC        |
| 26FDA2        | TTTTAGGATCAGGAGGTATCTAAGG          |
| 26FDD2        | TTTTAGGATCAGGAGGTATCTAAGG          |
| 34F           | CTGCCGCTATGACTCGGTCGAGATCCCGGCTAC |
| 34F5′D        | CTGCCGCTATGACTCGGTCGAGATCCCGGCTAC |
| 34FDD         | CTGCCGCTATGACTCGGTCGAGATCCCGGCTAC |
| 34F5′D1       | CTGCCGCTATGACTCGGTCGAGATCCCGGCTAC |
| 34F5′D2       | CTGCCGCTATGACTCGGTCGAGATCCCGGCTAC |
| 34F3′D4       | CTGCCGCTATGACTCGGTCGAGATCCCGGCTAC |
| 19 poly(A)-F  | GGGCGATTAAACATTGAGTGCACTCCCTCGATGATCCTAAGC |
| 19 poly(T)-F  | GGGCGATTAAACATTGAGTGCACTCCCTCGATGATCCTAAGC |
| 19 poly(A)-D  | GTACCGGAGGTATCCCGGATCGGACGTGAGCTCCG |
| 19 poly(T)-D  | GTACCGGAGGTATCCCGGATCGGACGTGAGCTCCG |

for 3 h in the presence of 1 mM isopropyl-β-D-galactopyranoside. Harvested bacteria were lysed by sonication, and the resulting soluble fraction was subjected to column chromatography on Affi-Gel Blue (Bio-Rad), hydroxyapatite (CHT-II from Bio-Rad), and Q-Sepharose (Amersham Biosciences) as described (24), followed by chromatography on an ssDNA cellulose column (Sigma) (25). The eluate from ssDNA cellulose was concentrated via Amicon Ultra-15 (molecular weight cutoff of 10,000; Millipore Corp.) and analyzed (Fig. 5A). T7 endo I was purchased from New England Biolabs.

Oligonucleotides were purchased from Integrated DNA Technologies and are listed in Table 1. See Ref. 16 for additional details regarding substrates. For biochemical assays (see below), the oligonucleotides containing an AP site were radiolabeled at the 5′ end using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs) as recommended by the manufacturer.

**AP Site Incision Assays**—Ape1 endonuclease activity was monitored essentially as described (16). In brief, unless otherwise indicated, Ape1 and 1 pmol of 32p-labeled F-containing ss or ds oligonucleotide substrates (10 μl final volume) were incubated at 37 °C under the previously defined, physiologically relevant reaction conditions (OPT buffer: 25 mM MOPS, pH 7.2; 100 mM KCl; 1 mM MgCl2; 1 mM dithiothreitol; and 50 μg/ml bovine serum albumin). Protein amount and incubation time are indicated in the figure or associated legend. Where RPA was included, RPA was pre-mixed with the substrate on ice for 20 min prior to the addition of Ape1 and the initiation of the reaction at 37 °C. Following the addition of stop buffer and heat inactivation at 95 °C for 10 min, an aliquot of the completed reaction was separated on a 15% polyacrylamide urea denaturing gel. Intact radiolabeled substrate and incised product were visualized and quantified using a Storm PhosphorImager (Amersham Biosciences) and accompanying software.

**DNA Secondary Structure Prediction**—Potential secondary structure for all oligonucleotides was determined using the RNAfold program at rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi. Structure predictions were run using RNA parameters and the default options.

**Native Gel Electrophoresis**—ss oligonucleotides were 5′-radiolabeled as described above. DNAs were then incubated for 30 min at 37 °C in OPT buffer plus 10% glycerol (see above). Oligonucleotides (0.5 pmol) were immediately loaded on a 12% nondenaturing polyacrylamide gel (19:1 acrylamide/bis) and electrophoresed at 160 V in 1× TBE for 2.5 h at room temperature. Gel images were obtained by standard PhosphorImager analysis.

**T7 Endo I Footprinting**—One μl of 5′-radiolabeled oligonucleotide (1 pmol) was added to 18 μl of ×1 New England Biolabs buffer 2 and incubated at 37 °C for 10 min. Reactions were then continued at 37 °C for 60 min in the presence of 1 μl of T7 endo I (i.e. 1–1.5 units of enzyme). DNA was subsequently precipitated by the addition of 0.5 ml of ethanol and 1 μl (20 μg) of glycogen, followed by centrifugation. The pellet was dried, resuspended in stop buffer, and heated at 95 °C for 5 min before electrophoresis on an 18% denaturing polyacrylamide sequencing gel. Images were obtained by standard PhosphorImager analysis.

