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THYMINE GLYCOL-DNA GLYCOSYLASE/AP ENDONUCLEASE OF CEM-C1 LYMPHOBLASTS: A HUMAN ANALOG OF ESCHERICHIA COLI ENDONUCLEASE III

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A thymine glycol-DNA glycosylase/AP endonuclease has been identified in human CEM-C1 lymphoblasts. The enzyme is active in the absence of divalent cations and has an apparent molecular size of approximately 60,000 daltons. The enzyme releases thymine glycol from osmium tetroxide-damaged DNA via an N-glycosylase activity and is associated with an endonuclease activity that mediates phosphodiester bond cleavage at sites of thymine glycol and apurinic sites. We propose that this enzyme, which we call redoxyendonuclease, is the human analog of a bacterial enzyme, E. coli endonuclease III, that recognizes oxidative DNA damage.

Oxidative damage to cellular DNA occurs continually and has been implicated in the degenerative processes leading to cancer and aging (1,2). Thymine glycol, a DNA damage product produced by ionizing radiation and other oxidizing agents, is recognized and acted upon by enzymes present in both prokaryotes and eukaryotes that possess DNA N-glycosylase (3,4,9,11,12), AP endonuclease (5,14-16,18,19) or combination N-glycosylase/AP endonuclease (6-8,13,17) activities. E. coli endonuclease III possesses an N-glycosylase that removes ring-saturated, ring-cleaved, and ring-contracted pyrimidines from oxidized and X-irradiated DNA (3,4,6-8) as well as a divalent cation-independent apurinic/apyrimidinic (AP) endonuclease activity that incises the phosphodiester backbone 3′ to the AP site (6-8,16,17).

Although several previous investigations have provided evidence suggesting that activities analogous to E. coli endonuclease III exist in human cells (5,9,19,20) none of these studies have previously demonstrated that a particular cellular preparation contains both N-glycosylase and AP endonuclease activities. We have employed DNA sequencing and HPLC methodologies to address the possibility that activities analogous to E. coli endonuclease III were present in human CEM-C1 lymphoblasts. We report here the partial purification of a human lymphoblast DNA glycosylase that releases thymine glycol from oxidized DNA and is associated with a divalent cation-independent AP endonuclease activity. It is likely that these combined activities are responsible for the observed endonucleolytic incisions at sites of thymine glycol when osmium tetroxide-damaged, end-labelled DNA fragments of defined sequence are employed

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as substrates. Our findings suggest that the human lymphoblast DNA glycosylase and AP endonuclease activities are analogous to E. coli endonuclease III.

MATERIALS AND METHODS

Purification of Human Lymphoblast Redoxyendonuclease.

Thymine glycol-DNA glycosylase AP endonuclease (redoxyendonuclease) was purified from 1.3 x 10^{10} human CEM-C1 lymphoblasts. Cell extracts were prepared and subjected to DEAE cellulose chromotography to remove nucleic acids (19). The DEAE cellulose flow-through fractions (unbound protein) were pooled (1.4 gm of protein), dialyzed against 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10 mM 2-mercaptoethanol (buffer A) plus 50 mM NaCl and 0.7 gm of protein was applied to a phosphocellulose column (Whatman P11, 3.6 x 36 cm) equilibrated in dialysis buffer. The column was washed extensively with dialysis buffer. Redoxyendonuclease was eluted with a 0.05-1.0 M NaCl gradient (600 ml/600 ml) plus buffer A. Redoxyendonuclease activity was monitored as described (19) and two peaks of activity centered at 0.3 M NaCl and 0.5 M NaCl were detected. Fractions containing the second peak of activity were pooled (56 mg of protein), concentrated and dialyzed against 0.1 M NaCl plus buffer A, 50% glycerol (storage buffer). An aliquot (10 mg) of this material was applied to a calibrated Sephadex G-100 (superfine) column (1.5 x 95 cm) and eluted with 15 mM KH_2PO_4 (pH 6.8), 10 mM EDTA, 10 mM 2-mercaptoethanol (buffer B) plus 0.5M NaCl. Fractions (2 ml) were monitored for DNA damage-specific endonuclease (Fig. 1). Fractions 39 to 42 exhibited the highest levels of redoxyendonuclease activity and were pooled, concentrated, and dialyzed against buffer B plus 0.1 M NaCl, 50% glycerol. This material (0.4 mg) was used in the thymine glycol endonuclease (Fig. 2), N-glycosylase (Fig. 3), and AP endonuclease (Fig. 4) experiments.

