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Redox Regulation of the DNA Repair Function of the Human AP Endonuclease Ape1/ref-1

MARK R. KELLEY and STEPHEN H. PARSONS

ABSTRACT

The second enzyme in the DNA base excision repair (BER) pathway, apurinic/apyrimidinic (AP) endonuclease or Ape1, hydrolyzes the phosphodiester backbone immediately 5′ to an AP site generating a normal 3′-hydroxyl group and an abasic deoxyribose-5-phosphate, which is processed by subsequent enzymes of the BER pathway. AP sites are the most common form of DNA damage, and the persistence of AP sites in DNA results in a block to DNA replication, cytotoxic mutations, and genetic instability. Interestingly, Ape1/ref-1 is a multifunctional protein that not only is a DNA repair enzyme, but also functions as a redox factor maintaining transcription factors, such as Fos, Jun, nuclear factor-κB, PAX (paired box-containing family of genes), hypoxia inducible factor-1α (HIF-1α), HIF-1-like factor, and p53, in an active reduced state. Ape1/ref-1 has also been implicated in a number of other activities, one of which is the activation of bioreductive drugs requiring reduction for activity. In this report, we present data supporting our findings that another level of posttranslational modification of Ape1/ref-1 that clearly affects the AP endonuclease activity is the reduction or oxidation of this protein. Furthermore, we show data demonstrating that at least one of the sites involved in this redox regulation is the cysteine amino acid found at position 310, immediately adjacent to the crucial histidine residue at position 309 in the DNA repair active site. These findings suggest that the Ape1/ref-1 protein may be much more intimately regulated at the posttranslational level than initially imagined. Antioxid. Redox Signal. 3, 671–683.

INTRODUCTION

APURINIC/APYRIMIDINIC (AP) sites are the most common form of DNA damage with some 20,000–50,000 sites produced in every cell each day under normal physiologic conditions (22, 26, 27). AP sites are generated from spontaneous and chemically initiated hydrolysis, ionizing radiation, UV irradiation, oxidative stress, oxidizing agents, and removal of altered, *e.g.*, alkylated, bases by DNA glycosylases (9, 23). For instance, direct alkylation of the base by electrophilic chemotherapeutic agents can result in AP sites as the alkylated base may be excised by specific DNA glycosylases (Fig. 1) (1–3, 44). The persistence of AP sites in DNA results in a block to DNA replication, cytotoxic mutations, and genetic instability (23). AP sites are primarily repaired by the DNA base excision repair (BER) pathway (Fig. 1).

The second enzyme in the BER pathway, AP endonuclease or Ape1, hydrolyzes the phosphodiester backbone immediately 5′ to an AP site (Fig. 1A). This incision generates a normal 3′-hydroxyl group and an abasic deoxyribose-5-phosphate, which is processed by subsequent enzymes of the BER pathway. Reactive oxygen species and free radicals, common products of ionizing radiation exposure, are capable of oxidizing the deoxyribose moiety producing 1′

Departments of Pediatrics (Section of Hematology/Oncology) and Biochemistry & Molecular Biology, Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202.

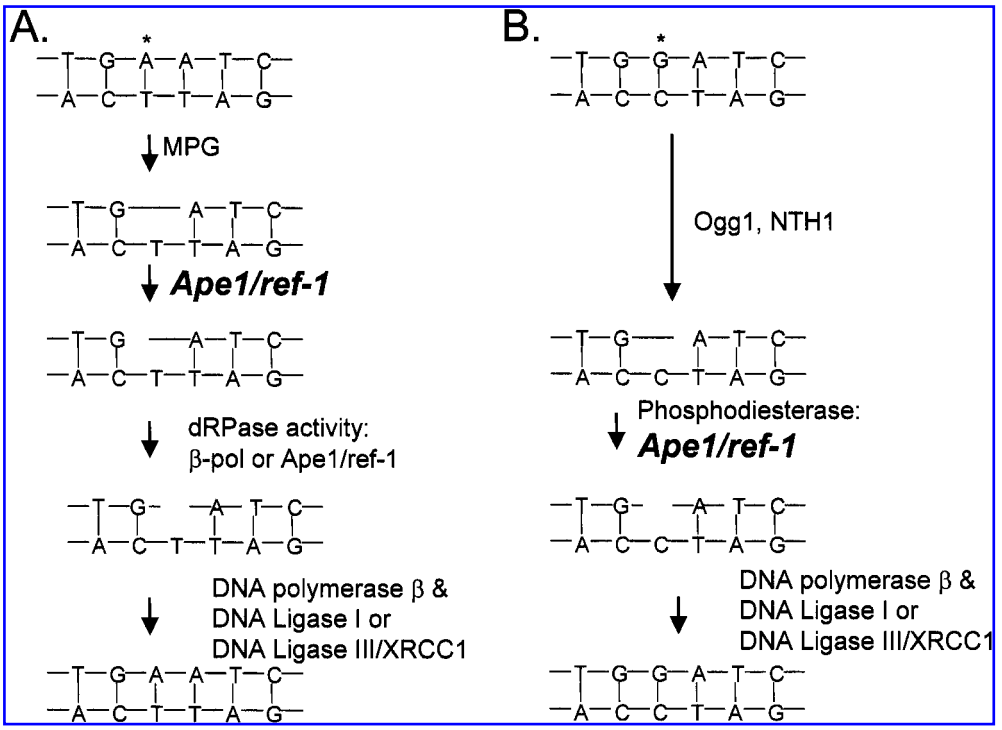

FIG. 1. BER pathways in mammalian cells. (**A**) Short patch BER with a simple glycosylase (class I glycosylase: MPG). (**B**) Short patch BER with a complex glycosylase (class II glycosylases: Ogg1, NTH).

and 4′ oxidized AP sites along with 3′-phosphate and phosphoglycolate species. As nearly all 5′-termini are 5′-phosphates, the 3′-ends presumably contain obstructive groups. These ends were shown to contain a mixture of 3′-phosphates and 3′-phosphoglycolates (16). If unrepaired, such strand breaks can lead to cell death by impeding repair synthesis or ligation, or can induce chromosomal rearrangements that lead to genetic changes (5, 8). Ape1 also contains repair activity for 3′-terminal oxidative lesions (9, 10, 24, 32, 33, 38). By hydrolyzing 3′-blocking fragments from oxidized DNA, Ape1 produces normal 3′-hydroxyl nucleotide termini necessary for DNA repair synthesis and ligation at certain strand breaks.

