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The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway

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ABSTRACT

In nucleotide incision repair (NIR), an endonuclease nicks oxidatively damaged DNA in a DNA glycosylase-independent manner, providing the correct ends for DNA synthesis coupled to the repair of the remaining 5′-dangling modified nucleotide. This mechanistic feature is distinct from DNA glycosylase-mediated base excision repair. Here we report that Ape1, the major apurinic/apyrimidinic endonuclease in human cells, is the damage-specific endonuclease involved in NIR. We show that Ape1 incises DNA containing 5,6-dihydro-2′-deoxyuridine, 5,6-dihydrothymidine, 5-hydroxy-2′-deoxyuridine, alpha-2′-deoxyadenosine and alpha-thymidine adducts, generating 3′-hydroxyl and 5′-phosphate termini. The kinetic constants indicate that Ape1-catalysed NIR activity is highly efficient. The substrate specificity and protein conformation of Ape1 is modulated by $MgCl_2$ concentrations, thus providing conditions under which NIR becomes a major activity in cell-free extracts. While the N-terminal region of Ape1 is not required for AP endonuclease function, we show that it regulates the NIR activity. The physiological relevance of the mammalian NIR pathway is discussed.

INTRODUCTION

DNA is a critical cellular target of reactive oxygen species such as $O_2{\cdot}^-$, H_2O_2 and OH·, which are constantly formed in cells by cellular metabolism and by exogenous factors such as ionizing radiation (IR) and certain drugs. 5,6-Dihydrothymidine (DHT), 5,6-dihydro-2′-deoxyuridine (DHU) and alpha-2′-deoxyadenosine (αA) constitute the major adducts detected in irradiated DNA (1–3). Importantly, αA and DHT are unique due to their strict requirement for anoxia during irradiation. The alpha-anomers of 2′-deoxynucleosides such as αA and alpha-thymidine (αT) are produced by abstraction of the anomeric hydrogen atom at C1′ by OH radicals (4,5). αA constitutes a moderate replication block both *in vitro* and *in vivo*, and generates exclusively a single nucleotide deletion *in vivo* (6). *In vitro*, ring-saturated pyrimidines are formed in DNA at rates higher than that of 8-oxoguanine (7). The fragmentation products of DHT strongly inhibits DNA synthesis and have been shown to be lethal lesions *in vivo* (8). 5-Hydroxy-2′-uridine (5ohU) arises from cytosine glycol, an unstable DNA adduct, followed by deamination and dehydration (3,9). DHU, another cytosine-derived product, is generated by IR under anoxic conditions (10). 5ohU and DHU resulting from C are miscoding lesions generating a C→T transition (11).

Base excision repair (BER), initiated by DNA glycosylases is thought to be the major pathway for the removal of most of these oxidized bases (12). Nevertheless, BER which requires the sequential action of two enzymes for proper incision of DNA, raises theoretical problems for the efficient repair of oxidative DNA damage, because it generates genotoxic intermediates such as apurinic/apyrimidinique (AP) sites, 2′-deoxyribose-5′-phosphate and/or 3′-blocking groups that must be eliminated before initiating DNA repair synthesis. Although some studies have shown that DNA glycosylase mutants show increased sensitivity to oxidative stress (13,14), the majority of genetic data indicate that the lack of DNA glycosylases does not render cells or mice more sensitive to oxidizing agents and IR (15–17). This is in striking contrast to the highly sensitive phenotype of AP endonuclease deficient bacterial, yeast and mammalian mutant cells towards oxidizing agents and IR (18–20).

Interestingly, in *Escherichia coli*, oxidative DNA damage such as αA is not repaired by DNA glycosylases/AP lyases but rather by the Nfo protein (endonuclease IV) in a DNA glycosylase-independent manner (21). Furthermore, we have shown previously that DHT, DHU and 5ohU residues can be

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processed by the alternative nucleotide incision repair (NIR) pathway that is initiated by one of two homologous AP endonucleases: the Nfo protein from *E.coli* and Apn1 protein from *Saccharomyces cerevisiae* (22). *Escherichia coli* DNA polymerase I (pol I) in the presence of dNTPs and/or human flap-endonuclease 1 (FEN-1) in the presence of proliferating cell nuclear antigen efficiently eliminate the 5′-terminal dangling DHU residue from the nicked duplex oligonucleotide as a mononucleotide (22). Thus, completion of NIR in eukaryotes would occur through the FEN-1-dependent long-patch repair pathway described in human cells (23). While we also presented evidence for the existence of a human NIR damage-specific endonuclease, its identity was not established and searches of the human genome database did not reveal any homologue of Nfo or Apn1. In this study, we have used HeLa S3 cells to purify to near homogeneity the endonuclease that incises DHU·G-containing duplex oligonucleotides 5′ of the lesion in a DNA glycosylase-independent manner. The enzyme has been purified to near homogeneity and identified as human apurinic/apyrimidinic endonuclease (Ape1), also known as Ref-1/HAP-1 (24).