DNA Damage-Specific Endonuclease Experiments.

The 112 bp (fragment A) and 201 bp (fragment B) Sal I-PvuII restriction fragments were generated from pUC18 and pUC19 (21), respectively and were ^{32}P 3' end-labelled and isolated as previously described (19). Fragment A was UV-irradiated (254 nm) with a dose of 10,000 J/m^2 and fragment B was reacted with osmium tetroxide as previously described (19). For AP endonuclease experiments, fragment B was ^{32}P 5' end-labelled and depurinated as described (17). These defined sequence DNA damage substrates were treated with either 1 M piperidine, 90°C, 30 min (hot alkali) or incubated with 2.3-4.5 μg of Sephadex G-100-purified human lymphoblast redoxyendonuclease or 1,000 units of E. coli endonuclease III (a gift from Dr. Richard Cunningham, SUNY, Albany) in 40 μl of 40 mM KCl, 15 mM KH_2PO_4 (pH 6.8), 10 mM EDTA, 10 mM 2-mercaptoethanol (reaction buffer) for 30 min at 37°C. Following incubation, DNA samples were processed, loaded onto denaturing 20% polyacrylamide-7M urea gels and subjected to electrophoresis and autoradiography as described (22). The purine (G+A) and pyrimidine (C+T)-specific DNA sequencing reactions (23) were performed for fragments A and B and the cleavage products run alongside the enzyme digests.

Thymine Glycol-DNA Glycosylase Experiments.

Poly (dA-[methyl-^{3}H]dT) was synthesized, reacted with osmium tetroxide as described (6), and used within 48 hours following preparation. Approximately 5 x 10^6 cpm (1.25 μg) of osmium tetroxide- damaged or undamaged poly (dA-[methyl-^{3}H] dT) was employed in each N-glycosylase assay containing 10 μg human lymphoblast redoxyendonuclease in reaction buffer (total volume, 60 μl). Following incubation for 60 min at 37°C, the reaction was terminated by the addition of 1 μg pUC19 carrier DNA and NaCl to a final concentration of 0.25 M. DNA was precipitated by addition of 120 μl 95% ethanol. Following centrifugation, the ethanol supernatants were lyophilized, resuspended in 12 μl deionized water, divided into three equal fractions and analyzed on a C_{18} reverse phase column (Alltech Adsorbosphere HS) eluted with either a 0-60% methanol gradient in 10 mM KH_2PO_4 (pH 4.5), flow rate 1 ml/min (solvent system A) or glass distilled water, flow rate 0.5 ml/min. (solvent system B). Authentic thymine glycol, prepared according to the procedure of Frenkel et al. (29), was added to each sample as an internal standard and the radioactivity in each eluted fraction (0.5 ml) was determined by scintillation counting. The elution of the thymine glycol standard, was determined by monitoring absorbance at 230 nm.

RESULTS AND DISCUSSION

Human CEM-C1 lymphoblasts contain a DNA damage-specific endonuclease activity (redoxyendonuclease) that is active in the presence of 10 mM EDTA and cleaves UV-irradiated DNA at sites of pyrimidines and osmium tetroxide-damaged DNA at sites of thymine glycol (19). These properties as well as an AP endonuclease are also possessed by E. coli endonuclease III (17-19,24) and a calf thymus enzyme (17-19). In addition, E. coli endonuclease III possesses an associated N-glycosylase activity that liberates damaged pyrimidines, including thymine glycol, from DNA damaged with ionizing radiation or other oxidizing agents (3,4,6-9,17). The goal of this study was to determine whether or not similarly associated N-glycosylase and AP endonuclease activities were properties of the human redoxyendonuclease. The size of human lymphoblast redoxyendonuclease was also determined.

Size Fractionation of Human Redoxyendonuclease.