**Standard and Competition EMSAs**—A DNA competitor-based EMSA was employed to evaluate the relative affinities of Ape1 for specific ss F-containing oligonucleotides. In brief, 50 fmol of radiolabeled ds 34F:34G were incubated with 0.5 ng of Ape1 (14 fmol) for 20 min in OPT buffer without MgCl2 and plus 5% glycerol. Subsequently, 100× (5 pmol) of the indicated ss substrate was added (final volume of 10 μl), and the incubation was continued for another 20 min on ice. The reaction was subsequently resolved on a 4% polyacrylamide nondenaturing gel at 4 °C in 0.5× TBE. Electrophoresis was carried out for 80 min at 10 V/cm gel. Radiolabeled DNA was visualized and quantified using standard PhosphorImager analysis (see above).

RPA DNA binding affinity was determined using a slightly modified EMSA. In brief, 20 fmol of labeled DNA substrate was incubated as above with the indicated amount of RPA. Where both RPA and Ape1 were included, Ape1 (60 fmol) was pre-mixed with the substrate for 20 min prior to the addition of RPA (see figure legend). Binding reactions were then analyzed as above.

**RESULTS**

**Dependence of Ape1 ss Incision Activity on DNA Secondary Structure**—Ape1 has been shown to effectively incise at AP sites in ssDNA (15), with its endonuclease efficiency seemingly influenced by the potential secondary structure of the oligonucleotide (16). To determine more explicitly the role of DNA secondary structure on Ape1 activity in vitro, incision assays were performed using DNAs unable to form secondary conformations, namely 19 poly(A)-F and 19 poly(T)-F (Table 1). As shown in Fig. 1, A (time course) and B (protein titration), Ape1 was unable to efficiently incise these ss
Ape1 ss Incision Activity

19-mer AP site-containing oligonucleotides, although the enzyme was fully capable of cleaving the positive control ss 34F DNA (see below). To exclude the possibility that 19 poly(A)-F and 19 poly(T)-F were improperly synthesized, complementary DNAs (19T and 19A) were generated and annealed accordingly, and the resulting duplexes were examined for Ape1 incision. As shown in Fig. 1C, although the ss versions of poly(A)-F and poly(T)-F were not cleaved, the ds conformations were converted to product at a rate >100-fold more rapid. To examine the possibility that nucleotide (nt) length was a limiting factor in incision of the 19-mer ss F-DNAs, poly(T) oligonucleotides were created (i.e. 26 poly(T)-F and 34 poly(T)-F; see Table 1), which have identical length and harbor an abasic residue at the equivalent position that ss 34F, 34F5/H1, and 34F5/H1/D consist of compact, intramolecular DNA secondary structure that promotes a more rapid mobility (Fig. 2B, left). In addition, each 34-mer exists as a single predominant detectable DNA species, without the existence of significant alternative DNA conformations. Similar results were obtained with the 26-mer AP-DNAs, with the ss oligonucleotides migrating more rapidly than 26 poly(T)-F and, in some instances, 19 poly(T)-F (Fig. 2B, right). Unlike the 34-mer s, however, three of the 26-mer s, 26F3/D, 26FDD, and 26FDD2, appeared to exist in alternative, secondary configurations that roughly comprised 23, 16, and 9% of the total DNA species, respectively. Most interestingly, the 26-mer that migrated most swiftly (i.e. 26F3/D, 26FDD, 26FDD1, and 26FDD2; Fig. 2B, right) were those predicted to possess the most compact (i.e. duplex) structure (Fig. 2A, bottom).

Although it is evident that each of the 26- and 34-mer AP site-containing oligonucleotides exist in a complex secondary form that is distinct from comparable linear ssDNA (and in some cases is unique from its sequence-altered counterparts; Fig. 2B), to gain a more precise picture of the intramolecular nature of the various substrates, we employed chemical and nuclease footprinting assays. Unfortunately, studies with either dimethyl sulfate (DMS) or diethyl pyrocarbonate, which did react with the DNA bases of the 34-mers as expected, did not uncover any obvious secondary conformation, which we hypothesized would reduce target base (namely guanine) reactivity. This result likely indicates that the short stretches of dsDNA present in the ss 34-mer oligonucleotides do not prevent DMS (supplemental Fig. 1) or diethyl pyrocarbonate (data not shown) modification. Thus, as an alternative means of probing for secondary conformations, we employed T7 endonuclease I (T7 endo I), a structure-selective enzyme that recognizes and cleaves perfectly matched DNA, cruciform DNA, and Holliday structures or junctions (27, 28).

