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Two distinct human DNA diesterases that hydrolyze 3′-blocking deoxyribose fragments from oxidized DNA

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ABSTRACT

Mammalian cells were investigated for enzymes that help correct oxidative damages in DNA. We focused on 3′-repair diesterases, which process DNA ends at oxidative strand breaks by removing 3′-blocking fragments of deoxyribose that prevent DNA repair synthesis. Two enzymes were found in a variety of mouse, bovine and human tissues and cultured cells. The two activities were purified to differing degrees from HeLa cells. One enzyme had the properties of the known HeLa AP endonuclease (M_r ~38,000, with identical substrate specificity and reaction requirements, and cross-reactivity with anti-HeLa AP endonuclease antiserum) and is presumed identical to that protein. The second activity did not interact with anti-HeLa AP endonuclease antibodies and had relatively less AP endonuclease activity. This second enzyme may have been detected in other studies but never characterized. In addition to the 3′-repair diesterase and AP endonuclease, this partially purified preparation also harbored DNA 3′-phosphatase and 3′-deoxyribose diesterase activities. It is unknown whether all activities detected in the second preparation are due to a single protein, although activity against undamaged DNA was not detected. The *in vivo* roles of these two widely distributed 3′-repair diesterase/AP endonucleases have not been determined, but with the characterizations presented here such questions may now be focused.

INTRODUCTION

Active oxygen species such as the superoxide radical ($O_2^{-}\cdot$), the hydroxyl radical ($HO\cdot$), and hydrogen peroxide (H_2O_2) are generated by aerobic metabolism (1, 2, 3) and are responsible for many of the DNA damages caused by ionizing radiation (4). Such damages include modified bases, abasic (AP) sites, and strand breaks with deoxyribose fragments at their termini (4–6). AP sites are also formed by spontaneous hydrolysis (7), and by a wide variety of mutagens (e.g., simple alkylating agents) as secondary products when damaged bases hydrolyze from DNA spontaneously or with the assistance of DNA N-glycosylases (8, 9).

The repair of abasic sites is initiated by AP endonucleases, the main forms of which hydrolyze the phosphodiester immediately 5′ to the AP site. These so-called Class II AP endonucleases produce a strand break with a normal 3′-hydroxyl nucleotide and a 5′-baseless sugar-phosphate (8–10). Repair is then completed when the sugar-phosphate is removed by another enzyme (11), and repair DNA synthesis and ligation seal the gap. Class II AP endonucleases can also remove various 3′-terminal groups from DNA, including phosphate monoesters, deoxyribose-5-phosphate esters, and phosphoglycolate esters, all of which block repair synthesis by DNA polymerase (12–16). The less-abundant Class I AP endonucleases cleave AP sites by β-elimination, which yields 3′-(*trans*-4-hydroxy-2-pentenal-5-phosphate; 3′-ddR5P) and a normal 5′-phosphorylated nucleotide (17). These 3′-ddR5P residues also block DNA synthesis, but can be removed by the Class II AP endonucleases (15, 18).

The Class II AP endonucleases that have been most well studied biochemically are exonuclease III (19) and endonuclease IV (20) of *E. coli*, and the Apn1 endonuclease of the yeast *Saccharomyces cerevisiae* (14, 15, 21). Their biological importance has been substantiated by molecular genetic studies that demonstrate critical roles for these enzymes in removing both alkylation-induced AP sites and 3′-blocking deoxyribose fragments that result from oxidative damage (13, 16, 22, 23).

Human Class II AP endonucleases have been purified from HeLa cells (24) and placenta (25), but a systematic analysis of 3′-repair activities has not been reported. In this study, we have used a synthetic DNA substrate (13) to characterize the two major diesterases of HeLa cells that remove 3′-blocking groups from DNA. One enzyme is identical to the previously identified HeLa AP endonuclease (24), while the second enzyme is a newly identified activity with a distinctive substrate specificity.

MATERIALS AND METHODS

General materials

[Uracil-^{3}H]dUTP (17 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Amersham Corp. [α-^{32}P]dUTP was prepared by deamination of [α-^{32}P]dCTP (see below). Ammonium sulfate (enzyme grade) was purchased from Schwarz/Mann. Phenylmethylsulfonyl fluoride, leupeptin,

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aprotinin, pepstatin A, and benzamidine were purchased from Sigma. DEAE cellulose DE52 was purchased from Whatman, Bio-Rex 70, Affi-gel blue agarose, and Affi-gel 10 from Bio-Rad, Ultrogel AcA54 from IBF, and phenyl-agarose and protein-A-agarose from Bethesda Research Laboratories. A prepacked Superose HR12 column was purchased from Pharmacia LKB Biotechnology, Inc. Poly [d(A-T)] was from Boehringer-Millipore. Fresh calf thymus was purchased from a local slaughterhouse.

Enzymes

E. coli exonuclease III and the large fragment of *E. coli* DNA polymerase I (Klenow enzyme) were purchased from New England Biolabs. *E. coli* uracil-DNA glycosylase and phage T4 endonuclease V were purified as described (13). *E. coli* endonuclease IV was a generous gift of J. D. Levin, purified as described (20). Small amounts of HeLa AP endonuclease and rabbit anti-AP endonuclease polyclonal IgG were generous gifts of S. Linn, University of California, Berkeley.

Cell lines

HeLa S3 cells were usually purchased from the MIT Cell Culture Center (Cambridge, MA), where they were harvested in exponential phase, after growth at 37°C in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum, 1% antibiotic solution, and 0.2% $NaHCO_3$. Human fibroblast cell lines were obtained originally from ATCC by J. B. Little, School of Public Health, Harvard University, whose laboratory subcultured them and generously supplied us with the indicated cell pellets. Lymphoblastoma HPB-ALL (T cell) extracts were a kind gift from R. Fishel (National Cancer Institute, Frederick, Maryland). The oxygen-resistant HeLa and CHO cells (26), and the mouse L5178Y lymphoblast cells, were supplied as frozen cell pellets, generous gifts of H. Joenje, (Free University, Amsterdam) and D.J. Chen (Los Alamos National laboratory), respectively.

