Purification and characterization of a novel human acidic nuclease/intra-cyclobutyl-pyrimidine-dimer-DNA phosphodiesterase

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A novel N-glycosylated, mannose-rich protein has been purified approx. 4000-fold from human liver in a seven-step procedure including ion-exchange chromatography and fractionation on concanavalin A–Sephadex G-75 and oligo(dT)–cellulose matrices. The molecular mass of the protein is 46 kDa when measured by gel filtration (i.e. under non-denaturing conditions) and 60 kDa by SDS/PAGE (i.e. under denaturing conditions). The protein possesses two DNA backbone-incising activities, namely, the random introduction of single-strand breaks in native DNA and the rupture of the phosphodiester linkage internal to cyclobutyl pyrimidine dimers, the major class of DNA lesions induced by solar UV rays. Both activities are optimal at pH 5.0 *in vitro*, although the non-specific nuclease displays appreciable activity at neutral pH, depending on the buffer composition. The protein has been named acidic nuclease/intra-cyclobutyl-pyrimidine-dimer-DNA phosphodiesterase (AN/IDP). As a nuclease, the protein ‘prefers’ a linear DNA structure over a covalently closed circular molecule and is

more proficient at digesting single-stranded than double-stranded DNA. The polynucleotide cleavage products of the nuclease contain 5′-OH and 3′-PO₄ termini, which are refractory to direct rejoining by DNA ligases. Depending on the substrate, the nuclease activity exhibits a temperature optimum of 50 °C or greater, and is neither stimulated by Mg²⁺ or Ca²⁺ nor inhibited by Zn²⁺. AN/IDP is present in human liver and in cultured human cells of both fibroblastic and lymphocytic origins. Intracellularly, the protein can be readily detected in both the cytosolic and nuclear fractions, although much more (approx. 3-fold) is found in the latter fraction. We propose that this bifunctional enzyme may be involved in both apoptotic DNA digestion and metabolism of cyclobutyl pyrimidine dimers in UV-irradiated human cells.

Key words: apoptosis, glycoprotein, nucleotide excision repair, UV damage, DNA metabolism.

INTRODUCTION

The ability to manipulate and modify the genetic material in an exacting and timely fashion is a fundamental endowment of all free-living organisms. The assorted DNA metabolic transactions (e.g. semi-conservative replication, homologous recombination and excision repair) are necessarily complex [1–3], and it is therefore not surprising that mammalian cells, for example, contain a diverse array of nucleases and other DNA-modifying enzymes [1–4]. Recently, a concerted effort has been made to obtain detailed mechanistic insight into a number of DNA metabolic processes, and to characterize the underlying nucleases and associated proteins [4–6]. In some cases considerable progress has been achieved. A case in point is nucleotide excision repair (NER), a universal and versatile error-correction system in which a lesion in DNA is first recognized and then removed within an oligomer, followed by gap-filling repair synthesis and strand ligation so as to restore the intact duplex [6,7]. In the current model of human NER [3,6,7], the early lesion-recognition and -excision steps are mediated by a multiheteromeric protein complex containing at least eight distinct gene products. Included in this complex are two helicases of opposite polarity to expose the damaged site and two endonuclease activities, specific for splayed-arm (Y-shaped) structures to mediate the lesion-flanking

strand incisions. In aggregate, no less than 30 polypeptides representing 12 gene products are required to execute the entire NER process *in vitro* on a ‘naked’ substrate [6,7], and more proteins are surely needed to deal with the added complexities of genomic chromatin [3,7].

By no means is our knowledge of all DNA metabolic transactions in mammalian systems as well-advanced as that of human NER. Indeed for some transactions, like the extensive digestion of genomic DNA that typically accompanies the late, cell fragmentation stage of apoptosis [8,9], our understanding is in its infancy. In this form of cell death, a variety of external stimuli, both physiological (e.g. growth factor withdrawal) and pathological (e.g. antineoplastic drug exposure), trigger a common ‘self-destruction’ pathway, which typically culminates in cleavage of chromatin into discrete, oligonucleosome-sized fragments [10,11]. The identity of the underlying endonucleases has been the subject of intense investigation in recent years. Of the many candidate enzymes, which have been implicated in apoptotic DNA digestion, perhaps the most surprising is a family of nucleases exemplified by DNase II [9,12]. Members of this family display acidic pH optima, and hence may be the nucleases that are activated in those experimental systems in which apoptotic induction is a prelude to intracellular acidification [9,12].