Ape1 is homologous to *E.coli* Xth protein (exonuclease III), and was first purified from human (HeLa) cells more than two decades ago by Kane and Linn (25). The cDNA encoding Ape1 was cloned 10 years later (26,27) and subsequently also identified as a redox-regulator of the DNA binding domain of Fos, Jun and several other transcription factors (28). Ape1 is a 35.5 kDa nuclear protein that plays a central role in both short-patch and long-patch BER and can substitute for exonuclease III in *E.coli* and for Apn1 in *S.cerevisiae* (29). In addition to its AP endonuclease activity, it exhibits other DNA repair activities: 3′→5′ exonuclease, phosphodiesterase, 3′-phosphatase and Rnase H, but it should be noted that these additional activities are much weaker than its AP endonuclease activity. Recently, it has been shown that Ape1 also has a 3′-mismatch exonuclease activity and so it might also be considered to be a proofreading enzyme (30).

Loss-of-function studies in mice indicate an absolute requirement for Ape1 in normal embryonic development and animal survival (20,31). Indeed, Ape1 is a multifunctional enzyme, involved in DNA repair, transcription regulation, oxidative signalling, etc. The physiological significance of the DNA repair function is supported by the fact that explanted homozygous Ape1 null blastocysts display increased sensitivity to γ-irradiation, indicating a deficiency in the repair of IR-induced DNA lesions (20). Furthermore, targeted antisense experiments in mammalian cells indicate that reduced Ape1 levels also increase cellular sensitivity to hydrogen peroxide, menadione, paraquat and IR, but not UV radiation, and Ape1 expression and activity levels correlate with chemo/radiosensitivity of tumour cells (32).

The ability of Ape1 to incise the DNA sugar–phosphate backbone 5′ to a bulky lesion such as 3,N^4-benzetheno-2′-deoxycytidine and 3,N^4-benzetheno-2′-deoxyadenosine has also been reported (33,34). However, these benzene-derived DNA adducts are neither a consequence of oxidative damage, nor substrates for any known DNA glycosylase. Therefore, this activity of Ape1 is not involved in the repair of oxidative DNA damage and was not considered an alternative, back-up function to DNA glycosylases. In the present work we show, for the first time, that Ape1 and human thymine glycol-DNA glycosylase (hNth1) have overlapping substrate specificities towards DHU, DHT and 5ohU residues. The novel NIR function of Ape1 serves as an alternative and back-up repair pathway to BER for oxidative DNA base damage. The potential importance of Ape1-NIR activity in cleansing genomic DNA of potentially mutagenic and cytotoxic lesions is discussed.

MATERIALS AND METHODS

DNA substrates

Oligonucleotides (17mer) containing either a single αA or αT adduct were prepared as described (21). All other oligodeoxyribonucleotides were purchased from Eurogentec (Seraing, Belgium), including those containing THF, DHT, DHU and 5ohU nucleosides and complementary oligonucleotides, containing either dA, dG, dC or T opposite the adduct. The oligonucleotide sequences were: 5′-AGCATTCGXGACTGGGT containing αA, αT or THF; d(AAATACATCGTCACCTGGGXCATGTTGCAGATCC) containing THF, DHU or 5ohU; d(TGACTGCATAXGCATGTAGACGATGTGCAT) containing DHT or DHU, where X is the position of the modified base. To generate oxidized bases, pUC19 plasmid DNA was treated with 10 mM $KMnO_4$ at 0°C for 5 min (35). The DNA samples were desalted on a Sephadex G50 column equilibrated in 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA. To generate fragmented guanine residues, pUC19 plasmid DNA was alkylated in buffer containing 0.05% dimethylsulfate, 50 mM sodium cacodylate, pH 7.0 and 1 mM EDTA at 23°C for 10 min. The reaction was stopped by the addition of an equal volume of 0.5 M 2-mercaptoethanol and immediately desalted through a Sephadex G50 column equilibrated in 10 mM Tris–HCl, pH 7.5 and 1 mM EDTA. Under these conditions, the guanine residues are methylated, yielding 98% N^7-methylguanine and 2% N^3-methylguanine (36). The N^7-methylguanine residues were converted to the ring-opened (me-FapyGua) form by incubating alkylated DNA in 60 mM phosphate buffer, pH 11.4 for 48 h at 23°C as described (36).

Enzymes and strains

Human (HeLa) cells were generously provided by Dr I. D. Hickson (University of Oxford, UK). HeLa whole cell-free extracts were prepared as described (22). Purification of the DHU-specific endonuclease activity from HeLa S3 cells is described in Supplementary Material. *Escherichia coli* Fpg, Nfo and Nth and human UDG, OGG1 and Ape1 proteins were purified as described (37). The AP endonuclease preparations were devoid of detectable AP-lyase and DNA glycosylase activities on substrates containing 8-oxoguanine, uracil, alkylpurines and other modified bases (data not shown). *Escherichia coli* strain BH110 (Δ*xth*, *nfo*::kan^R) a derivative of AB1157 was from laboratory stocks.

Mutagenesis

The DNA sequence encoding the truncated NΔ61-Ape1 protein was amplified by PCR from pIZ42 vector containing full-length Ape1 generously provided by Dr T. Izumi (University of Texas, Galveston) (38). The PCR product was subcloned into pET11a at NdeI/BamHI sites. The truncated protein was expressed in *E.coli* BH110 and purified to homogeneity using

chromatography on HiTrap™ Q-Sepharose, SP-Sepharose and heparin columns (Amersham Bioscience).