Substrates

Detailed accounts of the synthesis of the substrates based on poly[d(A-T)] can be found in Johnson and Demple (14, 15) and Levin and Demple (18). Briefly, [α-^{32}P] dCTP was deaminated by a modification of the method of Brynolf *et al.* (27), neutralized and incorporated into poly [d(A-T)]. The resulting polymer could then be treated with uracil glycosylase for the AP endonuclease assays (18), or treated sequentially with uracil glycosylase, T4 UV endonuclease (acting as a β-lyase), and $NaIO_4$ to yield the 3'-PGA substrate (13). Hydrolysis with NaOH instead of oxidation with $NaIO_4$ yielded a 3'-phosphate substrate (15). The incorporation of [α-^{32}P] dUTP was approximately 1 per 1,000 nucleotides, which corresponds to the maximum level of AP sites or 3'-PGA esters in the respective substrates.

Supercoiled plasmid pBR322 DNA was purified by standard methods (28). Partially depurinated pBR322 was prepared by incubation in 20 mM sodium citrate, pH 5.0, 0.2 M NaCl for 20 min at 70°C, followed by chilling on ice and dilution into neutral reaction buffers. The resulting DNA contained ~2 AP sites per plasmid molecule, as calculated from the extent of conversion of supercoiled molecules to relaxed circular molecules (29) by a saturating amount of *E. coli* endonuclease IV, quantitated by agarose gel electrophoresis, staining and densitometric scanning.

3'-Exonuclease activity against undamaged double-stranded DNA was also assayed. A substrate was made by cleavage of the Bluescript plasmid (Stratagene, La Jolla, CA) with the restriction enzyme SalI, followed by repair synthesis with [α-^{32}P] dATP and purification by gel electrophoresis.

Enzyme Assays

For all of the polymer-based assays, activity was determined at enzyme levels for which the reaction rate was in the linear range (typically, release of < 20% of the total ^{32}P present), and one unit of activity releases one pmol of [^{32}P]PGA per min at 37°C. The standard assay for 3'-PGA diesterase activity was performed in outline according to Levin *et al.* (20) and Johnson and Demple (14). Each 25-μl assay contained 50 mM Hepes·KOH, pH 7.5, 50 mM KCl, 100 μg/ml BSA, 10 mM $MgCl_2$ and substrate containing 1 pmol of labeled 3'-PGA. After addition of the enzyme sample, the mixes were incubated for 10 min at 37°C, chilled on ice and treated with trichloroacetic acid (TCA) and acid-washed Norit (30). After 10 min on ice, the samples were centrifuged and aliquots of the supernatant mixed with 10 volumes of aqueous scintillation fluor (Beckman Ready Value) and counted in a liquid scintillation counter.

For Class I and Class II AP endonuclease, each 25-μl assay contained 50 mM Hepes·KOH, pH 7.5, 50 mM KCl, 100 μg/ml BSA, 10 mM $MgCl_2$, 0.05% Triton X-100 and DNA polymer containing 1 pmol of AP sites (18). After addition of the enzyme samples and incubation for 10 min at 37°C, the samples were processed as described by Levin and Demple (18). An alternative method of assaying AP endonuclease activity was also used. Enzyme samples were added to 20 μl AP endonuclease buffer (see above) containing 0.2 μg partially depurinated pBR322 (an average total of 0.16 pmol AP sites). The mixes were incubated at 37°C for 10 min, and the reactions stopped by adding 5 μl electrophoresis sample buffer (1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cynol, and 50% glycerol) and heating at 70°C for 3 min. The samples were then run on a 0.7% agarose gel in 0.5×TBE and stained with ethidium bromide (28) After densitometric scanning, AP endonuclease activity was determined from the conversion from supercoiled to nicked circular molecules (29). The dRPase (11) and nonspecific 3'-exonuclease (15, 20) assays were carried out as described.

Immunological techniques

The HeLa AP endonuclease was purified from HeLa S3 cells as described below. For antibody production, 75 μg of the purified protein (pooled from several preparations) was emulsified in complete Freund's adjuvant, split into two aliquots, and injected subcutaneously into two female New Zealand rabbits. Three weeks after the primary injection, each rabbit was given two booster injections at 3-week intervals, each time with 25 μg of the purified HeLa protein emulsified in incomplete Freund's adjuvant. Blood was collected and the serum isolated two weeks after the second booster injection.

Enzymes were immunoprecipitated by mixing the indicated samples and antibody preparations in a total volume of 50 μl containing 50 mM Hepes·KOH pH 7.5, 50 mM KCl, and 100 μg/ml BSA. The mixtures were incubated at 0°C for 16–21 hr, then mixed with 25 μl of the stock protein-A-agarose slurry (diluted from the manufacturer's stock 1:1 with 50 mM Na_2PO_4, pH 7.5). After a further 12 hr at 0°C, the mixes were centrifuged at 4°C, and the supernatants assayed for enzyme activities.