Although recognizing that the substrate specificity of T7 endo I has not been thoroughly defined on ss oligonucleotides, our footprinting analyses generally support the existence of secondary structure as predicted for the 34- and 26-mer DNAs (Fig. 2A). In particular, in the case of 34F, the predominant nuclease band (24 nt in length) corresponds to a cutting site within the predicted unpaired 3’ loop, whereas the less prominent, yet significant, 13-nt band corresponds to incision immediately adjacent to the predicted 5’ stem-loop (Fig. 2C, left). Moreover, the major products of 13 and 24 nt seen with 34F5/D and 34F/D, respectively, are compatible with the above predicted 5’ and 3’ stem-loop structures. The 13-nt cleavage product observed with 34F5/D is also...
consistent with the theorized 5' stem-loop, although the observation of additional bands implies the existence of alternative DNA secondary structures not predicted (e.g. a potential stem-loop between T18–A19 and T22–A23; denoted by dots in Fig. 2A), or an effect of the AP site location. A minor band of 24 nt is observed with 34F5D and may reflect the presence of this 2-bp stem-loop as well, which again was not predicted by the RNAfold program. In the case of 34F5D1, the major bands of 14, 16, 17, and 18 nt could be explained by T7 endo I incision at sites 3' to the predicted stem-loop structure that may be influenced by the position of the AP site.

With respect to the 26-mers, the existence of the large ds region predicted for 26F3'D, 26FDD, 26FDD1, and 26FDD2 is supported by the generation of the prominent 5- and 6-nt products (Fig. 2C, right), which correspond to T7 endo I incision immediately 3' to the stem base (Fig. 2A). More importantly, the overall digestion patterns of 26F3'D, 26FDD and 26FDD1 are comparable, consistent with their similar mobility as seen in Fig. 2B (right). The predicted stem-loop structures of 26FD and 26FD2 are supported by the production of the 14–16-nt fragments, which are generated by cutting near the base of the evidently more stable 3-nt stem-loop structure. Significantly, the predicted structure of 26GFA was not confirmed by our footprinting studies, likely indicative of the weak stability of the predicted stem-loop structure, which consists of a short stem (2 nt) and a small loop (3 nt).
Our native gel electrophoresis experiments indicate the presence of secondary structure in each of the 26- and 34-nt F-containing oligonucleotides. The T7 endo I footprinting studies support that the proposed, favored conformations are by and large the predominant DNA species. We emphasize that to our knowledge routine methodologies to interrogate secondary structures of ssDNA oligonucleotides are not well established.

Effect of Secondary Structure on Ape1 Incision and Binding Activities—

With the above structural information in hand, we next evaluated the efficiency with which Ape1 cleaved the various ss F-containing oligonucleotides. As shown in Fig. 3A (time course reactions) and quantitatively reported in Table 2 (specific activities), 34F5’D was cleaved at an efficiency similar to 34F (<1.1-fold difference), whereas Ape1 incision of 34FDD was significantly slower (~7-fold reduced). Moreover, 34F5’D2 was converted to product at a rate ~50-fold slower than 34F5’D1 (which was incised at a rate similar to 34F; Table 2). The steady-state kinetic curves shown in Fig. 3A are consistent with the presence of a single major DNA species for each oligonucleotide substrate, as seen in the native gel electrophoresis studies (Fig. 2B). Time course experiments with the 26-mer substrates indicate that each of the sequence-modified DNAs were cleaved by Ape1 (i) more efficiently than the parental 26GFA oligonucleotide (Table 2) and (ii) primarily via near-linear kinetics (Fig. 3B). Notably, 26FD2, which most closely mimics the 5’ portion of 34F (Fig. 2A), was found to be the best substrate (among the 26-mers) for Ape1, with an ~50-fold improvement over 26GFA. Nonetheless, for reasons that are not clear (but may reflect the precise nt composition), all 26-mers were significantly poorer substrates (i.e. cleaved at a rate around 300-fold slower) in comparison to 34F, although 26FD2 was cleaved at an efficiency nearly identical to 34F5’D2 (summarized in Table 2).

The incision experiments in total suggest the following. (i) A 5’ duplex structure (with respect to the abasic site) is more favorable than a 3’ duplex, and ds structure on both sides is not essential for Ape1 cleavage (see 34F5’D versus 34FDD and see Ref. 13). (ii) A larger ss loop (i.e. 5 nt in 26FD2) is more favorable than a shorter loop (3 nt in 26FD).