Western blot analysis and immunodepletion

After fractionation by SDS–PAGE, proteins were transferred to Hybond nitrocellulose membrane (Amersham Biosciences). The rabbit pre-immune serum and antiserum against Ape1 were used at 1:1000 dilution, and the secondary antibody, anti-rabbit horseradish peroxidase conjugated (Roche) at 1:5000 dilution. Proteins were detected with enhanced chemiluminescence detection reagent (Amersham Biosciences) according to the manufacturer's instructions. In the immunodepletion experiments, 1 μg of HeLa S3 crude extract was pre-incubated with the polyclonal pre-immune serum or the antiserum against Ape1 at 1:100 dilution before enzyme assay.

Enzyme assays

The NIR activity assay standard reaction mixture (20 μl) contained 0.4 pmol of [3′-^{32}P]dCMP-end labelled DHU·G oligonucleotide duplex, 25 mM KCl, 20 mM HEPES/KOH, pH 6.8, 0.1 mg/ml BSA, 1 mM 2-mercaptoethanol, or 0.5 mM $MgCl_2$ and either 1 μg of crude extract, or 3 ng of partially purified HeLa-Ape1, or 5 nM of a pure protein, unless otherwise stated. To avoid non-specific nuclease activities in crude extracts, 0.3 mM $ZnCl_2$ and 0.1 mM EDTA were used instead of $MgCl_2$ for the NIR assays. The AP endonuclease assay standard reaction mixture (20 μl) contained 0.4 pmol of 5′-^{32}P-end labelled THF·G oligonucleotide duplex, 50 mM KCl, 20 mM HEPES/KOH, pH 7.6, 0.1 mg/ml BSA, 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 5 pM of a pure protein, unless otherwise stated. Reactions were performed at 37°C either for 15 min when using crude extracts, or 10 min for a pure protein, and stopped by adding 10 μl of 1.5% SDS/0.3 mg/ml proteinase K followed by incubation for 10 min at 50°C. The mixture was desalted by hand-made spin-down columns filled with Sephadex G25 (Amersham Biosciences) equilibrated in 5 M urea. Purified reaction products were heated at 65°C for 3 min and separated by electrophoresis in denaturing 20% (w/v) polyacrylamide gels (20:1, 7 M urea, 0.5× TBE). The gels were exposed to a Fuji FLA-3000 Phosphor Screen and analysed using Image Jauge V3.12 software.

Fluorescence assays

Changes in the intrinsic tryptophan fluorescence were monitored in buffer containing 180 nM Ape1 or Apn1, 20 mM HEPES/KOH, pH 6.8, 25 mM KCl at various concentrations of $MgCl_2$ at 4°C. The fluorescence emission was measured at 330 nm with excitation at 282 nm on an SFM-23/B spectrofluorometer (Kontron AG, Switzerland).

RESULTS

Identification of a human endonuclease that incises a DHU-containing oligonucleotide

We have purified an NIR incision activity from HeLa S3 cells using a 3′-labelled DHU-containing oligonucleotide to monitor the DNA glycosylase-independent incision of the oligonucleotide duplex. Although we reported previously only weak NIR activity in HeLa cell extracts, subsequent experiments showed that the addition of a divalent cation (0.3 mM $ZnCl_2$) greatly stimulated the NIR incision activity towards DHU and DHT adducts. Therefore, using these assay conditions, the NIR activity was eluted as a single symmetrical peak after seven chromatographic steps of protein purification, suggesting that it could be due to a single protein (see Supplementary Material). The most active fraction after heparin Sepharose chromatography (Fraction VII) was found to be essentially homogeneous and consisted of one polypeptide with an apparent molecular mass of 37 kDa (Supplementary Material, Fig. 1). The polypeptide was recovered from the gel, and a partial amino acid sequence was determined using quadrapole mass spectroscopy. The sequence matched that of human Ape1. To further substantiate the identity of the purified protein, western blot analyses were performed using antibodies specific to Ape1. As shown in Figure 1A the antibodies recognize specifically the major 37 kDa protein in Fraction VII (lane 3) that migrates similarly to the recombinant Ape1 (rec-Ape1) protein, which was over-expressed and purified from *E.coli* (lane 2). As expected, the Ape1 antibodies selectively inhibit NIR activity in HeLa cell extracts indicating that Ape1 is the major NIR initiating enzyme in human cells (Fig. 1B, lane 4).

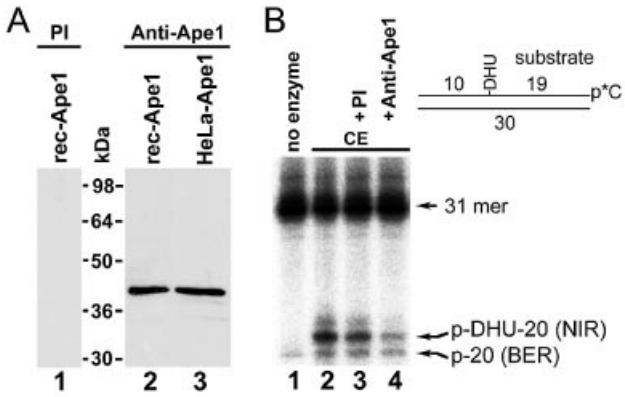

Figure 1. Human damage-specific endonuclease purified from HeLa cells is identical to Ape1. (**A**) Western blot analysis: pre-immune serum (PI) or antiserum specific to Ape1 (Anti-Ape1) was incubated with 10 ng of rec-Ape1 or HeLa-Ape1. (**B**) Substrate diagram with incision mode and immunodepletion experiment of the NIR activity for DHU·G in a cell-free extract (CE) of HeLa cells.