Abbreviations used: AN, acidic nuclease; conA, concanavalin A; DSC, double-stranded, circular; DSL, double-stranded, linear; DSO, double-stranded, open; IDP, intra-cyclobutyl-pyrimidine-dimer-DNA phosphodiesterase; NER, nucleotide excision repair; QMA, quaternary methylamine; XP, xeroderma pigmentosum.

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The impetus for the current work stems from our earlier inquiries into molecular mechanisms which enable cultured human cells to process (i.e. to repair, bypass or otherwise tolerate) cyclobutane dimers, which are formed between adjoining intrastrand pyrimidines in DNA on exposure to solar UV rays. Two of our observations, which were made by monitoring the metabolic fate of this predominant class of deleterious photoproducts in UV-irradiated fibroblasts, are pertinent here. First, when genomic DNA was isolated from post-UV-incubated xeroderma pigmentosum (XP) complementation groups A and D cells, and subjected to cyclobutane ring-splitting photoenzymic treatment [13], single-strand scissions were introduced at a significant incidence [14]. Secondly, when an enzymic hydrolysis/HPLC assay was employed to analyse the chemical composition of photolesions that had accumulated in excised oligonucleotide fragments during incubation of UV-treated normal fibroblasts, the excised cyclobutane dimers were often found to contain a ruptured interpyrimidine phosphodiester bond [15]. Together, these results indicated that: (i) in a fraction of the dimer-containing sites, both those retained in the genomic DNA of NER-defective XP cells and those in the excised oligomers of NER-proficient normal cells, the intradimer phosphodiester linkage is ruptured; and (ii) at such modified sites, the adjoining pyrimidine residues are held together solely by the cyclobutane bridge joining the two bases. By extension, it was surmised that human cells possess an enzyme, termed intra-cyclobutyl-pyrimidine-dimer-DNA phosphodiesterase (IDP), which is capable of catalysing this postulated intradimer backbone-cleavage reaction [14,16].

We report here that cultured cells of both fibroblastic and lymphocytic origins do indeed possess IDP activity. Using human liver as a source, extensive purification of the responsible enzyme – an N-glycosylated, mannose-rich, 46-kDa monomeric protein – is described, and partial characterization of its functional and physical properties is given. Intriguingly, the protein has a second DNA-incising activity, namely, the random introduction of single-strand breaks in native (undamaged) DNA, which, like the IDP activity, is optimal at pH 5.0. Hence the protein has been named acidic nuclease/IDP (AN/IDP). The salient features of both activities conferred by this novel protein, including an acidic pH optimum, lack of stimulation by Ca^{2+} or Mg^{2+} , and the presence of 5'-OH and 3'- PO_4 termini in the polynucleotide reaction products, are reminiscent of those of DNase II [9]. Accordingly, it is tempting to speculate that AN/IDP may be a member of the DNase II family of acidic nucleases with bi-functional involvement in both apoptosis-associated DNA fragmentation and metabolism of UV-induced cyclobutyl pyrimidine dimers.

EXPERIMENTAL

Materials

Reagents

All reagents were of the highest purity commercially available, most being of reagent grade.

Tissues

Human livers were obtained at autopsy from the Department of Pathology, University of Alberta (Edmonton, AB, Canada) and stored frozen in PBS/30% (v/v) glycerol at -80°C prior to use. Fresh bovine liver and spleen were donated by two local slaughterhouses (Gainers Ltd. and Alsask Beef Co., Edmonton, AB, Canada).

Enzyme substrates

[^3H]dT-labelled poly(dA)·poly(dT) (140 μM nucleotides; 127 d.p.m./pmol of dT) was prepared as described [13]. The average length of the co-polymer was approx. 350 bp. When UV irradiated, the co-polymer was diluted 2.5 times in distilled water and exposed to 2 kJ/m² of germicidal light (far UV, chiefly 254 nm) emitted at a fluence rate of 88 mW/m². The irradiated co-polymer was kept at 4 $^\circ\text{C}$. Plasmid (pBR322 and M13) DNA (Pharmacia Biotech Inc., Baie d'Urfe, QC, Canada) containing apurinic sites was prepared as described by Tomkinson et al. [17]. Subsequent analysis with T4 UV endonuclease revealed approx. 1.5 apurinic sites/plasmid. Linear forms of double-stranded pBR322 and single-stranded M13 plasmids were obtained by digestion with *Eco*RI (Pharmacia Biotech Inc.). High-molecular-mass native calf thymus DNA (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) was also employed.