Bacteria, yeast, or human cells lacking AP endonuclease repair activity are hypersensitive to agents (*e.g.*, alkylating or oxidizing) that induce the formation of AP sites (9). Moreover, targeted reduction of Ape1 protein by specific antisense RNA expression renders mammalian cells hypersensitive to methyl methanesulfonate, hydrogen peroxide (H_2O_2), and bleomycin, and recent studies have shown that radiosensitivity of cervical cancers is directly correlated to the levels of Ape1 activity (17, 29, 41). Ape1 levels have also been found to be elevated in a number of other cancers such as ovarian, cervical, prostate, rhabdomyosarcomas, and germ cell tumors (20, 21, 25, 36, 39, 46). Therefore, under a variety of situations, Ape1/ref-1 appears to be one of the rate-limiting steps in the BER pathway (19).

Interestingly, Ape1/ref-1 is additionally a multifunctional protein that not only is responsible for repair of AP sites, but also functions as a redox factor maintaining transcription factors in an active reduced state (Fig. 2) (45). In these initial studies, Ape1/ref-1 was given the name redox effector factor 1 (ref-1) and appears in the literature under this name, as well as APE, hAPE, HAP1, APEX, and others (12). Ape1/ref-1 has been shown to stimulate the DNA binding activity of numerous transcription factors that are involved in cancer promotion and progression, such as Fos, Jun, nuclear factor-κB (NFκB), PAX (paired box containing family of genes), hypoxia inducible factor-1α (HIF-1α), HIF-1-like factor (HLF), and p53 (12). Ape1/ref-1 has also been implicated

in the activation of bioreductive drugs requiring reduction in order to be active (31) and has been shown to interact with a subunit of the Ku antigen to act as a negative regulation of the parathyroid hormone promoter (7, 28), as well as part of the HREBP transcription factor complex (Fig. 2) (34).

Recent investigations into the posttranslational modification of Ape1/ref-1 by us (47) and others (13) have identified a number of potential phosphorylation sites scattered throughout the protein that also affect either the repair (47) or redox function of Ape1/ref-1 (13), albeit when studied *in vitro*. In this report, we present another level of posttranslational modification of Ape1/ref-1 that clearly affects the AP endonuclease activity of the protein. This modification is the reduction or oxidation of the protein. Furthermore, we show that at least one of the sites involved in this redox regulation is the cysteine amino acid found at position 310, immediately adjacent to the crucial histidine residue at position 309 in the repair active site (6, 11, 38, 42, 43). These findings suggest that the AP endonuclease activity of Ape1/ref-1 is regulated by its redox state.

MATERIALS AND METHODS

Reagents

Diamide, H_2O_2, Factor X protease, *N*-ethylmaleimide, dithiothreitol (DTT), Triton X-100, and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); [^{32}P]-dATP was from Amersham Life Science (Buckinghamshire, U.K.); and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), was from Molecular Probes (Eugene OR, U.S.A.).

Protein production and purification

Human Ape1/ref-1 wild-type and mutant proteins were overproduced using the vector pGEX-3X (Pharmacia Biotech, Uppsala, Sweden) system utilizing *BamH*I 5′ and *EcoR*I 3′ restriction sites. Lysis of *E. coli* cells containing the recombinant pGEX-Ape1/ref-1 construct and purification of GST-Fusion Ape1/ref-1 over a glutathione column were performed as previously described (15, 18, 47). The GST protein was cleaved from Ape1/ref-1 via Factor X protease digestion in 1× phosphate-buffered saline (135 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, pH 7.4). Protein (2.5–5.0 mg) was digested with 5.0 units of Factor X at 4°C for 16 h. Following digestion, samples were dialyzed into 1X phosphate-buffered saline and applied to a glutathione column. Unbound Ape1/ref-1 fractions were pooled and dialyzed into 10 m*M* Tris, pH 7.3, and applied to an S-Sepharose column. Protein fractions were eluted using a 100-ml, 0–100% 1 *M* NaCl salt gradient. Protein samples were resolved using a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and visualized via Coomassie Blue staining to determine purity. All protein samples used for study were ≥95% pure.

AP oligonucleotide assay for Ape1/ref-1 DNA repair activity

A ^{32}P-labeled 26mer oligonucleotide containing a tetrahydrofuran (F) residue at position 15 was utilized as previously described (Fig. 3) (15, 18, 47). Assays using 800 pg of Ape1/ref-1 protein were performed in a 10-μl reaction volume using 2.5 pmol of labeled double-stranded F oligonucleotide, in 50 m*M* HEPES, 50 m*M* KCl, 10 m*M* $MgCl_2$, 2 m*M* DTT, 1 μg/ml bovine serum albumin, 0.05% Triton ×-100, pH 7.5. Reactions were allowed to proceed for 15 min in a 37°C water bath and stopped by adding an equal volume of 96% formamide, 10 m*M* EDTA, xylene cyanol, and bromophenol blue. Five microliters of this 20-μl sample was separated with a 20% polyacrylamide gel containing 7 *M* urea. Gels were wrapped in plastic wrap and exposed to film for visualization. Reactions that displayed redox regulation were performed as above, but included the addition of oxidizing agents H_2O_2 and diamide.