Substrate specificity of Ape1

Since our previous study failed to detect Ape1-catalysed NIR (22), we analysed the substrate specificity of rec-Ape1 under the reaction conditions used for the purified protein from HeLa cells (HeLa-Ape1). As shown in Figure 2, rec-Ape1, along with Nfo and HeLa-Ape1, cleaved 5ohU·G, DHU·G, THF·T, αA·T, αT·A and DHT·A oligonucleotide duplexes (Fig. 2A and B, lanes 4 and 9 and lanes 3, 7 and 11, respectively, and data not shown). In addition, rec-Ape1 incises damaged supercoiled plasmid DNA containing either methylformamidopyrimidines or oxidized bases (Supplementary Material, Fig. 2). We did not observe any difference in the activity of the Ape1 proteins when either 0.3 mM $ZnCl_2$ or 0.5 mM $MgCl_2$ was used, in agreement with published data (39).

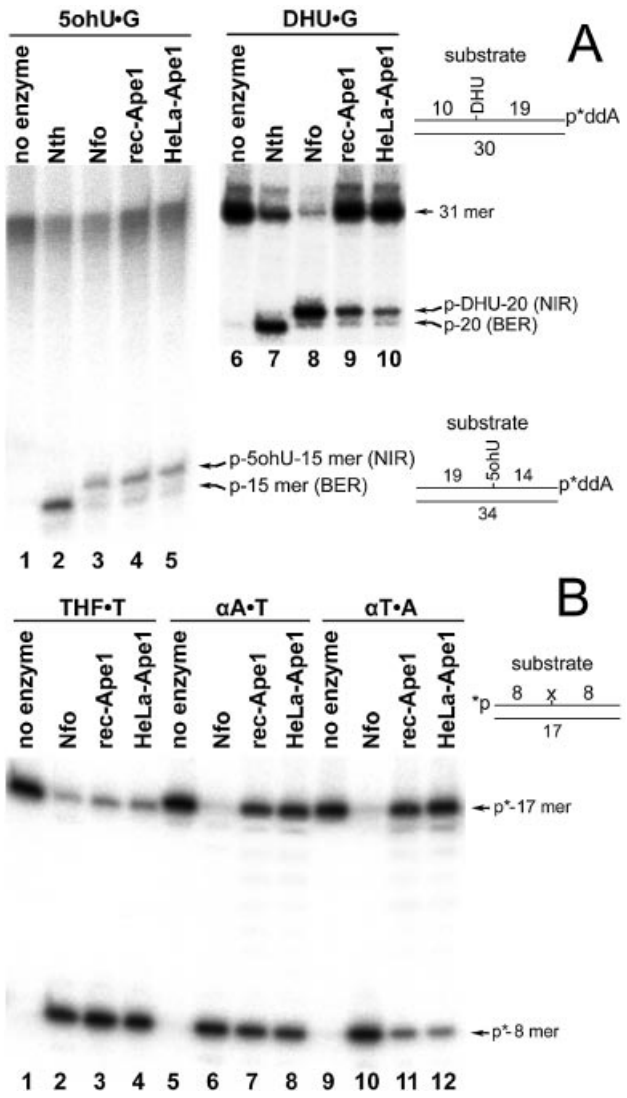

Figure 2. Comparison of substrate specificities of rec-Ape1 and HeLa-purified Ape1. Reactions were performed under standard NIR condition. (**A**) [3′-^{32}P]dCMP-labelled 5ohU·G and DHU·G; (**B**) 5′-^{32}P-labelled THF·T, αA·T and αT·A.

In order to determine the mechanism of Ape1-catalysed incision, the DNA fragments generated by the *E.coli* proteins Nth and Nfo were used as size markers. As shown in Figure 2A, Nth (a DNA glycosylase/AP lyase) generates 15 or 20mer fragments from 3′-labelled 5ohU·G and DHU·G oligonucleotides, respectively (Fig. 2A, lanes 2 and 7). In contrast, Nfo (an AP endonuclease) generates 16 and 21mer fragments containing the dangling base, from the same substrates (Fig. 2A, lanes 3 and 8). Thus, the migration pattern of Ape1-cleavage products corresponds to that of Nfo, indicating a DNA glycosylase-independent, endonucleolytic mechanism of action (Fig. 2A and B, lanes 4, 5, 9 and 10 and lanes 3, 4, 7, 8, 11 and 12, respectively). Taken together these results confirm that the NIR activity is indeed due to Ape1.

Optimal assay conditions for the NIR activity of Ape1

To characterize the biochemical properties of NIR incision by Ape1, we measured Ape1-mediated cleavage of THF·G and DHU·G in the presence of varying concentrations of $MgCl_2$. As shown in Figure 3A, inverse sigmoid dependencies of the two activities were observed in the concentration range 1–5 mM $MgCl_2$. Ape1 efficiently catalysed DHU·G incision even under very low magnesium concentrations (0.001 mM), but this activity was strongly inhibited by concentrations of $MgCl_2$ above 2 mM (Fig. 3A). Concentrations of at least 5 mM $MgCl_2$ were required for optimal AP endonuclease activity (Fig. 3A), in good agreement with published data (30,40). The pH and salt concentration profiles of Ape1 activities were also examined in the presence of 0.5 and 5 mM $MgCl_2$ for NIR and AP endonuclease activities, respectively. The results shown in Figure 3B reveal dramatic differences between the two activities: the AP endonuclease activity was optimal between pH 7.8 and 8.2, whereas NIR activity was optimal between pH 6.4 and 6.8. Furthermore, while AP endonuclease activity was maximal over a broad range of KCl concentrations (25–200 mM), the NIR activity decreased dramatically when the KCl concentration exceeded 50 mM (Fig. 3C). Taken together these data suggest that *in vivo*, the NIR and the AP endonuclease activities could be mutually exclusive depending on intracellular Mg^{2+} concentrations, pH and ionic strength.