FIGURE 3. Incision and binding of various AP site-containing 34- and 26-mers by Ape1. A, time course Ape1 incision studies using the 34-mers. Note that the amount of Ape1 used with each oligonucleotide was different: 10 pg (34F, 34F5’D, and 34F5’D1), 100 pg (34FDD), and 1 ng (34F5’D2). Time course Ape1 incision studies using the 26-mers. Note that the amount of Ape1 used was 5 ng (26GFA, 26F3’D, 26FD, 26FDD, 26FDD1, 26FDD2, and 26FD) or 2 ng (26FD2). See Table 1 for more information regarding the oligonucleotides and Table 2 for the comparative incision efficiencies. Data shown represent average and S.D. of three independent experiments. B, representative gel image of a typical DNA-competition EMSA. C, representative gel image of a typical DNA-competition EMSA. The relative percentage of bound radiolabeled ds 34F is indicated (relative to no competitor, see APE1 lane in C). Numbers represent the average and S.D. of three independent measurements and are listed in the same order as Panel C, starting with APE1. We note that the undamaged 26-mer competition effectiveness was similar to the abasic site-containing 26-mer oligonucleotides shown.

TABLE 2

| Substrates | Specific incision activity | Relative percent ds 34F bound to Ape1 in the presence of ss competitor DNA* |
|------------|---------------------------|---------------------------------------------------------------|
| 34F        | 3836 ± 919 g/μmol min    | 5.9 ± 8.8                                                    |
| 34F5’D     | 3612 ± 1148 g/μmol min   | 5.1 ± 6.1                                                    |
| 34FDD      | 568 ± 125 g/μmol min     | 37 ± 12                                                      |
| 34F5’D1    | 4793 ± 355 g/μmol min    | 57 ± 10                                                      |
| 34F5’D2    | 100 ± 15 g/μmol min      | 36 ± 11                                                      |
| 26GFA      | 4.7 ± 1.2 g/μmol min     | 69 ± 20                                                      |
| 26F3’D     | 12 ± 2.9 g/μmol min      | 106 ± 20                                                     |
| 26FD       | 13 ± 3.9 g/μmol min      | 110 ± 23                                                     |
| 26FDD1     | 5.5 ± 1.7 g/μmol min     | 106 ± 21                                                     |
| 26FDD2     | 11 ± 2.8 g/μmol min      | 98 ± 12                                                       |
| 26FD       | 16 ± 2.5 g/μmol min      | 103 ± 18                                                     |
| 26FD2      | 37 ± 4.6 g/μmol min      | 101 ± 27                                                     |

* The numbers are relative to the absence of ss DNA and are graphed in Fig. 3D.
implying that the neighboring 5’-duplex stability, which is likely adversely affected by the torsional strain created by a short ss region in the hairpin loop, is an important element in determining Ape1 effectiveness (see also the results with 26GFA and 34F). (iii) an AP site immediately adjacent to a 5’-duplex structure is a deterrent to Ape1 cleavage, as compared with an abasic lesion positioned 3 or 5 nt from the secondary conformation (compare for instance 34F5’D2 with 34F5’D and 34F5’D1), although such conclusions are tentative because the T7 endo I footprinting studies do not fully support the predicted secondary configurations for 34F5’D and 34F5’D2.

As a means of determining qualitatively whether the observed differences in Ape1 incision efficiency for the varying ss AP-DNAs (Table 2) were mainly the product of reduced substrate affinity, we employed a competitor-based EMSA. In these experiments, radiolabeled ds 34F:34G DNA (Table 1) was incubated with Ape1, and a 100-fold molar excess of unlabeled ssDNA (denoted) was then added to challenge for protein binding. As shown in Fig. 3, C and D, 100× unmodified ss 34G competed ~60% of the specific Ape1 AP site duplex binding, indicative of expected, nonspecific protein-DNA interactions (16, 29). The ss 34F and 34F5’D competed nearly 95% of Ape1 binding and represented the best, highest affinity competitors. The other 34-mer F-DNAs (34FDD, 34FD1, and 34F5’D2) showed antagonistic effects similar to nonspecific, undamaged 34G DNA. For reasons not completely clear, but that may have to do with the overall structure or length of the 34-mers relative to the 26-mers, all ss 34-mer DNAs exhibited by and large a stronger competition than the ss 26-mer F-containing oligonucleotides, consistent with the 34-mers generally being better substrates than the 26-mers. Thus, although the studies here suggest a broad, direct correlation between Ape1 incision efficiency and AP-DNA substrate affinity (i.e., the weaker binding ss substrates are cleaved at lower efficiency), our results also indicate that binding and incision can be separable and distinct events in the Ape1 reaction pathway (compare for instance 34F, 34F5’D1, and 34F5’D2; see Table 2). Finally, the poor competition by many of the ss AP site-containing DNAs (i.e., ≤50% at 100-fold molar excess) is generally consistent with Ape1 exhibiting a higher complex stability with and greater affinity for ds AP-DNA substrates (16).