Antibodies

Bovine liver AN/IDP protein (100 μg), purified as described below for its human counterpart, was mixed with RIBI adjuvant according to the manufacturer's suggestions (Cederline Laboratories, Hornby, ON, Canada). The resulting suspension was injected subcutaneously at five sites in the back and shoulder areas of white female New Zealand rabbits. Two booster injections, containing the same amount of protein and adjuvant, were administered after three and five weeks. One week after the second booster injection, blood was collected and the serum fraction containing anti-AN/IDP antibodies was isolated.

Cells and culture conditions

Normal human fibroblasts (strain CRL 1141; Institute for Medical Research, Camden, NJ, U.S.A.) were grown in Ham's F12 medium, supplemented with 10% (v/v) fetal calf serum, 100 units/ml of penicillin G and 100 $\mu\text{g}/\text{ml}$ of streptomycin sulphate. Human Epstein-Barr virus ('EBV')-immortalized lymphoblastoid cell lines GM 3714 and GM 1526C (American Type Culture Collection, Rockville, MD, U.S.A.) were propagated in RPMI 1640 medium supplemented with 15% (v/v) fetal calf serum, 100 units/ml of penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 1% glutamine until each culture reached confluence. All cultures were incubated at 37 $^\circ\text{C}$ in a humidified atmosphere of air/ CO_2 (19:1). All cell culture supplies were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.).

Methods

Preparation of human fibroblast extracts

CRL 1141 fibroblasts were harvested by trypsinization, suspended in ice-cold PBS, and collected by centrifugation. Cell pellets (8×10^7 cells) were taken up in 1–1.5 ml extraction buffer [10 mM Tris/HCl (pH 8.0), 0.32 M sucrose, 1 mM MgCl_2 , 1 mM β -mercaptoethanol, 1 mM PMSF and 0.5% Triton X-100], placed on ice for 30 min, and cleared by centrifugation (5000 g, 15 min) to obtain cell-free extracts, each containing 10–15 mg of protein.

Separation of nuclear and cytosolic fractions of cell lines

Lymphoblastoid cells were lysed in hypotonic buffer, whereupon nuclear and cytosolic fractions were separated by centrifugation [18]. Nuclei were incubated for 30 min in a high-salt buffer (0.3 M KCl, 10 mM Hepes/KOH, pH 7.4, 1 mM MgCl_2 , 1 mM dithiothreitol and 0.5 mM PMSF) and soluble proteins were recovered by centrifugation (10000 g, 10 min).

Isolation of nuclei from bovine liver and spleen

Each tissue was cut into small pieces and subjected to Dounce homogenization in a buffer containing 10 mM Tris/HCl, pH 7.4, 0.32 M sucrose, 3 mM MgCl₂ and 0.1 mM PMSF. After low speed centrifugation (1000 g, 10 min), the pellet was resuspended in 0.25 M sucrose containing 10 mM Tris/HCl, pH 7.4, 3 mM MgCl₂ and 0.1 mM PMSF, filtered through cheese cloth, and recentrifuged. The second pellet was then taken up in 2.2 M sucrose containing 10 mM Tris/HCl, pH 7.4 and 1 mM MgCl₂. This suspension was centrifuged (105000 g, 1 h), after which the pelleted nuclei were washed gently, resuspended and stored in a buffer containing 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 3 mM MgCl₂ and 0.1 mM PMSF.