Mg^{2+} induced conformational effects in Ape1

As the sigmoid dependencies induced by an effector are characteristic of allosteric regulation, we investigated magnesium induced conformational changes of Ape1 structure by measuring the intrinsic tryptophan fluorescence that results from modifications in the environment of one or more of the seven tryptophanyl residues in the Ape1 protein. The fluorescence emission at 330 nm for Ape1 and Apn1 was measured in the absence and presence of $MgCl_2$. As shown in Figure 3D, $MgCl_2$ titration led to an increase in fluorescence of Ape1 but not *S.cerevisiae* AP endonuclease 1 (Apn1), suggesting that Ape1 undergoes conformational change in the absence of DNA.

Steady-state kinetic studies and base-pair specificity of Ape1-NIR activity

To further characterize the substrate specificities of the Ape1 protein, the kinetic constants for the incision of THF·G and DHU·G and the influence of the base opposite were measured. Comparison of the kinetic constants (Table 1) shows that under reaction conditions optimal for AP endonuclease activity (5 mM $MgCl_2$ and pH 7.6), the THF residue is the preferred substrate for Ape1 (K_M 19 nM, k_{cat}/K_M 1600 $min^{-1}\mu M^{-1}$). However, under reaction conditions optimal for NIR activity (pH 6.8 and 0.5 mM $MgCl_2$), the k_{cat}/K_M values for the incision of THF·G, αA·T and DHU·G were in a similar range (45, 16 and 19 $min^{-1}\mu M^{-1}$). The apparent K_M and k_{cat}/K_M values measured for Ape1 and hNth1 when acting upon DHU·G indicate that *in vitro*, the AP endonuclease is somewhat more efficient than the DNA glycosylase/AP lyase (Table 1). Thus, the analysis of kinetic constants suggests that Ape1 can efficiently back-up the DNA glycosylases to repair DHU residues. As shown in Supplementary Material, Figure 3, Ape1 incised DHU, DHT, 5ohU and THF residues irrespective of the base opposite with similar efficiency. The apparently similar activity profiles of Ape1 towards different mismatches may reflect the fact that the enzyme recognizes a broad

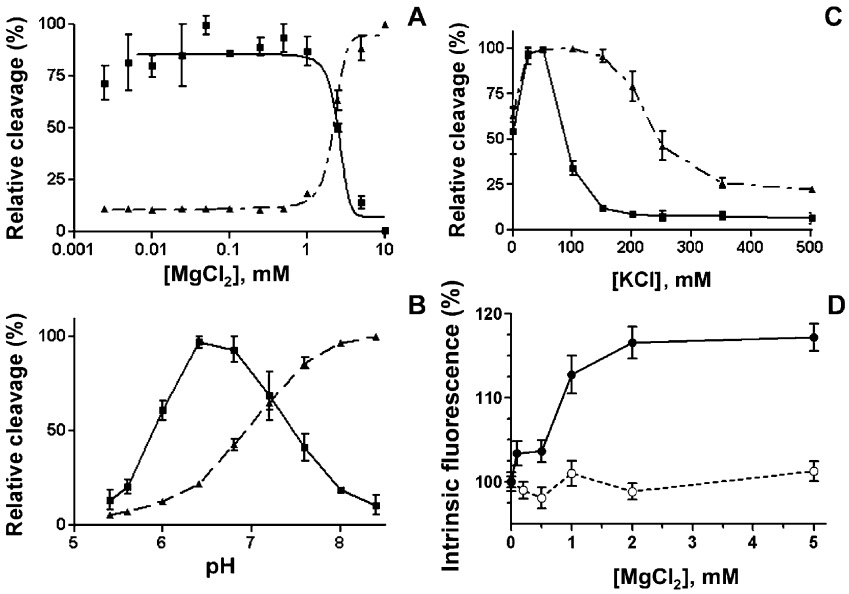

Figure 3. Activity profiles and conformational changes of Ape1. (A–D) Ape1 nucleotide incision (squares, straight line) and AP-endonuclease (triangles, dotted line) activities depending on Mg^{2+} concentrations (**A**), pH profile (**B**) and ionic strength (**C**). (**D**) Mg^{2+}-induced changes in the intrinsic tryptophan-fluorescence of Ape1 (filled circle) and Apn1 (empty circle).

Table 1. Kinetic constants of the human DNA repair proteins for the incision of duplex oligonucleotides containing a single modified residue

Protein	Substrate	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}\mu M^{-1}$)
Ape1[a]	THF·G	19 ± 2.0	30	1600
Ape1[b]	THF·G	2.7 ± 0.4	0.12	45
Ape1[b]	αA·T	7.2 ± 1.6	0.12	16
Ape1[b]	DHU·G	8.4 ± 1.8	0.16	19
hNth1[c]	DHU·G	47	0.6	13

[a,b]Enzyme activity was measured under standard reaction conditions for AP endonuclease[a] or NIR[b] assay (see Materials and Methods).
[c]Data taken from (50).

range of structures and is not sensitive to the variation in thermodynamic stability of the various mismatches.