Binding of RPA to ss and ds Oligonucleotide Substrates—RPA, which consists of three subunits of 70, 32, and 14 kDa (Fig. 4A), is the most abundant ssDNA-binding protein in mammalian cells (17). Before evaluating the effects of RPA on Ape1 ss and ds incision activity, we determined the binding affinity of this heterotrimeric complex for unmodified and AP site-containing oligonucleotide substrates (Table 1). In particular, RPA binding of ss and ds unmodified DNA (34COMP), as well as ss and ds AP site-containing DNA (34F), was assessed using an EMSA. We chose the 34-mer oligonucleotides, because they are effective ss and ds substrates for Ape1 (16). Consistent with prior reports (30, 31), our binding experiments indicate that human RPA has a higher affinity (>10-fold) for unmodified ss 34COMP DNA than for ds 34COMP:34G DNA (Fig. 4, B and C). When comparing ss 34COMP with ss 34F, we found that RPA bound either oligonucleotide with similar affinity, i.e., independent of the abasic damage. We also found that RPA exhibited an intermediate binding affinity for F-containing ds substrates that is ~5-fold weaker than the ss oligonucleotides (Fig. 4, B and C). Based on these studies, the apparent $K_d$ value for each DNA substrate (i.e., the concentration of RPA required to bind 50% of the DNA substrate) is estimated to be as follows: ss 34COMP and ss 34F, ~5 nM; ds 34F:34G, 25 nM; and ds 34COMP:34G, 67 nM (although 50% binding was never attained) (Fig. 4C). We note that our EMSA data are generally consistent with previous binding and footprinting studies (reviewed in Ref. 17), which indicate that RPA has a preference for ssDNA molecules and that RPA spans 8–30 nt of ssDNA per trimer, depending on its mode of DNA interaction (i.e., the conformational state of RPA (30, 32–34)), presumably explaining why we observe as many as four different protein-DNA complexes in the reactions here using the 34-mer oligonucleotide substrates (Fig. 4B).

Effect of RPA on the ss and ds Incision and Binding Activities of Ape1—Because RPA exhibits a binding affinity for ssDNA containing an AP site that is essentially identical to unmodified ssDNA (Fig. 4), we examined the effect of RPA on the Ape1 incision of ss 34F. These studies revealed an inhibitory effect of RPA. Specifically, inactivation of Ape1 (present in catalytic amounts) ss cleavage activity was RPA concentration-dependent, achieving ~100% inhibition at 2 pmol of RPA (Fig. 5A), a protein level roughly twice that of the oligonucleotide substrate concentration in the reaction (i.e. 1 pmol/10 μl). Heating of the RPA preparation (at 6 pmol) reversed this inhibition (see below as well), indicating that the RPA storage buffer did not appreciably contribute to the inactivation of Ape1 function.

To test whether the observed inhibition of Ape1 by RPA was substrate-specific, we performed additional incision studies using alternative ss F-containing oligonucleotide substrates. As shown in Fig. 5B, RPA exhibited a similar inactivation of Ape1 endonuclease activity with
Ape1 ss Incision Activity

FIGURE 5. RPA inhibits Ape1 ss AP site incision activity but not its ds activity. A, representative gel image showing the incision activity of Ape1 in the presence of RPA (left). S indicates intact ss 34F substrate; P indicates incised product. Each reaction was conducted for 5 min in the presence of 10 pg of Ape1 (0.28 fmol) and 1 pmol of DNA. RPA in the far right lane was heat-inactivated (denoted by the triangle). Quantitative analysis of three independent Ape1 incision reactions is shown to the right. Data represent relative averages and S.D. B, Ape1 incision assays with alternative ss F-containing substrates. Reactions were performed in the presence of 10 pg of Ape1 for 5 min with 34F' D1, and 250 pg of Ape1 for 1 h with 34F' D2 and 26FD2. DNAs were present at 1 pmol. RPA amounts are denoted, and reactions where RPA was heat-inactivated are indicated by the triangle. Numbers below each lane indicate the percent cleavage. S indicates substrate; P indicates product. C, representative EMSA gel image showing reduction of APE1 DNA binding by RPA (left). Experiment was performed with 60 fmol of Ape1; 0, 20, 60, 200, or 600 fmol of RPA; and 20 fmol of ss 34F DNA. Both the RPA- and Ape1-specific complexes are indicated. Quantitative analysis of three independent binding experiments is shown to the right. Data represent averages and S.D. D, assay of dG DNA incision by Ape1 (0.4 fmol) without or with RPA (60, 200, 600, and 2000 fmol). 700 fmol of duplex substrate was used in each reaction. Shown is a representative experiment from three individual reaction sets. Numbers below each lane indicate the percent cleavage. E, protein-competition EMSA. Experiment was performed with 10 fmol of Ape1, and 0, 60, 200, 600, or 2000 fmol of RPA. DNA was present at 20 fmol.