Determination of Ape1/ref-1 protein redox status in reactions displaying redox regulation

Eight nanograms of cleaved purified Ape1/ref-1 protein was exposed to oxidizing and reducing conditions and repair activity assayed as described above. Samples incubated in a 37°C water bath for 15 min under reducing or

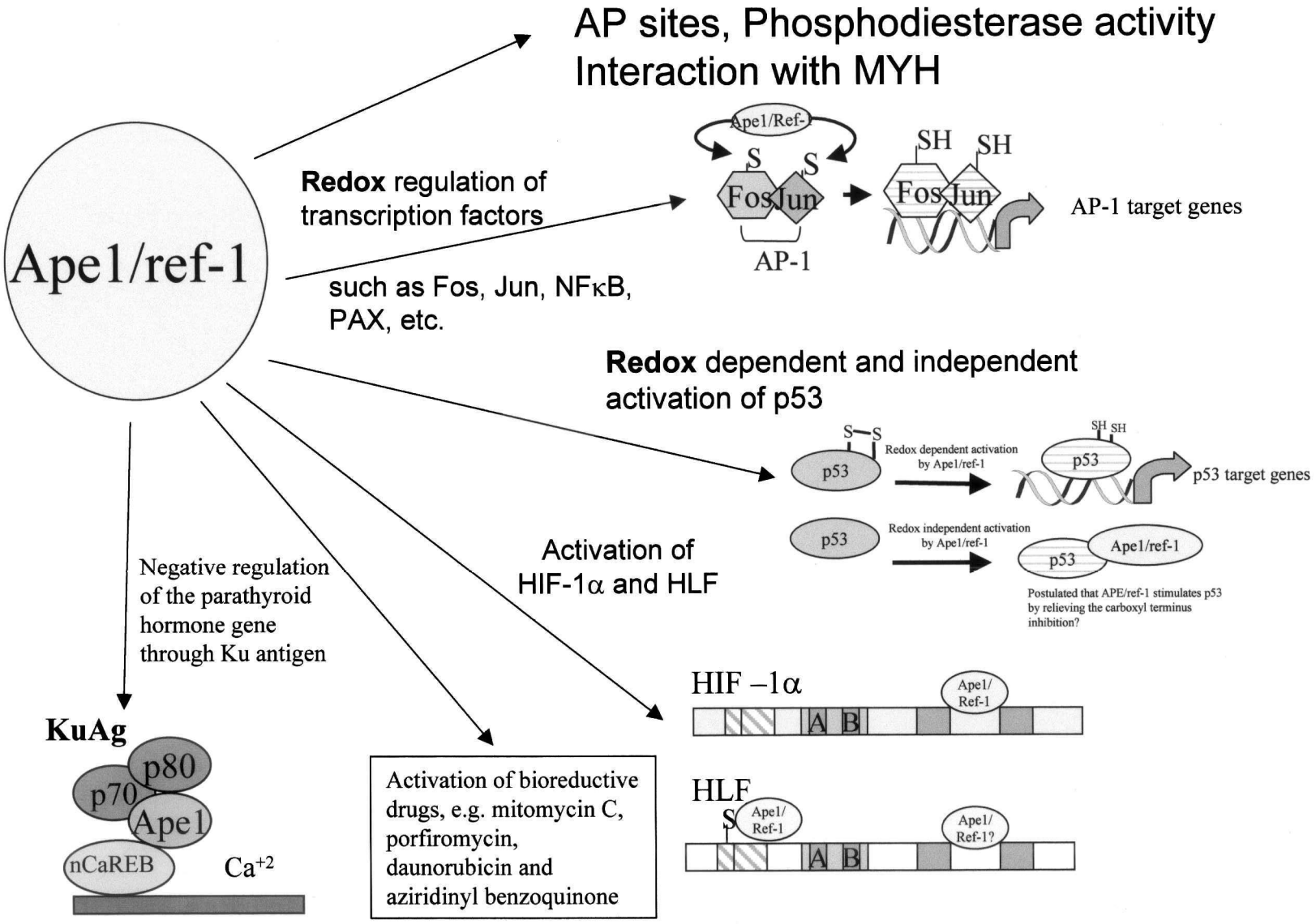

FIG. 2. Many functions of the Ape1/ref-1 protein. Ape1/ref-1 is a multifunctional protein that is involved in AP endonuclease repair activity, redox factor for numerous transcription factors (45), including some that are involved in cancer promotion and progression such as Fos, Jun, NFκB, PAX, HIF-1α, HLF, and p53 (12), and activation of bioreductive drugs (31) and has been shown to interact with a subunit of the Ku antigen to act as a negative regulation of the parathyroid hormone promoter (7, 28). It is also elevated in a number of cancers (12).