NIR activity in cell-free extracts

Reconstitution of DNA repair in whole-cell extracts suggests that BER is the major pathway for removal of oxidative DNA base damage (41,42). However, kinetic data indicate that both Ape1 and the DNA glycosylases/AP lyases can act directly on DHU·G. To understand which of the two pathways is responsible for repair of the majority of oxidative DNA base damage *in vivo*, we compared AP endonuclease and DNA glycosylase activities towards 3′-[^{32}P]dCMP-labelled DHU·G in HeLa cell-free extracts under different reaction conditions. As shown in Figure 4A, at pH 7.6, with or without 2 mM $MgCl_2$, we detect mainly a DNA glycosylase activity (lanes 3 and 4). Addition of 0.3 mM $ZnCl_2$ increases the NIR activity up to the level of the DNA glycosylase-dependent incision (lane 5). However, at pH 6.8, NIR was the major activity (lane 6). Similar results were obtained when 0.5 mM $MgCl_2$ was substituted for 0.3 mM $ZnCl_2$. Interestingly, the level of BER activity remained constant under the various reaction conditions (Fig. 4A).

To investigate whether the faster migrating fragments observed below the NIR reaction product in Figure 4A (lanes 5 and 6) might be due to nuclease degradation from the 5′ end of DHU·G, the HeLa cell-free extracts were incubated under various reaction conditions with the same 30mer oligonucleotide, but 5′-^{32}P-end-labelled. Under these conditions a 10mer fragment was obtained (Fig. 4B, lanes 6–11). As purified Nfo and Ape1 also generate 10mer products, it is likely that the substrate oligonucleotide was cleaved by an AP endonuclease present in the crude extract (Fig. 4B, lanes 4 and 5). This result excludes the possibility that the 3′-labelled NIR product generated by cell-free extracts is due to non-specific 5′→3′ exonuclease degradation and supports our belief that the migration pattern of incision products is consistent with the NIR mode of incision with subsequent 5′→3′ exonucleolytic degradation of the NIR fragment. These results also suggest that AP endonuclease and DNA glycosylase activities in cell-free extracts can vary depending on the reaction conditions showing that the respective activities are finely tuned.

The role of the redox-domain of Ape1 in NIR function

The DNA repair domain of Ape1, which is located in the C-terminal region, is highly conserved, sharing 28% sequence identity with Xth, the major AP endonuclease in *E.coli* (29). Therefore, we reassessed the activity of Xth towards oxidative DNA base damage under various reaction conditions. Within the $MgCl_2$ concentration range 0.01–10 mM and at pH 6.8 or 7.6, no Xth-mediated incision of DHU·G or αA·T was observed (data not shown). We reasoned that this could be due to the non-conserved N-terminal region of Ape1, which is absent in the prokaryotic homologue and known as the redox (Ref)-domain. To understand the role of the Ref domain in NIR activity we generated truncated Ape1 lacking the N-terminal 61 residues (NΔ61-Ape1) and measured the AP endonuclease and NIR activities. As shown in Figure 5 the AP

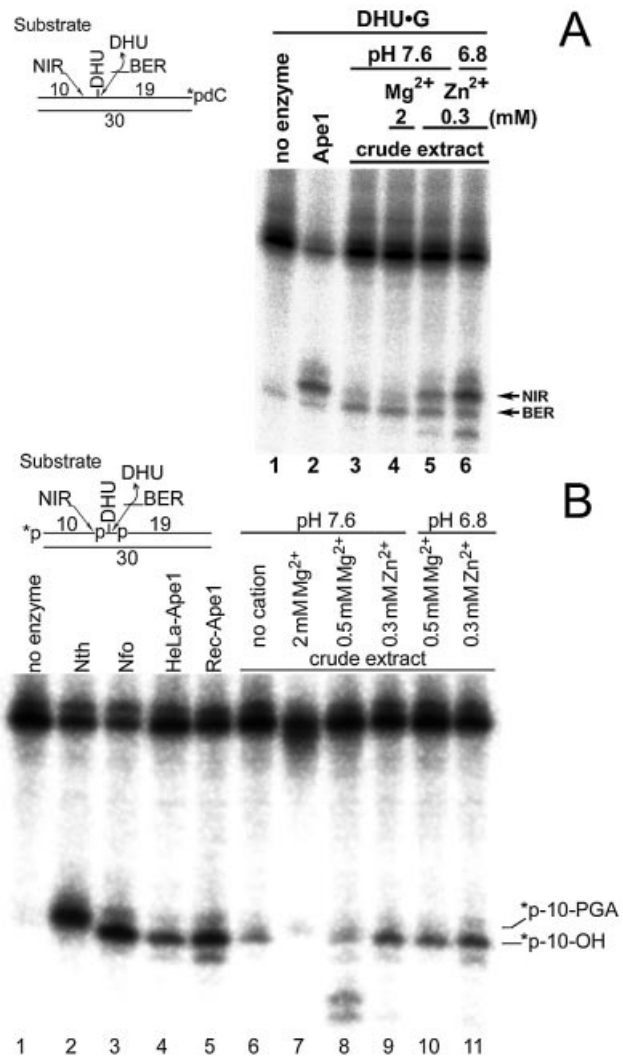

Figure 4. DNA repair assay using whole whole-cell extracts from HeLa cells. The extracts were incubated with 3′- (**A**) or 5′-labelled (**B**) DHU·G substrates under standard NIR or AP-endonuclease conditions with different concentrations of metal ions.