Enzyme Purification

S100. All operations were performed at 0–4°C, and all enzyme buffers contained 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 2 mM benzamidine and 1 mM PMSF. For crude extracts, 25 liters of HeLa cells (4×10^5 cells/ml) were harvested by low speed centrifugation (2000×g, 10 min) and washed twice with reversed phosphate buffered saline (8 g NaCl, 0.2 g KCl, 2.16 g $Na_2HPO_4\cdot7H_2O$, and 0.2 g KH_2PO_4/liter). The cells were resuspended with 5 packed cell volumes of buffer A (10 mM Hepes·KOH, pH 7.5, 10 mM KCl, 0.5 mM $MgCl_2$, and 1 mM DTT), kept on ice for 10 min, spun down as above and the supernatant aspirated off. The swollen cells were resuspended with 2 packed cell volumes of buffer A and transferred to a Dounce homogenizer, with which the cells were completely lysed in about 30 strokes. Cell lysis was monitored under a microscope with toluidine blue or trypan blue dye. After lysis, the cell debris and unbroken nuclei were spun down. The supernatant was adjusted to 0.4 M NaCl concentration and was centrifuged at 38,000 rpm in a Beckman 60 Ti rotor for 60 min. The supernatant from this high-speed spin, called the S100 fraction, was dialyzed for 5–8 hr against 20 volumes of buffer A containing 250 mM KCl. The dialyzed S100 was frozen quickly by immersion in liquid N_2 and was stored at −80°C.

After resuspension with Buffer A containing 0.4 M NaCl, the pellets produced by the low-speed (P10) and high-speed (P100) spins also contained 3′-PGA diesterase activity. However, ≤12% of the total activity was present in the P100 fraction. The P10 fraction contained activity almost equal to the S100 fraction, but > 95% of the 3′-PGA diesterase recovered from the P10 fraction chromatographed like peak II on Bio-Rex70 (data not shown; see below). For the joint purification scheme employed here, the S100 fraction was a more useful starting point because it contained roughly equal amounts of the two enzymes (see below).

DEAE-cellulose chromatography. A column (100 ml) of DEAE-cellulose DE52 was equilibrated with 50 mM Hepes·KOH, pH 7.5, 250 mM KCl, and 10% glycerol. The flow rate of this column was adjusted to 2 ml/min. S100 (150 ml) was loaded to the column and washed through with 50 ml of equilibration buffer. The 3′-PGA diesterase activity, which passed through the column, was collected. This pool was dialyzed overnight against 10 volumes of the Bio-Rex 70 equilibration buffer (50 mM Hepes·KOH, pH 7.5, 50 mM KCl and 10% glycerol). The dialyzed pool could be stored at −80°C for several months without significant loss of activity.

Bio-Rex 70 chromatography. A 250 ml Bio-Rex 70 column was equilibrated with 2 liters of equilibration buffer (see above). The dialyzed DE52 (180 ml) pool was applied to the column at a flow rate of 150 ml/hr. After washing with the same buffer until the eluate had A_{280} <0.1, a one-liter linear gradient of 50–500 mM KCl was then applied, at 120 ml/hr, and 15 ml fractions were collected. Two distinct 3′-PGA diesterase activity peaks were eluted: one beginning at 150 mM KCl and the other at 250 mM KCl (see below). For each peak, only the central fractions, containing ≥50% of the total activity, were pooled. The lower-salt and higher-salt pools were called Bio-Rex peak I and Bio-Rex peak II, respectively. These pools were separately concentrated using a Centriprep 30 filter apparatus (Amicon) with centrifugation at 3000 rpm for 4 hr. The concentrated Bio-Rex 70 pools were stored at −80°C.

Phenyl-agarose chromatography. Solid $(NH_4)_2SO_4$ was added to the Bio-Rex 70 peak I and peak II pools to 30% saturation. The pools were then stirred for 30 min at 0°C and centrifuged at 30,000×g for 30 min. Samples of Bio-Rex peak I (90 ml) or peak II (75 ml) supernatant were loaded on separate 20-ml phenyl-agarose columns pre-equilibrated with 50 ml of 50 mM Hepes·KOH, pH 7.5, 10% (vol/vol) glycerol, 30% saturated $(NH_4)_2SO_4$. The columns were run at a flow rate of 30 ml/hr. After washing with 50 ml of the equilibration buffer, 20 ml of each of the following buffers containing 50 mM Hepes·KOH, pH 7.5 and 10% glycerol were applied in succession: 20% saturated $(NH_4)_2SO_4$ and10% ethylene glycol; 15% saturated $(NH_4)_2SO_4$ and 20% ethylene glycol; 10% saturated $(NH_4)_2SO_4$ and 30% ethylene glycol; 5% saturated $(NH_4)_2SO_4$ and 40% ethylene glycol; 0% $(NH_4)_2SO_4$ and 50% ethylene glycol. The 3′-PGA diesterase activity of peak I was eluted during the 10% saturated$(NH_4)_2SO_4$-30% ethylene glycol step, and the activity of peak II was eluted during the 20% saturated $(NH_4)_2SO_4$-10% ethylene glycol step. No 3′-PGA diesterase was eluted in the other washes. These pools were concentrated by ultrafiltration as described above and stored at −80°C.

Gel filtration chromatography. An Ultrogel AcA54 column (2.2×200 cm) was equilibrated with 50 mM Hepes·KOH, pH 7.5, 150 mM KCl and 10% glycerol. The concentrated phenyl-agarose pools (5 ml) were applied to the AcA54 column in separate runs. The column was run at a flow rate of 27 ml/hr. The 3′-PGA activity of peak I was eluted at Ve/Vo = 1.16, and the activity of peak II was eluted at Ve/Vo = 1.56. The activity peaks were pooled and concentrated by ultrafiltration as described above. Aliquots (0.5 ml) of the concentrated phenyl-agarose pools were also applied to a Superose HR12 column fitted to a Pharmacia/LKB FPLC equilibrated with the same buffer used for AcA54. The flow rate on this column was set at 0.5 ml/min and 0.5 ml fractions collected. The 3′-PGA diesterase activity peak I was eluted at Ve/Vo = 1.37, and the activity peak II eluted at Ve/Vo =1.81.