Assay of IDP activity

The reaction mixture (total volume, 50 µl) contained 50 mM sodium acetate, pH 5.0, 0.2 mM MgCl₂, 1 mM β-mercaptoethanol, 6.8 µM UV-treated [³H]dT-labelled poly(dA)·poly(dT) co-polymer (127 d.p.m./pmol dT; equivalent to 5.6 pmol cyclobutyl pyrimidine dimers) and one of the following: 2 µg of protein from the tissue extract, whole cell extract, nuclear fraction, cytosolic fraction or each of fractions 1–4 inclusive (see purification of AN/IDP below), or 0.05 µg of each of fractions 5–7 inclusive. Reactions were carried out for 15 min at 37 °C, and terminated by the addition of 2.5 µl 1 M Tris/HCl followed by boiling for 3 min. Neutralized samples were then subjected to an enzymic digestion/HPLC assay [15] to distinguish intact from modified dimers. The following procedure was used: 5 units of DNase I, 25 units of staphylococcal nuclease, 5 units of calf alkaline phosphatase (Boehringer Mannheim, Dorval, QC, Canada) and 2.5 m-units snake venom phosphodiesterase (Sigma–Aldrich Canada Ltd.) were added to the neutralized samples and the reaction mixture was supplemented with 20 mM Tris/HCl, pH 7.6, 80 mM NaCl, 10 mM MgCl₂ and 4 mM CaCl₂ (total volume, 100 µl). After overnight incubation at 37 °C, the reaction products were analysed by HPLC (Waters 840 system; Waters Associates, Mississauga, ON, Canada) using a Whatman C₁₈ Partisil octadecylsilane ('ODS')-2 column (4.6 mm × 250 mm; Whatman Inc., Clifton, NJ, U.S.A.). To confirm the identification of the reaction products, 1 nmol authentic marker d-TpT'dT and 0.5 nmol unlabelled dT were added to each digested co-polymer sample prior to its injection on to the column. The column was pre-equilibrated with 50 mM sodium phosphate, pH 4.5 (Buffer I), and intact and modified dimer-containing trinucleotides were separated by the following elution scheme: Buffer I for 1 min; linear gradient, 0–80%, of Buffer II [50 mM sodium phosphate, pH 4.5, in 50% (v/v) methanol] in Buffer I for 30 min; and Buffer II for 5 min. Each elution buffer was run at a flow rate of 1 ml/min, and one-half ml fractions were collected and analysed for radioactivity. Intact dimers (d-TpT'dT) were eluted in 19.5 min, modified dimers (d-TpT > dT) in 27 min, and dT in 21.5 min. The radioactivity associated with each product was then expressed as the percentage of the total counts eluting from the column. These values were used to calculate the incidence of modified dimers and hence the number of intradimer backbone bonds cleaved. One unit of IDP activity was defined as the amount of protein which hydrolyses 1 pmol of intradimer phosphodiester linkages in 1 min at 37 °C under standard reaction conditions.

Assay of AN activity

This activity, which was first discovered when searching for a rapid and simple method to replace the enzymic digestion/HPLC

assay for detection of IDP activity (see the Results section), was monitored by measuring the release of trichloroacetic acid-soluble radioactive material from the poly(dA)·poly(dT) co-polymer. The reaction mixture was the same as that described above for IDP activity. After 15 min of incubation at 37 °C, the reaction was terminated by the addition of 90 µl of 20% (w/w) trichloroacetic acid followed by 100 µl of BSA (1 mg/ml). The precipitate, which formed during 10 min of incubation on ice, was separated by centrifugation (10000 g, 15 min), and a 0.15-ml sample of the supernatant (containing acid-soluble material) was analysed for radioactivity. Blank (no enzyme) samples typically contained less than 1% of the input radioactivity in the reaction mixture. One unit of AN activity was defined as the amount of enzyme which converts 10% of the substrate (i.e. 0.68 µM co-polymer) into acid-soluble oligonucleotides in 1 min at 37 °C under standard reaction conditions. In certain experiments pBR322 and M13 plasmids were selected as substrates for IDP activity studies, in which case the digestion products were separated by conventional 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Purification of AN/IDP from human liver

Unless stated otherwise, all operations were performed at 4 °C.

Step I: tissue extraction. Thawed human liver (100 g) was cut into small pieces and placed in two volumes (w/v) of Buffer A [20 mM Tris/HCl, pH 7.0, 20 mM NaCl, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 1 mM PMSF and 1 µg/ml of leupeptin (Sigma; St. Louis, MO, U.S.A.)] supplemented with 0.25 M sucrose. The suspended tissue was subjected to 5 × 15-s bursts in a Sorvall Omni Mixer Homogenizer (DuPont Canada, Markham, ON, Canada) operating at setting 5. Triton X-100 (0.1%) was added to the homogenate, whereupon the suspension was magnetically stirred for 30 min and cleared of tissue debris by centrifugation (10000 g, 15 min). The supernatant is henceforth referred to as Fraction 1 (whole tissue extract, 250 ml).