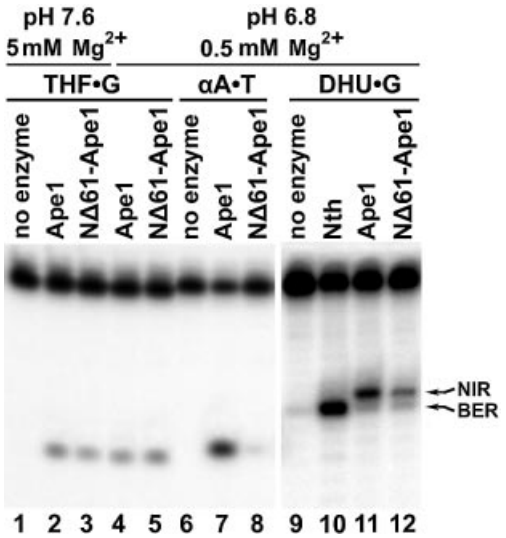

Figure 5. DNA repair assay of the N61Δ-Ape1 mutant and wild-type Ape1 proteins. Reactions were performed under standard AP-endonuclease (lanes 1–3) or NIR (lanes 4–12) conditions using 3′-labelled oligonucleotide substrates. In the case of THF·G duplex concentrations of Ape1 and N61Δ-Ape1 were 5 (lanes 2 and 3) and/or 50 pM (lanes 4 and 5).

endonuclease activity of NΔ61-Ape1 is similar to that of Ape1 (lanes 3 and 5 versus lanes 2 and 4). However, NΔ61-Ape1 displayed a dramatic loss of activity towards αA·T (lane 8) and a significant reduction in activity towards DHU·G (lane 12), compared with the full-length protein (lanes 7 and 11). Interestingly, in contrast to full-length Ape1, we detected a significant decrease in the NIR activity of NΔ61-Ape1 (~1.5-fold) in the absence of 2-mercaptoethanol (not shown). Taken together these results indicate an important role for the N-terminal region of Ape1 in NIR function.

DISCUSSION

Oxygen radicals generated as by-products of cellular metabolism cause extensive damage to DNA. Oxidative DNA damage have been postulated to be the major type of endogenous damage leading to human degenerative disorders including cancer, cardiovascular disease and brain dysfunction. Following the acquisition of the nucleus during the formation of the eukaryotic cell, oxidative metabolism was confined to the mitochondria, thus creating pseudo-anoxic conditions and protecting the genome located in the nucleus (43). Since the eukaryotic nucleus is a very poorly oxygenated cellular compartment (44), oxidative DNA damage preferentially formed under anoxic conditions might result in significant biological consequences *in vivo*. Interestingly, cancer cells can grow under reduced oxygen-supply conditions, and tumour hypoxia is associated with poor prognosis and resistance to radiation therapy (45). In contrast to oxidatively damaged bases such as 8-oxoguanine and thymine glycol, formation of 5,6-dihydropyrimidines and alpha-anomeric 2′-deoxynucleotides requires strict anoxic condition (1,2). This might suggest that the latter lesions are biologically important DNA damage formed in the eukaryotic nucleus *in vivo*.

In NIR, an Nfo/Apn1-like damage-specific endonuclease directly nicks DNA containing free radical-induced base lesions acting as a back-up repair pathway to BER (22). As the identity of the human functional homologue of Nfo was unknown, we proceeded to purify the enzyme responsible for the incision of DHU·G oligonucleotide duplexes from HeLa cell extracts. Following seven chromatographic steps, we identified this activity as belonging to Ape1 (Fig. 1). To characterize the substrate specificity of Ape1, we compared the activity of HeLa-purified and recombinant proteins towards various free radical-induced base lesions. The DNA duplexes containing DHT·A, DHU·G, 5ohU·G, THF·T, αA·T and αT·A were readily incised by both Ape1 proteins. Like Nfo and Apn1, Ape1 nicks DNA on the 5′ side of the damaged base, leaving the dangling damaged nucleotide on the 5′-terminus of the downstream reaction product (Fig. 2). Taken together the results show that Ape1 is involved in the NIR pathway for oxidative DNA base damage. In contrast to both *E.coli* and humans, the major AP endonuclease of the budding yeast is Apn1, an Nfo homologue (46). While the reason for

the reversal in AP endonuclease prevalence is not understood, here we provide evidence that Ape1 is in fact the mammalian functional counterpart of Apn1, rather than *E.coli* Xth. Thus, in eukaryotes the major AP endonuclease is also involved in the DNA glycosylase-independent repair pathway.

Dramatic differences in reaction conditions were found for AP endonuclease and NIR activities of Ape1 (Fig. 3). Thus, the AP endonuclease activity was maximal between pH 7.8 and 8.2, while NIR activity was maximal between pH 6.4 and 6.8 (Fig. 3B), conditions that are also optimal for AP site–DNA binding (47). Likewise, AP endonuclease activity was maximal over a broad range of KCl concentrations (25–200 mM), whereas NIR activity decreased dramatically when the KCl concentration exceeded 50 mM (Fig. 3C). Interestingly, optimal conditions for NIR activity are similar to those for the 3′→5′ exonuclease activity of Ape1 observed on matched, 3′-mispaired and nucleoside analogue β-L-dioxolane-cytidine terminated nicked DNA (30).