Affi-gel blue agarose chromatography. A 1-ml Affi-gel blue agarose column was equilibrated with 10 ml of 50 mM Hepes·KOH, pH7.5, 50 mM KCl, 1 mM EDTA, 0.05% Triton X-100. A sample (1 ml) of the concentrated of peak II pool from the AcA54 column was diluted such that the KCl concentration was 50 mM and applied to the Affi-gel column. The flow rate on this column was set at 1 ml/min, and 0.5 ml fractions were collected. After washing with 5 ml of equilibration buffer, followed by 5 ml of equilibration buffer containing 5 mM ATP and 1 mM NAD^+, a further 5 ml of equilibration buffer was applied, followed by a 20 ml linear gradient of 50–500 mM KCl. The 3′-PGA activity was eluted at 140–180 mM KCl. The pool was concentrated by ultrafiltration as described above and stored at −80°C.

RESULTS

3′-Repair activities in mammalian cell extracts

A synthetic DNA polymer containing ^{32}P-labeled 3′-PGA was used to assay mammalian cells for enzymes that repair DNA 3′-termini bearing deoxyribose fragments. The substrate and assay were originally developed to characterize DNA repair diesterases from *E. coli* (13) and yeast (14). These microbial enzymes release free [^{32}P]-PGA to generate 3′-hydroxyl ends

Table 1. 3'-PGA diesterase levels in crude extracts of cultured mammalian cells

Extract	3'-PGA diesterase (units/mg)
HeLa/20% O_2 (Human)	6.6
HeLa/80% O_2 (Human)	8.5
CHO/20% O_2 (Hamster)	4.7
CHO/80% O_2 (Hamster)	5.3
GM3348 Normal human fibroblast	0.31
GM3847 Ataxia	0.52
GM3058 Ataxia	0.41
GM5823 Ataxia	0.39

[a] For the HeLa and CHO cells, the% O2 given is the oxygen level to which the cells were adapted for growth (26). 'Ataxia' refers to fibroblasts derived from patients with the genetic disease ataxia telangiectasia (31).

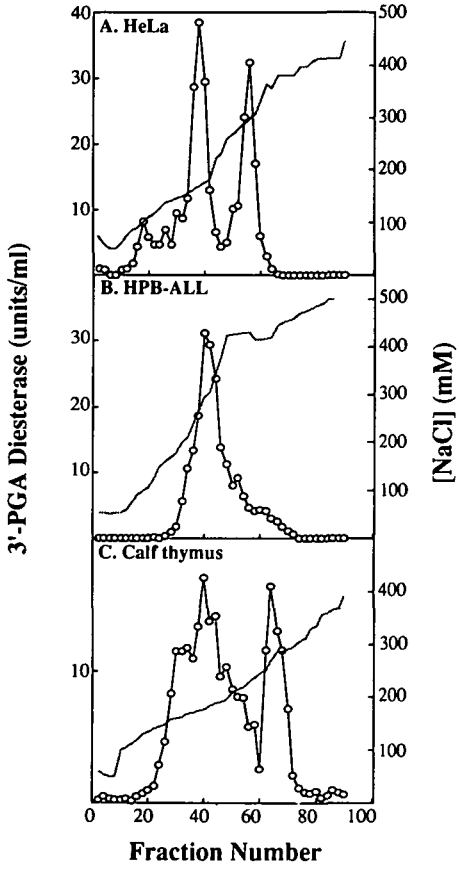

Fig. 1. Bio-Rex 70 chromatography of HeLa, HBP-ALL and calf thymus extracts. The dialyzed DE52 pools were applied to individual Bio-Rex 70 columns and developed as described in Methods. 3'-PGA activity was assayed as described in the text. (A) HeLa extract; (B) HBP-ALL extract; (C) calf thymus extract. Note that the steepness of the salt gradients varies somewhat among the experiments. Thus the main peak in panel B corresponds to the second peaks in panels A and C.

(13, 14, 20). Application of this assay to extracts from a variety of cultured mammalian cells revealed significant 3'-PGA diesterase activity in the presence of 10 mM $MgCl_2$ (Table 1).

Since 3'-PGA diesterases act on oxidative damage, we examined whether the level of enzyme was correlated with any cellular phenotypes. HeLa or CHO cells selected to resist high O_2 concentrations (26) had < 1.5-fold elevated 3'-PGA

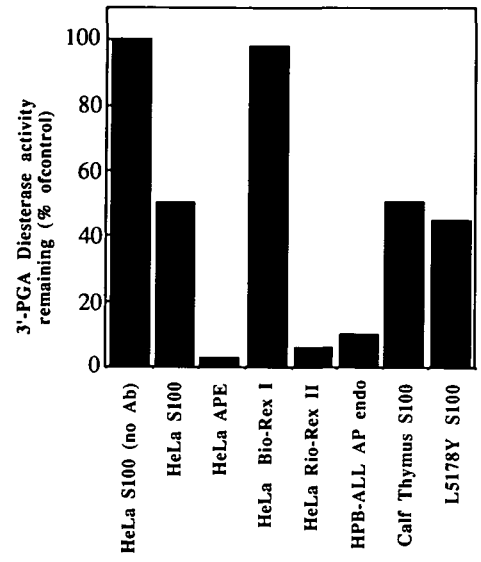

Fig. 2. Immunoprecipitation with anti-AP endonuclease antiserum. Samples (5 μl of cell extract as indicated) were mixed with 10 μl rabbit anti-AP endonuclease antiserum in a total volume of 50 μl and processed as described in the text. **HeLa S100,** Fraction I (see Table 2); **HeLa APE**, purified AP endonuclease (fraction 5b); **HeLa Bio-Rex peak I** (fraction 3a); **HeLa Bio-Rex peak II** (fraction); **HPB-ALL AP endo**, fraction 5b isolated from HPB-ALL extract; **Calf Thymus**, extract of calf thymus; **L5178Y S100**, mouse cell crude extract. The limit of detection in these experiments was equivalent to 5% residual activity.

diesterase activity in crude extracts (Table 1). This activity is thus apparently not the critical factor for the oxygen-resistant phenotype. Cells from patients with the genetic disease ataxia telangiectasia exhibit hypersensitive to ionizing radiation (31). This radiosensitivity is apparently not due to a deficiency in 3'-PGA diesterase (Table 1).