Step II: acetone precipitation. An equal volume of acetone, held at –20 °C, was added to Fraction 1 over 30 min with gentle stirring. After stirring for an additional 15 min, the precipitate was collected by centrifugation (10000 g, 15 min) and resuspended in Buffer A. The suspension was cleared of undissolved particular matter by centrifugation (10000 g, 15 min) to obtain Fraction 2 (acetone precipitate, 100 ml).

Step III: quaternary methylamine (QMA)-accel chromatography. A QMA-accel column (1.6 cm × 40 cm, 80-ml bed volume; Waters Associates) was equilibrated with Buffer A, and Fraction 2 was applied to the column at a rate of 2 ml/min. Since the active material did not bind to the column, the flow-through fractions (200 ml total) were collected, precipitated with acetone as described above, and resuspended in Buffer B (25 mM Mes/NaOH, pH 6.0, 50 mM NaCl, 1 mM MgCl₂ and 1 mM PMSF) to provide Fraction 3 (QMA-accel, 50 ml).

Step IV: CM-accel chromatography. Fraction 3 was loaded, at the rate of 1 ml/min, on to a CM-accel column (1.6 cm × 20 cm, 40-ml bed volume; Waters), which had been pre-equilibrated with Buffer B. The bound protein was washed with three bed vol. of the column buffer, and a 200-ml linear gradient, 50–500 mM NaCl, was applied in the same buffer. Fifty 4-ml fractions were collected at 1 ml/min. Sixteen active fractions (peak activity eluted between 250–300 mM NaCl) were pooled, the pH was adjusted to 7.5, and the concentration of NaCl was increased to 0.5 M. This pooled material constituted Fraction 4 (CM-accel, 64 ml).

Step V: Concanavalin A (conA)–Sephacrose chromatography. Upon equilibrating a conA–Sephacrose column (0.9 cm × 10 cm,

12-ml bed volume; Pharmacia Biotech Inc.) with Buffer C (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1 mM $MnCl_2$). Fraction 4 was applied to the column at the rate of 0.5 ml/min. Under these loading conditions, the active material binds to the column. Following a 50-ml wash with Buffer C, retained proteins were eluted with a 50-ml linear gradient of 0–1 M mannose in Buffer C, followed by 50 ml of 1 M mannose in the same buffer. The latter (25, 2-ml fractions) were collected at 0.5 ml/min. Active material, which eluted in fractions 5–25 of the mannose-containing buffer, was combined and concentrated to 2 ml in an Amicon ultrafiltration unit with a PM-10 membrane (Amicon, Oakville, ON, Canada). This concentrated material is Fraction 5 (conA-Sepharose, 2 ml).

Step VI: Sephadex G-75 chromatograph. Fraction 5 was applied to a Sephadex G-75 gel filtration column (1.6 cm $\times$ 60 cm; Pharmacia), which had been pre-equilibrated with Buffer D (10 mM Tris/HCl, pH 7.4, 300 mM NaCl, 1 mM $MgCl_2$ and 1 mM β -mercaptoethanol) and the column was developed in the same buffer. Two-ml fractions were collected at the rate of 0.5 ml/min. To determine the molecular mass of the native form of AN/IDP, the column was calibrated with independent runs of the following markers (Pharmacia Biotech Inc.): Blue Dextran (2000 kDa), phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa). The native protein, which eluted in six fractions immediately before the ovalbumin peak, was concentrated using a Centricon-30 unit (Amicon). The concentrated material (3 ml) was dialysed overnight, with one change of Buffer E [50 mM sodium acetate, pH 5.2, 5% (v/v) glycerol and 1 mM β -mercaptoethanol] to obtain Fraction 6 (Sephadex G-75, 4 ml).

Step VII: oligo(dT)-cellulose chromatography. Fraction 6 was loaded, by gravity, on to an oligo(dT)-cellulose type 3 column (0.6-ml bed volume; Collaborative Biomedical Products, Bedford, MA, U.S.A.) which had been pre-equilibrated with Buffer E. The affinity column was washed twice, first with 12 ml of Buffer E containing 50 mM NaCl, and next with 6 ml of Buffer E supplemented with 200 mM NaCl. The active material was then eluted in 3 ml of Buffer D and dialysed for 2 h against 2 l of Buffer F [50 mM sodium acetate, pH 5.2, 20% (v/v) glycerol and 10 mM β -mercaptoethanol] to give Fraction 7 [oligo(dT)-cellulose, 2.5 ml]. This fraction was split into 100- μ l aliquots which were either used immediately or stored at -20°C .