In agreement with previous data for AP endonuclease activity of Ape1 (25,40) over the concentration range 1–5 mM, Ape1-mediated cleavage of THF·G and DHU·G shows a sigmoid dependency on $MgCl_2$ (Fig. 3A). Furthermore, $MgCl_2$ titration led to an increase in conformation related fluorescence of Ape1 but not of Apn1 (Fig. 3C). Based on this it is tempting to speculate that Ape1 could be an allosteric enzyme, regulated by the Mg^{2+}-effector. Although, initial structural studies indicated that the active site of Ape1 is unlikely to undergo radical structural changes upon Mg^{2+} binding (48,49). A more recent study, on the X-ray structure of the full-length Ape1 protein crystals made at different pH reveals two metal ions bound 5 Å apart in the active site at pH 7.5 (47), whereas only one metal ion is bound at acidic pH. In addition, the loop regions of Ape1, consisting of residues 100–110 and 120–125, exhibit significant structural variation (47). Therefore, we hypothesize that Mg^{2+}-dependent conformational change regulates catalytic activity of Ape1 and may channel the repair of oxidative DNA damage to either the BER or NIR pathways. However, structural studies of the NIR complex are required to fully investigate these phenomena.

Analysis of the kinetic data presented in Table 1 indicates that the primary substrate for Ape1 is a THF residue and although reduced under the NIR-conditions, the AP endonuclease activity of Ape1 is still robust. The k_{cat}/K_M value of Ape1 acting upon DHU is 1.5-fold more efficient than the value for hNth1 (50), indicating that DHU might be processed equally well by both BER and NIR (Table 1). Overall, a comparison of the kinetic parameters for the BER and NIR activities demonstrates that Ape1 can efficiently back-up the DNA glycosylases to repair DHU adducts.

To determine the balance between BER and NIR pathways, we measured activities towards DHU·G in HeLa cell-free extracts under different reaction conditions. We found that, at pH 6.8 in the presence of 0.3 mM $ZnCl_2$, NIR was the major activity in the extracts (Fig. 4A, lane 6). We propose that under certain conditions the extremely high cellular concentration of Ape1 (3.5×10^5–7×10^6 molecules/cell) (51) may direct the processing of oxidative DNA damage to the NIR pathway *in vivo*. A 5′→3′ exonucleolytic degradation of the NIR fragment can be seen in cell-free extracts (Fig. 4A, lanes 5 and 6). This activity might be due to FEN-1. Indeed, FEN-1 exonuclease activity is stimulated at low Mg^{2+} and monovalent salt concentrations (52). However, to exclude the possibility that the NIR fragment emerges from non-specific 5′→3′ exonuclease activity, we used a 5′-labelled DHU·G duplex oligonucleotide in our assay. As expected, incubation of the 30mer oligonucleotide in HeLa cell-free extracts under different reaction conditions gave rise to a 10mer fragment retaining 5′-label (Fig. 4B, lanes 6 and 8–11). These results exclude the possibility that the NIR fragment generated by the purified Ape1 proteins and cell-free extracts is due to a 5′→3′ non-specific exonuclease halting at the modified site. Taken together the results suggest that the repair mode for oxidative DNA damage *in vivo* depends upon the intracellular environment and concentration of a given repair enzyme.

As the first 61 N-terminal residues of Ape1 are indispensable for redox but not for AP endonuclease activity (38,53), we generated a truncated Ape1 lacking the N-terminal 61 amino acids (NΔ61-Ape1) and assayed it for AP endonuclease and NIR activities. Although NΔ61-Ape1 exhibited no decrease in THF·G incision, we observed a 2.5- and 20-fold decrease in NIR activity towards DHU·G and αA·T, respectively, compared with wild type Ape1 (Fig. 5) (38). The results indicate that the redox-domain of Ape1 regulates the NIR activity, thus providing insight for an additional role of the Ref domain. Importantly, in known Ape1 crystals the N-terminal 35–42 residues were truncated or not visible in the electronic density maps and the final models of Ape1 (47,48). In conclusion, it is tempting to speculate that the Ref domain was acquired during evolution by an AP endonuclease to repair oxidative damage to both DNA bases and proteins.

Evolutionary conservation of two types of AP endonucleases with overlapping substrate specificity [Xth and Nfo in *E.coli*, Apn1 and Apn2 in yeast, and Ape1 and Ape2 in human cells (29)], highlights the existence of two alternative repair pathways for oxidative DNA damage. However, the biological impact of the different Ape1-DNA repair activities, including NIR, needs further investigation. As Ape1-initiated NIR can excise alpha-anomeric nucleotides, DNA adducts that are not substrates for BER, we suggest that NIR targets oxidative DNA damage formed under natural (anoxic) conditions. Therefore, as NIR and BER also share many common substrates, we propose that they work in concert to cleanse genomic DNA of potentially mutagenic and cytotoxic lesions. This in part, may explain the lack of readily discernable phenotype of DNA glycosylase-deficient mice (17) and the increased susceptibility to oxidative stress of Ape1 heterozygous null mutant mice (54).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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