HeLa cells had the highest specific activity of all the cultured human cells tested and were chosen as the starting material for enzyme purification. Conventional purification techniques were used to isolate the 3'-PGA diesterases from HeLa extracts. More than 95% of the diesterase activity passed through a DEAE anion-exchange column at 250 mM KCl, which served to remove nucleic acids and some proteins. The DE52 pass-through was dialyzed and then loaded onto a Bio-Rex 70 cation-exchange column. Two major activity peaks were eluted using a linear salt gradient: peak I beginning at ~150 mM KCl and peak II at 250–300 mM KCl (Fig. 1A). Chromatography of the DEAE fraction on phosphocellulose also revealed two distinct peaks (data not shown). The two phosphocellulose peaks were less well-separated than on Bio-Rex and therefore less suitable for further fractionation.

The two activity peaks on Bio-Rex 70 were about equal from HeLa extracts, but an extract from T lymphoblast (HPB-ALL) cells yielded predominantly peak II on the Bio-Rex 70 column (Fig. 1B). We do not know whether the low level of the peak I enzyme is a general property of T-cells or specific to the HPB-ALL line. Bio-Rex 70 chromatography of a calf thymus extract also produced two major peaks, but peak I was very broad (Fig. 1C). The broadness of the calf thymus peak I may be due to the presence of multiple species, perhaps the result of protein degradation. Indeed, the peak I activity of calf thymus was unstable. Extracts from human placenta also revealed two activity

Table 2. Parallel purification of HeLa AP endonuclease/3'PGA diesterase activities

Fraction	Total[a] protein (mg)	3'-PGA diesterase specific activity (units/mg)	Total 3'-PGA diesterase activity (units)	AP endonuclease[b] specific activity (units/mg)
1. Crude extract/S100	756	9.2	7000	
2. DE52	620	10.9	6800	
3a. Bio-Rex70/peak I	50	37.6	1870	
4a. Phenyl-agarose/peak I	6.25	144	900	
5a. FPLC superose/peak I	0.3	1400	420	10,600
3b. Bio-Rex70/peak II	21	137	2880	
4b. Phenyl-agarose/peak II	2.5	840	2100	
5b. FPLC superose/peak II	0.36	1860	670	
6b. Affi-gel blue/peak II	0.035	4800	170	800,000

[a]Estimated by the method of Bradford (42).
[b]Determined using the synthetic polymer (21).

peaks upon Bio-Rex 70 chromatography (data not shown). The extracts from ataxia telangiectasia fibroblasts and oxygen-resistant HeLa and CHO cell lines (Table 1) also yielded two activity peaks upon fractionation on small phosphocellulose columns, but the levels of the two peaks were not significantly different from their normal counterparts (data not shown). It is likely that mammalian cells generally contain these two activities.

Immunological Characterization

Purified HeLa AP endonuclease (24) was found to act not only as a Class II AP endonuclease but also as a 3'-PGA diesterase (32). It was therefore likely that one or both of the two peaks from HeLa cells resolved on Bio-Rex 70 corresponded to this AP endonuclease. In fact, polyclonal antibodies against the HeLa AP endonuclease (24) cross-reacted with peak II but not with peak I, as judged by immunoprecipitation (data not shown).

Polyclonal rabbit antisera against the 3'-PGA diesterase purified from peak II in the present work (see Methods and text below) also recognized the AP endonuclease specifically in cell extracts. While over 90% of the peak II activity from HeLa cells was immunoprecipitated by such an antiserum, no immunoprecipitation was seen for the peak I activity (Fig. 2). The antibodies immunoprecipitated only ~50% of the 3'-PGA diesterase activity from crude extracts of HeLa cells, a mouse cell line (L5178Y) and calf thymus (Fig. 2), and from extracts of human placenta (data not shown), consistent with the presence of a second distinct 3'-PGA diesterase in mammalian cells. The lack of immunological cross-reactivity indicates that the two activities are not related by proteolysis.

Physical Properties

The apparent identity of the Bio-Rex peak II material with the known HeLa AP endonuclease caused us to adopt the reaction conditions already determined for that enzyme (24). We confirmed that the 3'-PGA diesterase activity in the pooled Bio-Rex peak II had the expected requirement for Mg^{2+} (data not shown).

Additional basic studies were performed on the Bio-Rex peak I 3'-PGA diesterase, which can be summarized as follows. The activity displayed an absolute requirement for Mg^{2+}, with ~10 mM $MgCl_2$ in the assay buffer being optimal, ~30% activity observed at 0.1 mM $MgCl_2$ and <1% of maximal activity detected in the absence of $MgCl_2$. The optimum pH for the reaction was 7.0–7.6, as determined using Tris3HCl and 3-[N-morpholinopropane sulfonic acid as the buffers (data not shown).