To elucidate more directly the mechanism for RPA inhibition, protein-competition EMSAs were performed. These studies revealed that RPA out-competed Ape1 for binding to the AP site-containing ss 34F DNA (Fig. 5C). Specifically, in reactions where the Ape1 concentration was held constant (i.e. 60 pmol/10 μl), increasing the concentration of RPA to roughly 10-fold over the DNA substrate (i.e. 200 fmol of RPA and 20 fmol of ss 34F) resulted in complete abolishment of the preformed Ape1-DNA complexes in favor of the newly formed RPA-DNA complexes. Moreover, when RPA (200 fmol) was premixed with 34F (20 fmol) at a saturating amount (i.e. at 100% bound AP-DNA), subsequent addition of Ape1 (up to 2 pmol) failed to give rise to any observable Ape1-DNA complexes, although an Ape1-DNA complex was seen in the absence of RPA (data not shown). Such results further support the notion that RPA inhibition is mediated via a direct, stable interaction with the ss AP-DNA molecule, which ultimately prevents efficient Ape1 recognition/incision. This conclusion is in line with our following observations: (i) RPA and Ape1 do not physically interact in vitro (supplemental Fig. 2), and (ii) E. coli single-stranded DNA-binding protein inhibits Ape1 ss incision activity as well (data not shown). Finally, we emphasize that no supershift was observed in the presence of both proteins relative to the protein alone reactions (Figs. 4 and 5C), indicating that Ape1 and RPA form distinct and independent complexes with DNA, also consistent with the lack of a direct physical interaction.

We next examined whether RPA had an effect on Ape1 ds AP site incision activity. As shown in Fig. 5D, the ds AP endonuclease function of Ape1 was unaffected by increasing amounts of RPA, and no impact was seen even at ~5000- and 3-fold molar excess heterotrimer (i.e. 2000 fmol of RPA) in comparison to Ape1 and ds AP-DNA, respectively. Consistent with Ape1 ds incision activity being unaltered by RPA (Fig. 5D), the heterotrimer did not affect Ape1 complex stability even when present at a 100- and 200-fold molar excess with respect to AP-DNA and Ape1 as assessed using a protein competition EMSA (Fig. 5E). In total, the findings here support the conclusions that RPA inhibition is not mediated by a direct protein-protein interaction and that the relative affinity of the two proteins for the specific DNA substrate in question dictates which protein factor is capable of binding most effectively.
Ape1 ss Incision Activity

FIGURE 6. RPA inhibits Ape1 incision at an AP site within a fork but not a bubble substrate. A, ds fork substrate (42F-fork:42Comp) contains an AP site in the ss region, 11 nt from the duplex junction. B, ds bubble substrate (42F-11bubble:42Comp) contains an AP site centrally located in an 11-nt bubble region. Details regarding the oligonucleotides are reported in Ref. 16. For the reactions here, 1 pmol of substrate, and 150 pg (fork) or 30 pg (bubble) of Ape1 were used; * indicates no Ape1. Amount of RPA employed is indicated. Reactions were performed at 37 °C for 5 min. Representative incision gel is shown to the left, with the intact substrate the upper band and the incised product the lower band. Quantitative analysis of three independent Ape1 incision reactions is shown to the right. Data represents relative averages and S.D.

(i.e. RPA to ssDNA and Ape1 to ds AP-DNA), and thus the biochemical outcome.

RPA Inhibits Ape1 Incision of Fork but Not Bubble Structures—To explore further the specificity of RPA inhibition, previously characterized biologically relevant substrate mimics were employed (16). Specifically, the effects of RPA on Ape1 incision at an AP site positioned within the ss region of a fork duplex (replication/recombination mimic) or within an 11-nt bubble structure (transcription mimic) were examined. These studies (performed essentially as described above) revealed that the heterotrimeric complex RPA inhibits Ape1 activity on fork duplexes but has no or little effect on enzymatic incision of bubble structures (Fig. 6). As detailed above (Figs. 2 and 3), it is anticipated that the nature of the ss region extending from the fork duplex (i.e. length and sequence context) will dictate the efficiency with which Ape1 cleaves and the extent of RPA inhibition.