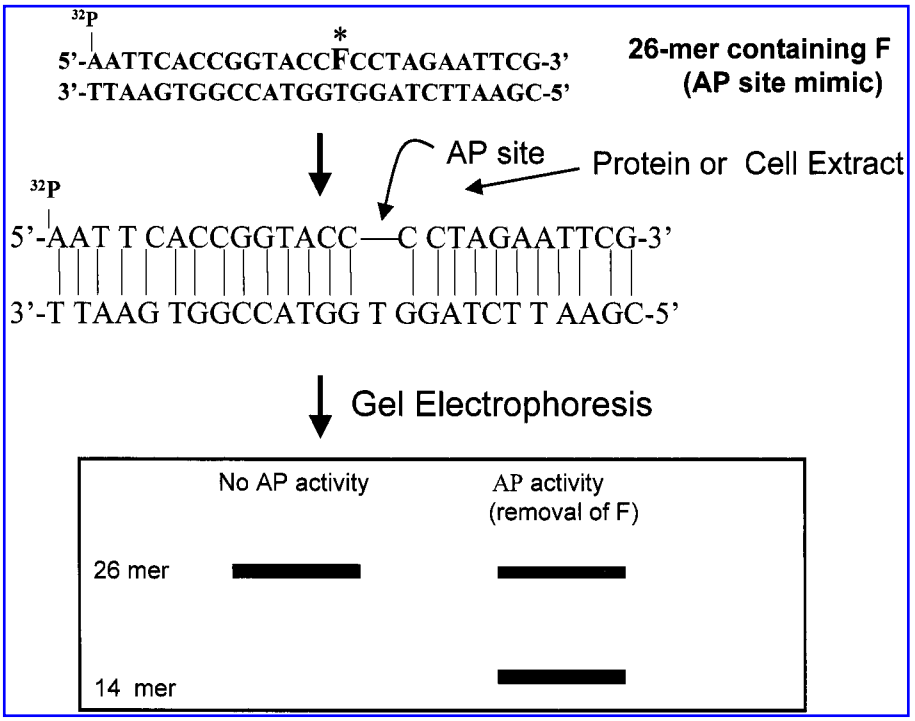

FIG. 3. AP oligonucleotide endonuclease assay. Radiolabeled 24mer oligonucleotide containing a tetrahydrofuran AP site mimic (F) at position 15 in the labeled strand is reacted with the protein or extract in assay buffer (see Materials and Methods). After the reaction is stopped, the fragments are denatured and analyzed on a DNA sequencing gel. If there is no AP endonuclease activity at the AP site, the unreacted product migrates as a 26mer, whereas AP endonuclease activity is shown as a 14mer.

oxidizing conditions were precipitated using 1/10 volume of 100% trichloroacetic acid. Protein samples were recovered by centrifugation for 10 min at 10,000 rpm. Protein pellets were resuspended in a 0.1% sodium dodecyl sulfate/0.67 *M* Tris-HCl, pH 8.0, solution, containing 15 m*M* AMS. The 15-μl alkylation reactions were allowed to proceed for 2 h in a 37°C water bath. Following alkylation, the reactions were applied to an SDS-PAGE gradient gel 4–20% to resolve protein bands (FMS Bioproducts, Rockland, ME, U.S.A.). Subsequent transfer, western hybridization, and chemiluminescent detection utilizing monoclonal Ape1/ref-1 antibodies (Novus Biologicals, Littleton, CO, U.S.A.) were performed as previously described (15, 35, 47).

Site-directed mutagenesis of Ape1/ref-1

Cysteine-to-alanine changes were obtained using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.). Oligonucleotide design and application in PCR reactions were performed as suggested in the QuickChange instructions. The pGEX-3X vector containing the Ape1/ref-1 cDNA was used for template in our mutagenesis PCR reactions. Colonies transformed with products of the site-directed mutagenesis PCR were isolated and grown, and purified DNA was sequenced to verify that only the specified mutation was present.

K_m, V_{max}, and turnover determination for wild-type and mutant Ape1/ref-1 proteins

AP endonuclease activity assays were performed as described above with the exception of varying F oligonucleotide substrate concentration. Substrate concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, and 16 μM were used to determine kinetic activities of wild-type and mutant Ape1-/ref-1 proteins. Band intensities were quantified using Sigma Scan software (SPSS Inc., Chicago, IL. U.S.A.). The sum intensity was used to estimate picomole amounts of cleaved and uncleaved substrate. The activity of wild-type and mutant protein was calculated in pmol of product/min. Once the activities of

the protein samples were derived, they were applied to the Cleveland Kinetics program to obtain K_m and V_{max} values. Turnover numbers were calculated based on the estimated V_{max} values, obtained from the kinetics program, divided by picograms of Ape1/ref-1, to give pmol of product/pg of Ape1/ref-1/min.

RESULTS

AP endonuclease oligonucleotide assays were performed testing whether wild-type Ape1/ref-1 protein activity was inhibited by the addition of varying concentrations of the oxidizing agents H_2O_2 and diamide. Functional AP endonuclease repair activity was determined by the amount of cleaved 26mer oligonucleotide. Cleaved product is observed as a 14mer fragment on a denaturing acrylamide gel, and uncleaved oligonucleotide correlating to no activity was observed as a 26mer fragment. The addition of 10 m*M* H_2O_2 with 2 m*M* DTT in our assay demonstrated no detectable activity, whereas the addition of 10 m*M* H_2O_2 with 40 m*M* DTT restored the activity completely (Fig. 4). In a similar fashion using diamide as our oxidizing agent, we observed no detectable activity in reactions containing 3 m*M* diamide with 2 m*M* DTT, whereas the addition of 10 m*M* diamide with 40 m*M* DTT also completely restored activity (Fig. 4).

To determine if the inhibition of activity was caused by redox regulation or simply by degradation of Ape1/ref-1 protein, we performed an AMS alkylation assay. In this assay, the AMS moiety was covalently bound to reduced cysteine residues. The addition of this molecule resulted in a gel shift in which reduced proteins migrated more slowly than oxidized proteins. We repeated the experimental conditions, as described above and shown in Figs. 4, 6, and 7, to determine if inactivity of Ape1/ref-1 correlated with oxidation of the protein. Results of this assay indicated that Ape1/ref-1 was not degraded by treatment with oxidizing agents, but clearly demonstrated that inactivity was related to the oxidation state of the protein; reduced proteins were active and oxidized proteins were not (data not shown).