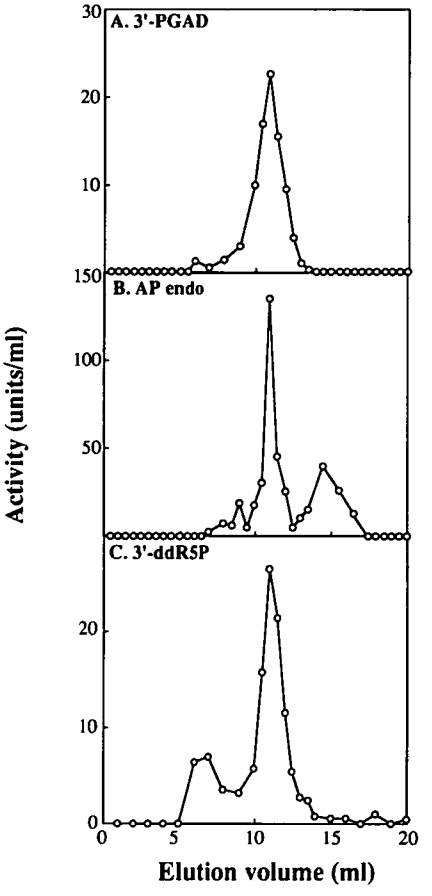

Fig. 3. FPLC superose HR12 gel filtration chromatography. A sample (0.5 ml) of Fraction 4a (see Table 2) was injected and chromatographed as described in the Methods. Each fraction (0.5 ml) was assayed for 3'–PGA diesterase (A), hydrolytic (Class II) AP endonuclease (B), or 3'-ddR5P diesterase activity (C).

Similarly, the optimal concentration of KCl was determined to lie within a broad range (50–150 mM; data not shown), and 50 mM KCl was chosen for the standard buffer for the peak I enzyme.

Further purification of the HeLa peak I and II activities was necessary for understanding their enzymatic characteristics. Table 2 summarizes a joint purification for these enzymes from HeLa

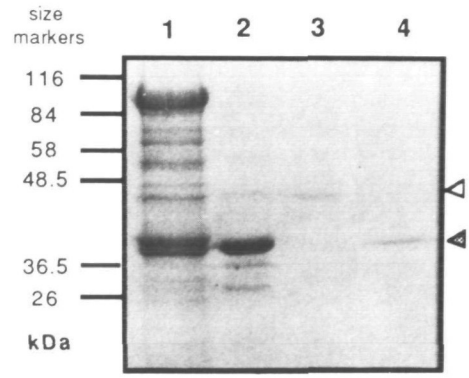

Fig. 4. SDS polyacrylamide gel electrophoresis of AP endonuclease and 3′-PG-A diesterase preparations. Samples and amounts of protein loaded: **Lane 1**, peak II phenyl-agarose pool (Fraction 4b of Table 2), 9 μg; **Lane 2**, peak II AcA54 fraction (Fraction 5b of Table 2), 4.5 μg; **Lane 3**, peak I Affi-gel blue pool (further chromatography of Fraction 5a; see text), 0.5 μg; **Lane 4**, peak II Affi-gel blue pool (Fraction 6a of Table 2), 0.5 μg. The gel contained 12% acrylamide and 0.32% bisacrylamide, and was stained with Coomassie Brilliant Blue. The symbols on the the right of the figure indicate the positions of the major polypeptides in Fraction 5a (open caret) and 6b (crosshatched caret).

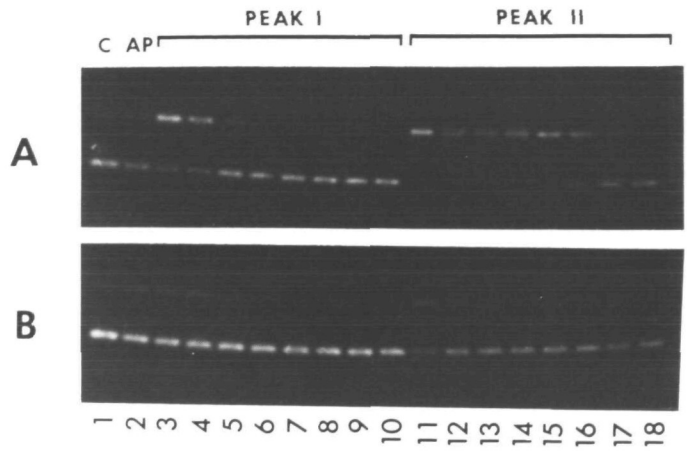

Fig. 5. AP endonuclease activities on supercoiled plasmid DNA. Incision at AP sites was assayed by following the conversion of supercoiled plasmid DNA (lower band in each panel) to relaxed circular molecules (upper band in each panel) as described in the text. (A) DNA containing AP sites; (B) Undamaged DNA. *Lanes*: 1, undamaged DNA, no enzyme; 2, AP DNA, no enzyme; 3–10, incubation with 3′-PGA diesterase (Fraction 5a: 5×10^{-2}, 2.5×10^{-2}, 5×10^{-3}, 2.5×10^{-3}, 5×10^{-4}, 2.5×10^{-4}, 5×10^{-5}, 2.5×10^{-5} units, respectively); 11–18, incubation with AP endonuclease (Fraction 6a: 1.5×10^{-2}, 7.5×10^{-3}, 1.5×10^{-3}, 3×10^{-4}, 1.5×10^{-4}, 7.5×10^{-5}, 3×10^{-5}, and 1.5×10^{-5} units, respectively). The enzyme units given here are expressed as 3′-PGA diesterase activity.

cells. After hydrophobic interaction chromatography on phenyl-agarose (Table 2), peak I activity was eluted from both AcA54 (data not shown) and Superose HR12 gel filtration columns at a position corresponding to a globular protein of 48–60 kDa (Fig. 3). Additional purification of this material on a small-scale Affi-gel blue column yielded a preparation containing a major protein of M_r ~45,000 (Fig. 4), although in poor yield. This fraction also contained 3–4 other polypeptides not visible in Fig. 4.