Partially purified human lymphoblast redoxyendonuclease was size-fractionated on a calibrated Sephadex G-100 (superfine) column. Redoxyendonuclease activity was monitored by incubating an aliquot (10 μl) of each fraction with heavily UV-damaged, 3' end-labelled DNA fragment A followed by analysis of the incubation mixtures on DNA sequencing gels (Fig. 1). Redoxyendonuclease-specific cleavage of UV-damaged DNA substrates occurs primarily at positions of cytosine and less frequently at positions of thymine (17-19). The same positions of cleavage were also observed following incubations with E. coli endonuclease III (not shown). The chemical nature of the photoproducts recognized by these enzymes is not known at present, but they probably correspond to monobasic hydration products of cytosine and thymine (17,24). Under the experimental conditions employed, the DNA sequencing assay is specific for detecting redoxyendonuclease as the particular endonucleolytic properties of the enzyme can be precisely monitored. Human redoxyendonuclease-mediated DNA cleavage was centered around fraction 41 and corresponded to an activity eluting at an apparent molecular size of 60,000 $\pm$ 6,000 daltons (Fig. 1). The apparent size of the human redoxyendonuclease is considerably larger than the reported size of 25,000 daltons for E. coli endonuclease III (3). A Drosophila DNA glycosylase that releases both urea and thymine glycol from oxidized DNA also possesses a molecular size (40,000 daltons) significantly larger than E. coli endonuclease III (11). These results suggest that the human lymphoblast enzyme is larger than E. coli endonuclease III, although it is conceivable that the enzyme eluted as a dimer during gel filtration.

Thymine Glycol-DNA Endonuclease Activity of Human Redoxyendonuclease.

The base specificity of DNA cleavage of the human lymphoblast redoxyendonuclease was compared to E. coli endonuclease III under conditions where the nature of the DNA base damage was known. Osmium tetroxide-damaged 3' end-labelled DNA was employed as a lesion-specific substrate containing thymine glycol (5,6-dihydroxy- 6-hydrothymine) (25). This substrate was reacted with human redoxyendonuclease, E. coli endonuclease III, or hot alkali and the resulting DNA cleavage products were analyzed on a DNA sequencing gel (Fig. 2). Identical endonucleolytic DNA cleavage patterns at positions of thymine glycol

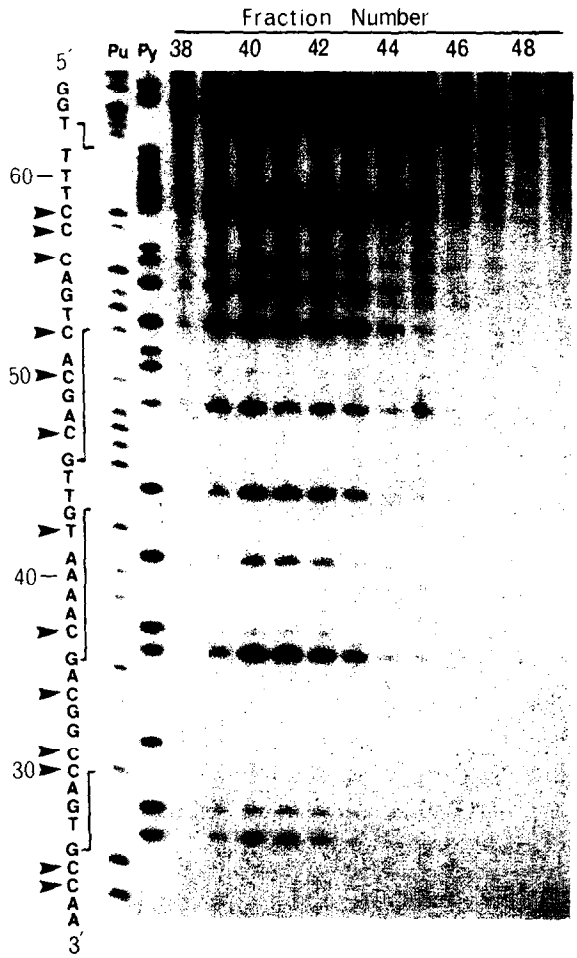

Figure 1. Size fractionation of human lymphoblast redoxyendonuclease. Pooled, concentrated fractions obtained from phosphocellulose chromatography were size fractionated on Sephadex G-100 (Materials and Methods). An aliquot (10 μl) of each fraction (2 ml) was incubated with UV-irradiated 3' end-labelled DNA fragment A and the reaction products were analyzed on a DNA sequencing gel followed by autoradiography (Materials and Methods). Redoxyendonuclease activity was centered around fraction 41 and corresponded to an apparent size of approximately 60,000 daltons. Fractions 39,40,41 and 42 correspond to elution of species with apparent molecular sizes of 66,000, 62,000, 60,000, and 54,000 respectively. Approximately 30,000 cpm of processed incubation sample was loaded into each lane. The purine (Pu) and pyrimidine (Py)-specific DNA sequencing reactions were included in the far-left lanes. Base numbering starts from the 3' end-labelled terminus. Arrows denote the positions of enzyme incision at sites of pyrimidine base damage.