To elucidate further which amino acids may be involved in the redox regulation of Ape1/ref-1, we initiated site-directed cysteine-to-alanine mutations of residues in the redox domain (C65) or the repair active site of Ape1/ref-1 (C310) (Figs. 5–8). When we subjected the Ape1/ref-1-C65A protein to diamide oxidation conditions identical to those used

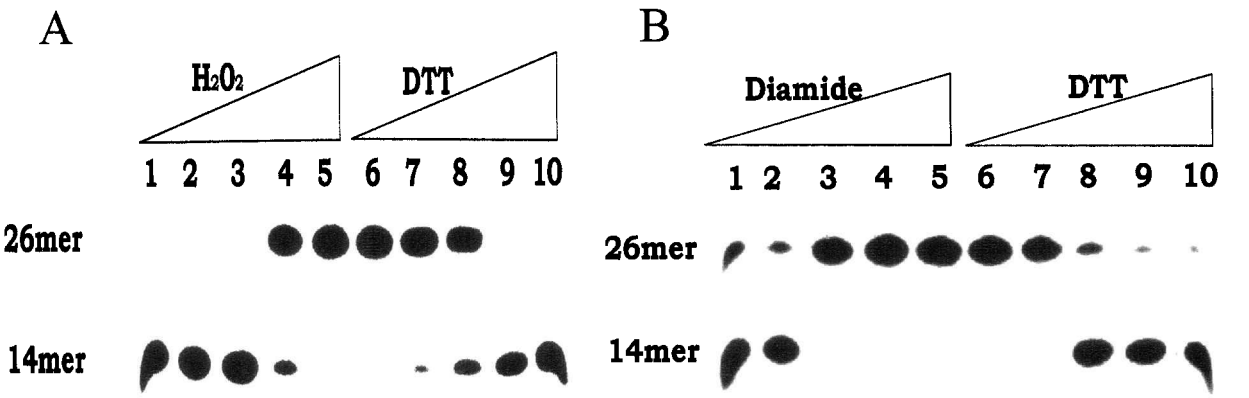

FIG. 4. AP endonuclease assay of wild-type Ape1/ref-1 for redox regulation of the DNA repair activity. (**A**) Ape1/ref-1 (800 pg) was treated with either H_2O_2, DTT, or both and reacted with the F 26mer labeled oligonucleotide. Lanes 1–5 all had 2 m*M* DTT in the reaction with increasing concentration of H_2O_2. Lane 1, no H_2O_2; lane 2, 1.25 m*M* H_2O_2; lane 3, 2.5 m*M* H_2O_2; lane 4, 5 m*M* H_2O_2; lane 5, 10 m*M* H_2O_2. Lanes 6–10 all had 10 m*M* H_2O_2 and increasing concentrations of DTT. Lane 6, 5 m*M* DTT; lane 7, 10 m*M* DTT; lane 8, 15 m*M* DTT; lane 9, 20 m*M* DTT; lane 10, 40 m*M* DTT. (**B**) Ape1/ref-1 (800 pg) was treated with either diamide, DTT, or both and reacted with the F 26mer labeled oligonucleotide. Lanes 1–5 all had 2 m*M* DTT in the reaction with increasing concentration of diamide. Lane 1, no diamide; lane 2, 0.1 m*M* diamide; lane 3, 3 m*M* diamide; lane 4, 5 m*M* diamide; lane 5, 10 m*M* diamide. Lanes 6–10 all had 10 m*M* diamide and increasing concentrations of DTT. Lane 6, 5 m*M* DTT; lane 7, 10 m*M* DTT; lane 8, 20 m*M* DTT; lane 9, 30 m*M* DTT; lane 10, 40 m*M* DTT.

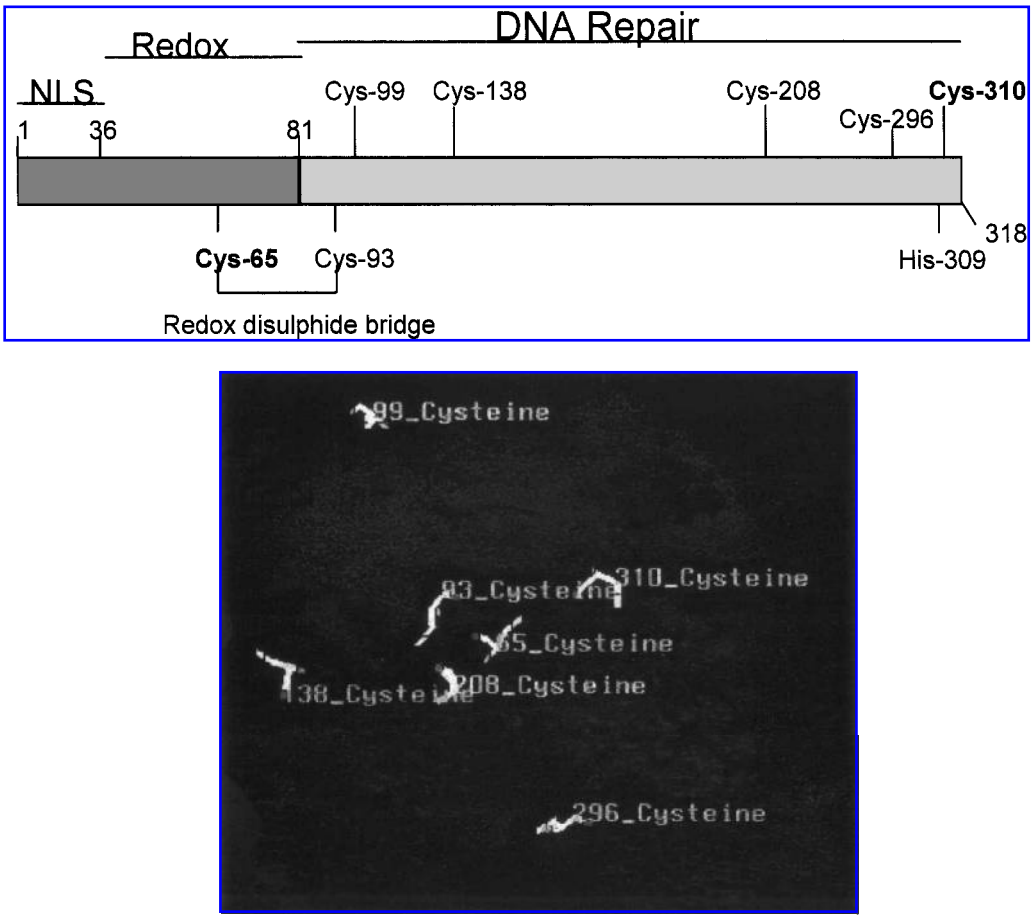

FIG. 5. Cartoon of Ape1/ref-1 illustrating the redox and repair domains and mutated cysteines. (**Top**) The C65 is involved in Ape1/ref-1 redox function, and C310 is located in the endonuclease active site immediately adjacent to the crucial H309 amino acid (12). The C65 and C93 form a disulfide bridge that is implicated in the redox function of Ape1/ref-1. (**Bottom**) Three-dimensional location of the seven cysteine residues in Ape1/ref-1 based on the known structure of Ape1/ref-1 (see references in 12). NLS, nuclear localization signal; repair domain, 81–318; redox domain, 1–81.