The peak I enzyme was too labile to purify beyond the gel filtration step, even though substantial efforts were made to minimize proteolysis (e.g., by the inclusion of multiple protease inhibitors). The Superose HR12 pool of peak I lost over 50% of its activity within one hour after chromatography, even though the pooled fractions were concentrated by ultrafiltration immediately after elution from the column. This enzyme activity was not recovered after loss even when possible stabilizing or activating factors such as metals (Mg^{2+}, Co^{2+}, Mn^{2+} or Zn^{2+}) or nucleotides (ATP, GTP or cAMP) were added to the gel filtration pool or the assay mixtures (data not shown). The reason for this instability remains unknown. The peak I enzyme activity (Fraction 3a) was also heat-labile: 50% of its 3′-PGA diesterase activity was lost during incubation at 37°C for 40 min. Upon incubation with 1 mM β-mercaptoethanol, 50% of the peak I 3′-PGA diesterase activity was lost in only 10 min at 37°C; less than 6% of the activity remained after 40 min. In contrast, the peak II enzyme retained over 90% of its activity after incubation at 37°C for 90 min, even when incubated with 10 mM β-mercaptoethanol.

After hydrophobic interaction chromatography on phenyl-agarose, the peak II activity was eluted from AcA54 and Superose HR12 gel filtration columns at a position corresponding to a globular protein of 35–40 kDa (data not shown). The peak II enzyme was stable enough to be purified to >90% homogeneity by chromatography on Affi-gel blue agarose column, which yielded ~35 mg of the purified material (Table 2 and Fig. 4). Taking into account the approximately equal contributions of the two enzymes to the total 3′-PGA diesterase of Fraction I, Fraction 5a represents a purification factor of ~300-fold, and Fraction 6b a factor of ~1000-fold (Table 2).

Enzymatic Specificities

Neither Fraction 5a nor 5b contained significant 3′-exonuclease activity against undamaged duplex DNA (<0.03% of 3′-PGA diesterase; data not shown). The Bio-Rex peak I activity after phenyl-agarose chromatography (Fraction 4a) contained 3′-PG-A diesterase, hydrolytic (Class II) AP endonuclease and 3′-ddR5P diesterase activities that co-chromatographed upon gel filtration using Superose HR12 (Fig. 3). The Class II AP endonuclease activity of the major peak was not immunoprecipitated by the anti-AP endonuclease antibodies (data not shown). However, minor activity peaks, one probably corresponding to the major AP endonuclease (Fig. 3B), were also observed. The same cochromatography of several activities was seen when Fraction 4a was applied to an AcA54 gel filtration column, although the yield of activity was in that case much lower (data not shown). Fraction 5a was also tested for dRPase (11) and Class I AP endonuclease (β-lyase; ref. 18), which were absent from most preparations of this enzyme. After phenyl-agarose chromatography of the Bio-Rex peak II (Table 2, Fraction 4b), the 3′-PGA diesterase, Class II AP endonuclease and 3′-ddR5P diesterase activities also copurified upon gel filtration (data not shown). The ratio of AP endonuclease to 3′-PGA diesterase was about 170:1 in Fraction 6b and about 7:1 in Fraction 5a (Table 2).

The enzymes being investigated here were identified and monitored by their activity against a synthetic polymer containing 3′-esters and AP sites. Both enzyme preparations were also tested for their ability to attack AP sites in a natural supercoiled plasmid DNA (Fig. 5A). The purified peak II enzyme contained substantial AP cleavage activity (AP endonuclease:3′-PGA

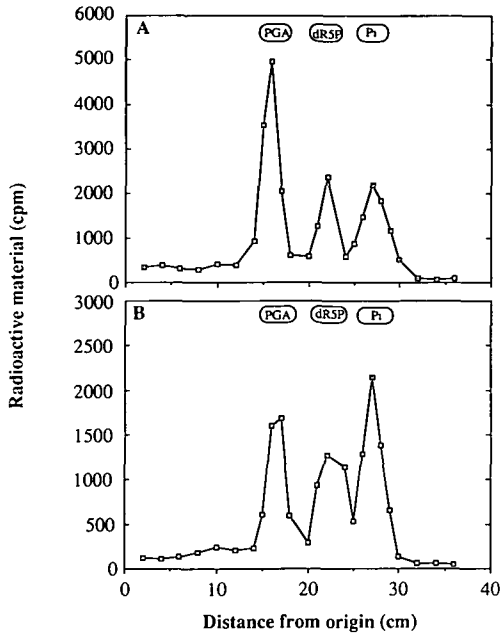

Fig. 6. Paper chromatography of products released from a mixed substrate by AP endonuclease and 3'-PGA diesterase. The reactions contained substrate with 2 pmol total of 3'-PGA, 3'-ddR5P and 3'-phosphate in 50 μl. AP endonuclease (0.03 unit) or 3'-PGA diesterase (0.05 unit) was added, and the mixtures incubated at 37°C for 10 min and processed as described in Methods. Norit-nonadsorbed reaction products were chromatographed on Whatman 3MM paper as described by by Johnson and Demple (15) Markers (PGA, dR5P, and P_i (inorganic phosphate)) were visualized with a reagent (15). (A) AP endonuclease (Fraction 5b); (B) 3'-PGA diesterase (Fraction 5a).

diesterase ~75:1). The peak I enzyme exhibited a much lower level of AP endonuclease (AP endonuclease:3'-PGA diesterase ~1:5). These results are consistent with those obtained using the polymer substrate (Table 2), which the peak I enzyme may prefer over the plasmid substrate. Alternatively, the lower AP endonuclease activity of the Peak I enzyme in the plasmid-based assay (compared to the synthetic substrate) could results from instability of the enzyme upon dilution. Neither enzyme preparation had significant endonuclease activity against undamaged plasmid DNA, except at the very highest enzyme levels employed (Fig. 5B). From these data, we estimate the nonspecific endonuclease activities of the preparations to be ú1% for peak I and ≤2% for peak II, relative to their AP endonuclease activities.