DISCUSSION

Ape1 exhibits a robust ss AP site incision activity that in some instances is more powerful than its essential ds incision function (16). This fact, and the significant discrepancy of Ape1 AP endonuclease activity previously observed with two ss abasic substrates (i.e. 26GFA and 34F), prompted us to identify which DNA structural elements may influence Ape1 ss incision efficiency. Moreover, we aimed to understand how the ss endonuclease activity of Ape1 might be regulated in vivo, exploring specifically the effect(s) of the major ssDNA-binding protein RPA on a variety of transaction intermediates. Our studies herein reveal that the endonuclease activity of Ape1 is poorly operational on ss AP site-containing polyadenine or polythymine oligonucleotides (Fig. 1), clearly indicating a crucial role for DNA sequence context and associated secondary structure in determining Ape1 cleavage efficiency. In addition, RPA was found to be a selective negative regulator of Ape1 ss AP site incision activity, likely indicative of a key means of modulating this endonuclease function in vivo.

As documented in Fig. 2B, each of the ss F-containing 26- and 34-mer oligonucleotides takes on an intramolecular DNA secondary structure that fosters a more rapid electrophoretic mobility shift relative to the corresponding poly(T)-F-DNAs. T7 endo I footprinting analyses (Fig. 2C) indicate (acknowledging the limitations of the method) that the nature of the secondary structure, with a few exceptions, generally mimics that predicted by the RNAfold algorithm (Fig. 2A). Thus, in combination with the incision results presented in Fig. 3, A and B, the overall findings suggest that the elements which most prominently determine Ape1 effectiveness are as follows. (i) The position (i.e. 5’ or 3’) of the duplex structure with respect to the abasic site, and its length (i.e. the number of complementary nt within the ds region). (ii) The location of the AP site with respect to the potential secondary conformation (i.e. the number of nt the abasic lesion is from the duplex or hairpin-loop structure). (iii) The size of the ss region within the hairpin loop (i.e. the number of unpaired nt); for more specifics, see under “Results.” All told, the data emphasize the importance of DNA secondary structure in determining the capacity of Ape1 to associate and/or incise at abasic lesions in ssDNA regions, which undoubtedly exist in the genome.

Our incision results in conjunction with the competition-based EMSAs (Fig. 3, C and D) suggest that binding affinity and cleavage efficiency of the various ss AP-DNA molecules can be correlated in a broad sense. That is, the typically higher affinity 34-mer substrates were incised more efficiently than the generally lower affinity 26-mers by Ape1 (summarized in Table 2). However, some deviation from this rule did exist. In particular, 34F and 34F’D1 exhibit similar incision efficiencies, yet display an ~10-fold difference in binding affinity as determined by the competition experiments (Table 2). Moreover, 34F’D1 and 34F’D2, while exhibiting a similar competitor effectiveness, display an ~47-fold difference in AP site incision proficiency. These specific examples agree with prior studies that indicate that binding and incision (i.e. catalysis) by Ape1 can be distinct and separable events that take place during phosphodiester bond cleavage (16, 35). Thus, in total, our data emphasize that many factors, including nt sequence context, DNA secondary and tertiary structure, and overall flexibility/dynamic properties of DNA, play an important role in determining the effectiveness of Ape1 as both an ss and ds AP endonuclease.

Most biochemical studies to date have characterized the activity(ies) of DNA repair proteins in the absence of other cellular factors. Specifically, how major DNA-binding proteins may affect the processing functions of repair enzymes has gone largely unexamined. For instance, we (and others) have studied the in vitro ss and ds AP site incision activity of Ape1 primarily in the absence of other cellular proteins (13, 16, 23, 36–38). More recent studies, however, have begun to explore the effects of chromatin structure on DNA repair efficiency, and early analyses indicate that nucleosome assembly can influence the aptitude of BER enzymes (39, 40). In particular, uracil-containing dsDNA reconstituted into nucleosomes is cleaved by the uracil DNA glycosylase UNG and Ape1 at a rate ~10-fold slower than naked DNA (40).