with the wild-type Ape1/ref-1 protein, the repair activity of the C65A mutant was identical to the normal, wild-type activity shown in Fig. 6. We concluded for this experiment that the C65 was not involved in the redox control of Ape1/ref-1 repair activity, and thus one or more of the other six cysteines in the protein must be involved.

To complement the above experiment, we wanted to test whether an alteration in the cysteine found in the repair active site, C310, would have an effect on the redox control when changed to alanine. When Ape1/ref-1-C310A was tested under oxidizing conditions, no inhibition of activity in reactions containing 10 m*M* H_2O_2, with 2 m*M* DTT, or in reactions containing 5 m*M* diamide, with 2 m*M* DTT, was observed (Fig. 7). Reaction mixtures containing, 10 m*M* H_2O_2, 40 m*M* DTT, or 5 m*M* diamide, 40 m*M* DTT, however, demonstrated stimulation in activity (Fig. 7). We also repeated similar experiments with fresh preparations of both the wild-type Ape1/ref-1 and C310A protein at the same time in order to rule out any discrepancies due to variation in protein preparations, reagent bias, or differences in the activity of the oligonucleotide substrate. As can be seen in Fig. 8, we have observed identical results as previously determined for both proteins.

To try and understand what biochemical mechanisms are influencing the phenotypes observed in our mutants, we determined the K_m, V_{max}, and turnover numbers of wild-type Ape1/ref-1 enzyme, under reducing conditions, and the Ape1/ref-1-C310A mutant en-

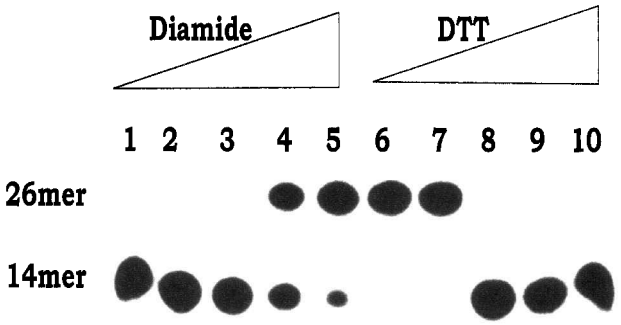

FIG. 6. The Ape1/ref-1 C65A mutant has DNA repair activity after treatment with diamide. C65A-Ape1/ref-1 (800 pg) was treated with either diamide, DTT, or both and reacted with the F 26mer labeled oligonucleotide. Lanes 1–5 all had 2 m*M* DTT in the reaction with increasing concentration of diamide. Lane 1, no diamide; lane 2, 0.1 m*M* diamide; Lane 3, 3 m*M* diamide; lane 4, 5 m*M* diamide; lane 5, 10 m*M* diamide. Lanes 6–10 all had 10 m*M* diamide and increasing concentrations of DTT. Lane 6, 5 m*M* DTT; lane 7, 10 m*M* DTT; lane 8, 20 m*M* DTT; lane 9, 30 m*M* DTT; lane 10, 40 m*M* DTT.

zyme under reduced, 20 m*M* DTT, and oxidized, 2 m*M* DTT, 10 m*M* H_2O_2, conditions (Table 1). The data presented above demonstrated that the Ape1/ref-1-C310A was not inhibited by oxidizing environments (Figs. 7 and 8). However, the Ape1/ref-1-C310A mutant was also stimulated by the reducing 40 m*M* DTT, 10 m*M* H_2O_2, environment. The K_m for the Ape1/ref-1-C310A mutant under oxidizing conditions increased over the reduced condition. The V_{max} and turnover values for the Ape1/ref-1-C310A mutant decreased in response to the oxidizing environment. It appears that the Ape1/ref-1-C310A mutant displayed stimulation because it was kinetically slower in the oxidized environment than in the reduced environment, whereas the K_m changed only slightly.

DISCUSSION

The recognition and repair of AP sites, whether by the 5′ endonuclease activity of Ape1/ref-1 or by the combined glycosylase/lyase activity cutting 3′ of the AP site followed by the phosphodiesterase activity of Ape1/ref-1, are crucial for either the immediate survival of a cell or the prevention of mutation accrual. A large amount of interest and experimentation has been focused on the BER pathway and the AP endonuclease reaction in particular (12). This activity has been studied in a wide variety of organisms, all the way from *E. coli* to humans, with a fair degree of conservation. However, a couple of crucial points became apparent in all these analyses. First, most of the studies, particularly those using mammalian preparations of AP endonuclease, whether purified or produced through recombinant techniques, have used enzyme that is always maintained in a reducing environment. Without