were observed for both enzymes and were confirmed by comparison to the chemical cleavage pattern obtained with hot alkali treatment, a procedure that results in chemical scission of DNA at sites of thymine glycol (26). These results confirm an initial observation with the human lymphoblast enzyme purified through the phosphocellulose chromatography step (19). We conclude that both human redoxyendonuclease and E. coli endonuclease III are identical with respect to their ability to cleave DNA at sites of thymine glycol.

Thymine Glycol-DNA Glycosylase Activity of Human Redoxyendonuclease

We wished to determine whether or not a thymine glycol DNA-glycosylase activity was associated with the thymine glycol endonuclease activity observed

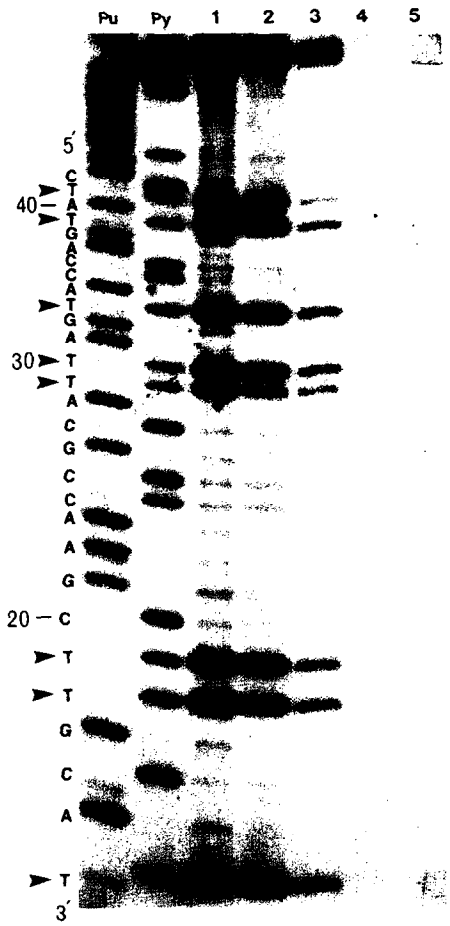

Figure 2. Thymine glycol endonuclease activity of human lymphoblast redoxyendonuclease. 3′ End-labelled DNA fragment B was damaged with osmium-tetroxide (Materials and Methods). Damaged (lanes 1-3) and undamaged (lanes 5 and 6) DNA samples were treated with hot alkali (lane 1), human redoxyendonuclease (lanes 2 and 4) or E. coli endonuclease III (lanes 3 and 5). Analysis of treated samples on a DNA sequencing gel and autoradiography was as described in Materials and Methods. Base numbering and DNA sequencing as in Fig. 1. Arrows denote the positions of DNA strand scission at sites of thymine glycol.

for human lymphoblast redoxyendonuclease. Osmium tetroxide-damaged poly (dA-[methyl-^{3}H]dT) was utilized as the DNA damage substrate and incubated with human lymphoblast redoxyendonuclease. N-glycosylase-mediated release of thymine glycol was monitored by HPLC analysis of the ethanol-soluble enzyme digestion products in two different solvent systems (Fig. 3). The retention times for authentic thymine glycol were 5.0 min (solvent A, Fig. 3A) and 10.0 min (solvent B, Fig. 3B). Human lymphoblast redoxyendonuclease liberated radioactive material from osmium tetroxide-damaged poly (dA-[methyl-^{3}H]dT) DNA that eluted with retention times of 5.0 min and 10.0 min for solvent systems A and B, respectively. These results indicate that a thymine glycol-DNA glycosylase activity is associated with human lymphoblast redoxyendonuclease. In this respect, human lymphoblast redoxyendonuclease is similar to E. coli endonuclease III (3,6-9,17), a calf thymus enzyme (12,17), an enzyme present in HeLa (9) and mouse plasmacytoma (13) cells, and M. luteus gamma endonuclease (27).