The labeled products released by the 3'-diesterase activities of the two enzyme preparations (Fractions 5a and 6b) were analyzed using a partially oxidized polymer that contained 3'-PG-A, 3'-ddR5P and 3'-phosphate. Both the purified peak II (data not shown) and the partially purified peak I activity (Fig. 6) released all three 3'-blocking damages from this mixed substrate. The 3'-phosphatase and 3'-PGA diesterase activities measured in this way for the peak II enzyme paralleled those previously reported (32) for the HeLa AP endonuclease of Kane and Linn (24). The HeLa AP endonuclease is therefore a combined phosphodiesterase and phosphomonoesterase like the AP endonucleases of *E. coli* (13, 20) and yeast (15). The partially purified Bio-Rex peak I preparations also contained both the two 3'-diesterase and the 3'-phosphatase activities (Fig. 6).

DISCUSSION

Using a synthetic DNA substrate that contains labeled 3'-terminal fragments of deoxyribose, we have identified two distinct 3'-repair diesterases present in cell-free extracts of various mammalian tissues and cultured cells. These activities were physically separable by various procedures and did not cross-react immunologically. Although 3'-diesterase activities of this type act on oxidative damages DNA, the levels and ratios of the two activities were not significantly affected in cell lines selected for growth in high levels of O_2, or in cells from patients with the radiation-sensitive disease ataxia telangiectasia. The two activities were extensively purified from HeLa cells, one to near homogeneity, the other only incompletely purified. The first enzyme was by several criteria identical with the AP endonuclease identified by Kane and Linn (24). Thus, that enzyme was a polypeptide of M_r ~38,000 that exhibited a 100- to 200-fold higher level of hydrolytic (Class II) AP endonuclease than of 3'-PGA diesterase activity and cross-reacted immunologically with the AP endonuclease. Both the AP endonuclease purified by us and that of Kane and Linn (24) had a level of 3'-phosphatase similar to their 3'-PGA diesterase activities (32). The second enzyme has apparently not been explicitly identified in previous work. That activity has a Stoke's radius consistent with a globular protein of M_r 50,000–60,000, and harbors a level of hydrolytic AP endonuclease activity only 7-fold higher than its 3'-PGA diesterase. We have provisionally named this second activity 3'-PGA diesterase.

The work described here represents a fundamental step toward characterizing an important DNA repair pathway in mammalian cells, the excision of oxidatively damaged deoxyribose. As in other organisms, one of the key activities in HeLa cells is also the major cellular AP endonuclease. Various mammalian AP endonucleases in addition to the HeLa enzyme (this work; ref. 24) have been characterized. These include the major activities from human placenta (25), calf thymus (33) and cultured mouse cells (34). All three of these enzymes are similar to the HeLa AP endonuclease: they are proteins of Mr ~28,000–39,000, and act as Mg^{2+}-requiring, hydrolytic AP endonucleases. The placental, calf thymus and mouse enzymes cross-react with anti-HeLa AP endonuclease antibodies (35), and the N-terminal 20 residues of the calf thymus enzyme (33) are nearly identical to the sequence found for the HeLa enzyme (35). The cloned cDNA encoding the bovine AP endonuclease (36) specifies a protein strongly homologous to the human AP endonuclease (35). It is likely that essentially the same enzyme is found widely in mammalian tissues.

The 3'-PGA diesterase identified here may have been detected in other work on AP endonucleases (37, 38). Since these other investigators did not further characterize these 'minor' AP endonucleases, it cannot be ascertained whether the 3'-PGA diesterase we are dealing with here is related to any of those enzymes. Similarly, since the 3'-PGA diesterase described here (Table 2, Fraction 5a) is not yet completely purified, it is uncertain whether all the activities detected in the preparation are due to the same protein.

Multifunctional AP endonuclease/3'-repair diesterase enzymes from *E. coli* and *S. cerevisiae* have been characterized extensively and function in repair of both oxidative and alkylation DNA damages (13, 16, 22, 23). In the microbial cases, 3'-repair diesterases remove 3'-blocking groups that prevent DNA repair synthesis at oxidative strand breaks (13, 16, 23). If true for the

mammalian enzymes, such a function would involve them in cellular resistance to various agents that damage DNA via free radicals. The AP endonuclease activities would also initiate the repair of abasic sites resulting from many reactions (7, 8, 9). Mammalian AP endonuclease/3′-repair diesterases may therefore also participate in multiple DNA repair pathways, a point that must be established via genetic studies.

For all the well-characterized microbial enzymes, the specificities for AP sites and 3′-PGA esters are about equal. This contrasts with the situation in HeLa cells, where one enzyme dominates as an AP endonuclease, but the 3′-repair diesterase function is shared between at least two proteins. The specific activity of the HeLa AP endonuclease for AP sites (Table 2) is ~10-fold higher than found for the microbial enzymes. Despite these differences, the HeLa AP endonuclease is related to a bacterial counterpart, exonuclease III; the structural gene encoding the human enzyme was cloned using as a screen the specific antisera raised against the enzyme isolated in this work (35). The availability of the cDNA for the AP endonuclease gene *APE* provides an essential tool for determining that enzyme's physiological function, for example, by generating antisense expression constructs. Efforts are underway to isolate the cDNA encoding the 3′-PGA diesterase identified here to provide an additional critical tool for dissecting the *in vivo* roles of these proteins.

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