We investigated here the effect of RPA, the major ssDNA-binding protein in eukaryotes (17), on the incision activity of Ape1 using various DNA substrates. We report that RPA inhibits the AP endonuclease activity of Ape1 on ss AP-DNA (Fig. 5, A–C), although the heterotrimer...
does not interact with the human Ape1 protein directly (supplemental Fig. 2). Thus, given that (i) RPA exhibits high affinity for and complex stability with ssDNA molecules, regardless of the AP damage (Fig. 4), (ii) RPA competes away Ape1 binding to ss 3′AF in EMSAs (Fig. 5C), and (iii) E. coli single-stranded DNA-binding protein has a similar inhibitory effect of Ape1 cleavage activity, we conclude that inhibition is achieved by RPA coating ssDNA and obstructing Ape1 complex formation with the substrate (or by replacing Ape1 on ss abasic DNA). This inference is consistent with the known apparent KD values of the two proteins for ss and ds AP-DNAs that are as follows: Ape1, ~2 nM for ds AP-DNA and ≥7 nM for ss abasic DNA (16); RPA, ≥67 nM for ds AP-DNA and ~5 nM for ss substrates (Fig. 4). Moreover, because RPA can unwind DNA in the absence of ATP, i.e. it is helix-distabilizing (41–43), it is quite possible that this heterotrimer disrupts the secondary structure of ssDNA, creating a substrate poorly recognized by Ape1.

It is noteworthy that RPA binding was unaffected by the presence of a single AP site in ssDNA, particularly because other DNA-binding proteins, such as sequence-specific transcription factors and telomere repeat binding proteins, have been shown to be adversely impacted by the presence of oxidative lesions, including abasic damage (44, 45). Related to this topic, prior work has shown that RPA complex stability with ss added DNA is largely dependent on the nature of the DNA modification, where certain bulky lesions can negatively impact RPA binding affinity (46–49).

RPA has been reported previously to participate in BER. For instance, RPA physically interacts with the DNA glycosylases MYH (50), UNG (51), and NEIL3 (52) and stimulates the enzymatic activity of DNA ligase I (53). Although not essential for in vitro long-patch BER (54), some reconstitution experiments using either cell extracts (55) or purified proteins (56) have suggested that RPA promotes the long-patch BER process, which involves strand displacement synthesis and the incorporation of typically 2–7 nt. We report here that RPA neither physically interacts with Ape1 (supplemental Fig. 2) nor has any impact on Ape1 incision activity of ds AP-DNA substrates (Fig. 5, D and E), suggesting that RPA plays no significant role in regulating the initial steps of classical AP site-directed BER. Nonetheless, RPA was found to bind to AP site-containing dsDNA more efficiently than undamaged dsDNA, albeit with less affinity than ss oligonucleotides (Fig. 4), perhaps suggesting that RPA recognizes instability around the abasic lesion. Such enhanced damage-specific ds binding affinity has been seen with ultraviolet- and cisplatin-damaged duplex DNAs (46, 57–60). It is conceivable that RPA encourages long-patch BER as a helix-distabilizing factor by unwinding the AP-DNA duplex upon binding and promoting extended polymerization after strand incision.

In total, Ape1 exhibits a powerful ds AP site repair activity, as well as the ability to incise at AP lesions in either ss or dsDNA is influenced appreciably by reaction conditions (namely salt concentrations). We detail herein a novel mechanism for regulating Ape1 ss cleavage activity that involves DNA secondary structure (i.e. nt sequence context) and the cellular factor RPA. Although RPA does not modulate Ape1 cleavage activity on classical ds AP-DNA BER substrates, RPA binds to and inhibits Ape1 incision activity on ss nontemplate-containing abasic DNA. We propose that this inhibitory effect (i) involves RPA coating of ss AP-DNA, and possibly dissolution of any complex secondary structure, (ii) prevents promiscuous strand cleavage of nontemplated ssDNA stretches, and (iii) specifically suppresses the formation of ds breaks at regions of ongoing replication (namely those sites that are paused or stalled), where AP site-containing ss regions may assume secondary conformations that are readily cleaved by Ape1. Consistent with this notion, RPA inhibited the incision activity of Ape1 at abasic sites positioned within the ss portion of a model fork substrate designed to mimic a characteristic replication intermediate (Fig. 6A). It seems reasonable after all to predict that recombinegenic ds break products would be more harmful to genetic efficacy than the initial single nt lesion. Moreover, RPA did not inhibit Ape1 incision activity at AP sites within an 11 nt unpaired bubble structure (Fig. 6B), suggesting an unanticipated selectivity of RPA inhibition. Thus, the picture as a whole suggests that whereas RPA has no regulatory effect of Ape1 activity on classic ds BER substrates, or on transcription-type intermediates, this essential replication complex strongly and specifically inhibits cleavage of AP sites within nontemplated ss regions (see model outlined in supplemental Fig. 3). Studies are underway to define the roles of other cellular proteins on modulating Ape1 activities using different biologically relevant complex DNA structures, such as transcription mimics. Results within may also have implications for immunoglobulin gene diversification (61).

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