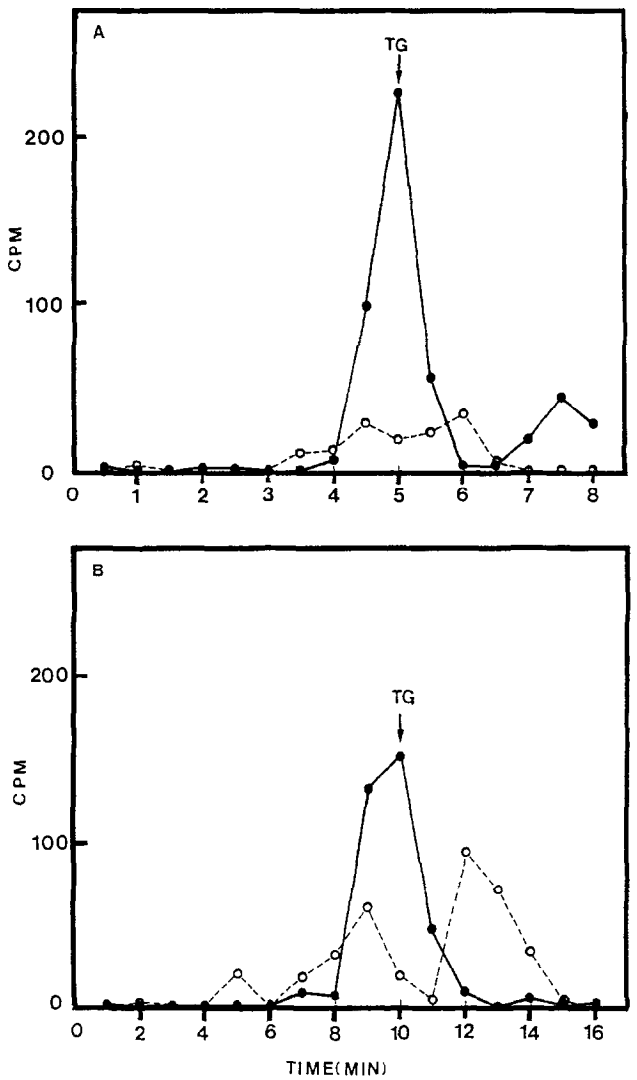

Figure 3. HPLC analysis of ethanol-soluble human lymphoblast redoxyendonuclease (N-glycosylase) reaction products. Ethanol-soluble reaction products from incubation of human redoxyendonuclease with osmium-tetroxide-damaged (●—●) or undamaged (o---o) poly(dA-[methyl-^{3}H]dT) were subjected to HPLC analysis in solvent system A (panel A) or B (panel B) as described in Materials and Methods. Arrows indicate the positions where authentic thymine glycol (TG) was eluted from the column. Background radioactivity from analyses of mock incubations of either damaged or undamaged DNA containing no enzyme were subtracted from the values obtained from the incubations with enzyme.

Human lymphoblast redoxyendonuclease incubations with osmium tetroxide-damaged poly(dA[methyl-^{3}H]dT) produced a second peak of radioactivity (retention time 7.5 min) that eluted after the thymine glycol peak in solvent system A, but was not detected in solvent system B. Additional peaks of radioactivity have also been observed for the HPLC analyses of osmium tetroxide-damaged poly (dA[methyl-^{3}H]dT) incubated with E. coli endonuclease III, a calf thymus endonuclease, and HeLa cell extracts (9,17). In addition, incubations of human lymphoblast redoxyendonuclease with undamaged DNA produced several peaks of