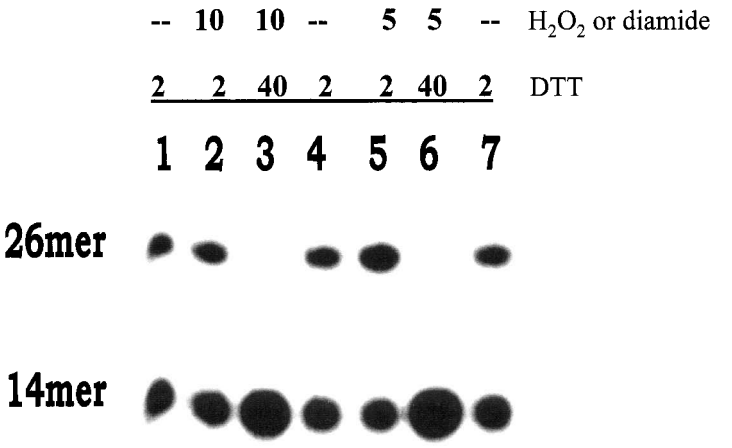

FIG. 7. AP endonuclease assay of Ape1/ref-1 C310A mutant testing the involvement of amino acid C310 in the redox control of Ape1/ref-1 repair activity. Lane 1, 800 pg of C310A, 2 m*M* DTT; lane 2, 800 pg of C310A, 2 m*M* DTT, 10 m*M* H_2O_2; lane 3, 800 pg of C310A, 40 m*M* DTT, 10 m*M* H_2O_2; lane 4, 800 pg of C310A, 2 m*M* DTT; lane 5, 800 pg of C310A, 2 m*M* DTT, 5 m*M* diamide; lane 6, 800 pg of C310A, 40 m*M* DTT, 5 m*M* diamide; lane 7, 800 pg of C310A, 2 m*M* DTT.

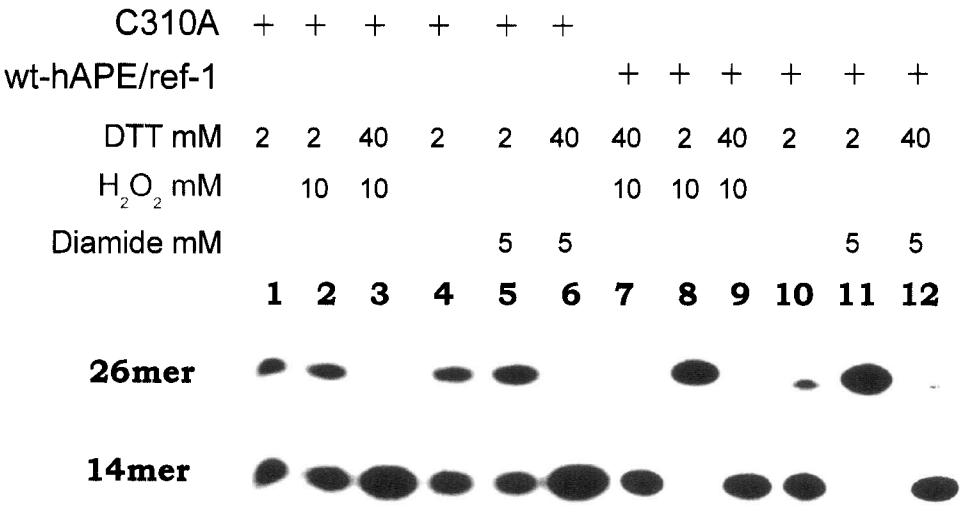

FIG. 8. Direct comparison of wild-type Ape1/ref-1 and Ape1/ref-1–C310A protein on the redox control of repair activity. Lane 1, 800 pg of C310A, 2 m*M* DTT; lane 2, 800 pg of C310A, 2 m*M* DTT, 10 m*M* H_2O_2; lane 3, 800 pg of C310A, 40 m*M* DTT, 10 m*M* H_2O_2; lane 4, 800 pg of C310A, 2 m*M* DTT; lane 5, 800 pg of C310A, 2 m*M* DTT, 5 m*M* diamide; lane 6, 800 pg of C310A, 40 m*M* DTT, 5 m*M* diamide; lane7, 800 pg of wild-type Ape1/ref-1, 40 m*M* DTT; 10 m*M* H_2O_2; lane 8, 800 pg of wild-type Ape1/ref-1, 2 m*M* DTT, 10 m*M* H_2O_2; lane 9, 800 pg of wild-type Ape1/ref-1, 40 m*M* DTT, 10 m*M* H_2O_2; lane 10, 800 pg of wild-type Ape1/ref-1, 2 m*M* DTT; lane 11, 800 pg of wild-type Ape1/ref-1, 2 m*M* DTT, 5 m*M* diamide; lane 12, 800 pg of wild-type Ape1/ref-1, 40 m*M* DTT, 5 m*M* diamide.

exception, the assays are performed in the presence of DTT or β-mercaptoethanol. Second, a large number of these studies have used recombinantly produced Ape1/ref-1, and particularly protein produced in *E. coli* expression systems. These two factors have contributed to the lack of observing crucial posttranslational control features of Ape1/ref-1 function. This is beginning to be illuminated with the previous work showing that phosphorylation plays a role in the regulation of the repair (47) or redox (13) function of Ape1/ref-1, and our data presented in this article.

It has also been a perplexing situation that Ape1/ref-1 is fairly abundant in mammalian cells [3.5×10^5 to 7×10^6 (9)], which has previously been used as a justification that it cannot be the rate-limiting protein in the BER pathway (4). However, a number of recent findings have begun to shed light on this observation. As mentioned above, it appears that Ape1/ref-1's repair and redox function can be under the control of phosphorylation (13, 47). It has also been shown that the repair and redox domains are mutually exclusive such that one can have molecules of Ape1/ref-1 that are redox-proficient and repair-deficient or redox-deficient and repair-proficient, respectively (13, 47). When placed in the context that one could also then have Ape1/ref-1 molecules that are phosphorylated, the number of potential combinations increases dramatically: therefore, our observations that an additional mechanism of Ape1/ref-1 control of function, *i.e.*, redox regulation, adds to the growing list of important posttranslational modifications and configurations of Ape1/ref-1 that could exist in a cell; and also, the fact that the role Ape1/ref-1 plays in interactions with other proteins in the cell appears to grow continuously (Fig. 2). For example, recent studies have demonstrated that Ape1/ref-1 also interacts with the human MutY homologue (MYH), a mammalian mismatch repair protein that is involved in the recognition and removal of 7,8-dihydro-8-oxodeoxyguanines:adenine mismatches (30).