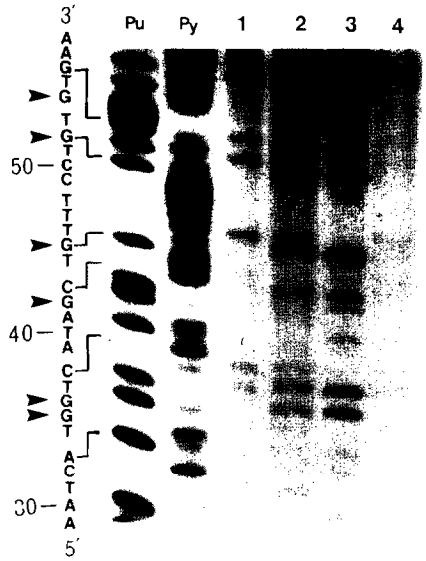

Figure 4. AP endonuclease activity of human lymphoblast redoxyendonuclease. 5′ End-labelled DNA fragment B was depurinated as described (17). Depurinated DNA (lanes 1-4) was treated with E. coli endonuclease III (lane 1), human redoxyendonuclease (lane 2), hot alkali (lane 3) or reaction buffer alone (lane 4). Analysis of treated samples on a DNA sequencing gel, autoradiography, and DNA sequencing was as described (Materials and Methods). Base numbering is from the 5′ end-labelled terminus. Arrows indicate the positions of DNA strand scission at apurinic sites.

radioactivity that eluted with retention times different from that of thymine glycol and were not observed with osmium tetroxide-damaged DNA (Fig. 3A, B). The nature of these additional peaks is unknown at present.

AP Endonuclease Activity of Human Redoxyendonuclease.

E. coli endonuclease III cleaves osmium tetroxide- damaged DNA at sites of thymine glycol via a two-step, thymine glycol-DNA glycosylase/AP endonuclease mechanism (6-8,17). Presumably, the N-glycosylase reaction creates an AP site that is subsequently acted upon by the AP endonuclease, cleaving the phosphodiester bond 3′ to the AP site (7,8,17). Since the human lymphoblast redoxyendonuclease also mediates phosphodiester bond cleavage at sites of thymine glycol and possesses a thymine glycol-DNA glycosylase activity, a similar two-step mechanism involving an AP endonuclease activity might be expected. We tested the ability of human lymphoblast redoxyendonuclease to cleave 5′ end-labelled DNA fragment B at apurinic sites in the presence of 10 mM EDTA (Fig. 4). DNA was subjected to mild acid treatment, a procedure that produces depurinations primarily at positions of guanine (28) that are revealed following hot alkali treatment and analysis of the scission products on a DNA sequencing gel (Fig. 4, lane 3). Hot alkali-induced scission of DNA at AP sites produces cleavage products that contain 3′ terminal phosphoryl groups (17,23). Human redoxyendonuclease cleaved depurinated DNA at sites of guanine as did E. coli endonuclease III (Fig. 4, lanes 1 and 2). A comparison of the DNA cleavage products

produced by endonuclease III and human redoxyendonuclease revealed that although both enzymes cleave DNA at apurinic sites, they differ with respect to the exact nature of the 3' terminus of the scission products. This difference is revealed as a shift in the electrophoretic mobilities of the scission products created by the two enzymes. The mobilities of the human redoxyendonuclease scission products are consistent with a species containing a 3' terminal phosphoryl group, whereas endonuclease III scission products possess a 3' terminal base free sugar or modified sugar (7,8,17). In this respect, human redoxyendonuclease possesses an AP endonuclease activity identical to that of a calf thymus enzyme (17).

CONCLUSIONS

The results of the thymine glycol DNA-endonuclease (Fig. 2), N-glycosylase (Fig. 3), and AP endonuclease (Fig. 4) experiments support the notion that human lymphoblasts contain an enzyme that is analogous to *E. coli* endonuclease III (6-8), a calf thymus (17) and mouse plasmacytoma (13) DNA repair enzyme, and *M. luteus* gamma endonuclease (27). The bovine and bacterial enzymes possess a broad substrate specificity directed against damaged pyrimidines and act via an N-glycosylase/AP endonuclease mechanism (3,4,7,8,17,18). The human enzyme also possesses a broad substrate specificity (19, and unpublished results) and probably acts via a similar mechanism. Although the N-glycosylase and AP endonuclease activities are associated with partially purified human redoxyendonuclease, absolute proof that both activities are associated with the same protein molecule must await purification of the enzyme to homogeneity. The finding that CEM-Cl lymphoblasts also contain these activities, further extends the species distribution to humans for a conserved DNA repair enzyme that may mediate a crucial role in maintaining the genetic integrity of a cell responding to numerous and varied sources of active oxygen species.

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