We report here the first observations that Ape1/ref-1 DNA repair function can be altered by a change in the redox status of the protein. Normal Ape1/ref-1 protein is completely inhibited under oxidizing conditions. We also demonstrate that one of the cysteines involved in this redox control of repair function is the cysteine at position 310. This cysteine is immediately adjacent to the histidine at position 309 that is crucial for the repair activity of

Table 1. Kinetics of Wild-Type and C310A Mutant Ape1/ref-1 Under Oxidizing or Reducing Conditions

	Redox status	*Wild-type*	*C310A*
K_m (μM)	Reduced	26.4 ± 1.2	40.4 ± 4.4
V_{max} (pmol/min)	Reduced	8.9 ± 0.7	11.8 ± 2.3
Turnover number (pmol of product/ pg of protein/min)	Reduced	0.015	0.0196
K_{cat}	Reduced	5.6×10^{-3}	4.9×10^{-3}
K_{cat}/K_m ($M^{-1}s^{-1}$)	Reduced	0.212×10^3	0.121×10^3
K_m (μM)	Oxidized	NA	98.42
V_{max} (pmol/min)	Oxidized	NA	10.0 ± 1.1
Turnover number (pmol of product/ pg of protein/min)	Oxidized	NA	0.0167
K_{cat}	Oxidized	NA	10.0 ± 1.1
K_{cat}/K_m ($M^{-1}s^{-1}$)	Oxidized	NA	0.0167

Ape1/ref-1 (12, 14, 37, 42). We show that if the C310 is changed to an alanine, the Ape1/ref-1 protein is now active regardless of the redox environment. Furthermore, the cysteine at position 65, which has been shown to be involved in the redox domain of Ape1/ref-1 by forming a disulfide bridge with cysteine 93 (2, 3, 40), when changed to an alanine, the resulting protein performs endonuclease activity at a level similar to wild-type Ape1/ref-1 protein. This implies that the C65 amino acid is not involved in the redox regulation of the repair domain and is in keeping with the two domains being completely exclusive.

We have also attempted to define some kinetic changes in the mutant C310A protein compared with the wild-type protein under oxidizing and reducing conditions. We observed that the K_m for the oxidized Ape1/ref-1-C310A mutant increased slightly over that for the reduced Ape1/ref-1-C310A and that the V_{max} and turnover values for the Ape1/ref-1-C310A mutant decreased in response to the oxidizing environment. Furthermore, the Ape1/ref-1-C310A mutant was stimulated by the reducing 40 mM DTT, 10 mM H_2O_2 environment. It appears that the Ape1/ref-1-C310A mutant displayed stimulation because it was kinetically slower in the oxidized environment than in the reduced environment, whereas the K_m changed only slightly. Given these results, we postulate that the negative charge associated with oxidized cysteine residues may explain the significantly increased K_m values for substrate under oxidizing conditions. The net negative charges associated with these residues would function to repel binding of negatively charged DNA molecules, while greatly increasing the speed of disassociation, which was reflected in the greatly increased V_{max} and turnover numbers. The location of these negative charges with respect to the active site could explain the differences in the kinetic values observed in these experiments.

Intriguingly, the stimulation of the Ape1/ref-1-C310A mutant activity under reducing conditions is of interest, implying that the rate of endonuclease cleavage and, therefore, repair could be influenced by the local redox microenvironment within the nucleus, particularly surrounding the area where Ape1/ref-1 is functioning. This stimulation cannot be observed with the wild-type Ape1/ref-1 protein because it is inactive under oxidizing conditions. This result will be of interest when the effect of this mutant on cellular function and DNA repair after transfection into human cell lines is investigated.

Clearly, there are a number of studies that need to be initiated in order to unravel further the apparent complex regulation of the Ape1/ref-1 repair function under reducing and oxidizing conditions. We have begun a series of experiments to mutate the other five cysteine residues individually and in combinations to see if other cysteines are involved in this redox regulation (Fig. 5). Preliminary data suggest that at least one other cysteine is involved in this redox control of Ape1/ref-1 repair function (data not shown). It is also imperative that the status of Ape1/ref-1 be determined under *in vivo* cellular conditions. Furthermore, the transfection of these various mutants into mam-

malian cells, such as the Ape1/ref-1-C310A protein, which is always active regardless of its redox status, may help to explain some of the many roles and regulation of the Ape1/ref-1 protein. It is also necessary to determine the redox status of Ape1/ref-1 that has been found to be elevated in a variety of cancers (21, 25, 35, 36, 39, 46), but the actual form of the Ape1/ref-1 protein (*e.g.*, reduced, oxidized, reduced and phosphorylated, oxidized and phosphorylated, reduced in the redox domain and oxidized in the repair domain, etc.) in these previous studies is unknown, and the determination of the status of Ape1/ref-1 in these cancers may provide clues as to the role Ape1/ref-1 is playing in their initiation, progression, and drug resistance.

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ABBREVIATIONS

AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; AP, apurinic/apyrimidinic; Ape1, apurinic/apyrimidinic endonuclease; BER, base excision repair; DTT, dithiothreitol; F, tetrahydrofuran; HIF-1α, hypoxia inducible factor-1α; HLF, HIF-1-like factor; H_2O_2, hydrogen peroxide; NFκB, nuclear factor-κB; PAX, paired box containing family of genes; ref-1, redox effector factor 1.

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Address reprint requests to:
Mark R. Kelley, Ph.D.
Jonathan and Jennifer Simmons
Professor of Pediatrics
Herman B. Wells Center for Pediatric Research
Riley Hospital for Children, Rm. 2600
702 Barnhill Dr.
Indianapolis, IN 46202

E-mail: mkelley@iupui.edu

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