Purification of DNase II from bovine spleen

A commercial preparation of this enzyme (Sigma-Aldrich Canada Ltd.) was dissolved in Buffer C and applied to a conA-Sepharose column (see Step V above). Active material was eluted with Buffer C containing 1 M mannose and collected fractions were concentrated, as described in Step V.

Digestion of AN/IDP with N-glycosidase F (EC 3.5.1.52)

Fraction 6 (Sephadex G-75) was desalted on a Bio-Gel P-6 column (6-ml bed volume; Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and 50 μ l of the purified enzyme, containing approximately 1.5 μ g of protein, were mixed with 1 μ l of 3 $\times$ concentrated Laemmli sample buffer [19] and 0.06 unit of N-glycosidase F (Boehringer Mannheim). The reaction was carried out overnight at 37°C and the products were analysed by SDS/PAGE.

Enzymic labelling of AN/IDP-generated strand termini

Desalted Fraction 6 preparation (0.03 μ g) was incubated with 1 μ g of pBR322 DNA for 10 min under conditions as described

above for the IDP assay, except that the reaction volume was reduced to 20 μ l. After neutralization and enzyme denaturation, samples were treated with either T4 polynucleotide kinase (Pharmacia Biotech Inc.) in the presence of [γ - ^{32}P]ATP (3.7×10^{13} Bq/mmol; Amersham Canada Ltd., Oakville, ON, Canada) or terminal deoxynucleotidyl transferase (Pharmacia Biotech Inc.) in the presence of [α - ^{32}P]CTP (3.7×10^{13} Bq/mmol; Amersham Canada Ltd.) according to the manufacturer's recommendations. The plasmid DNA was then precipitated by ethanol and analysed by standard agarose gel electrophoresis and autoradiography.

SDS/PAGE and Western blotting

SDS/PAGE, 10% (w/v) acrylamide, was performed according to Laemmli [19] and Western blotting as described by Towbin et al. [20]. After electrophoretic separation, proteins were transferred (1 h at 60 mA) to Hybond C nitrocellulose membrane (Amersham Canada Ltd.), using a semi-dry system (Gelman Sciences, Ann Arbor, MI, U.S.A.) and Towbin buffer [20]. Immunocomplexes were formed during 1 or 3 h in the presence of rabbit anti-AN/IDP serum (500–1000-fold diluted) and detected with goat anti-rabbit IgG coupled to horseradish peroxidase (1000-fold diluted), employing a colour development kit (Bio-Rad Laboratories Ltd.).

Protein determination

Protein content was determined throughout using Bradford reagent (Bio-Rad Laboratories Ltd.).

RESULTS

Detection of IDP activity in human cell-free extracts

To maximize the possibility of observing the putative IDP activity in protein extracts of human cells, careful consideration was given to the selection of both the enzyme substrate (i.e. DNA containing pyrimidine dimers) and the assay for detecting the occurrence of the intradimer backbone-nicking reaction. [^3H]dT-labelled poly(dA)·poly(dT) was chosen as the substrate, since only one type of dimer (i.e. Thy–Thy homodimer) is introduced and its formation is induced efficiently upon exposure of the co-polymer to UV rays. In selecting the enzymic digestion/HPLC method [15] for monitoring IDP activity, particular attention was paid to its unique ability to fulfil the following two requirements: (i) the capacity to detect the novel reaction, that is, to distinguish a pyrimidine dimer with a severed, as opposed to an intact, internal phosphodiester linkage; and (ii) the capability to do so with great sensitivity, that is, to detect IDP activity in the human cell-free extract independent of the level of nonspecific nucleases active on the vast excess of undamaged, in comparison to damaged, residues in the UV-treated substrate. Accordingly, the radioactive co-polymer was UV-irradiated and incubated with a cell-free extract of normal human (CRL 1141) fibroblasts containing the postulated intradimer-incising activity, after which the sample was heated (100°C for 3 min) to inactivate all proteins. Two incubation mixtures, one lacking the protein extract and the other containing a heat-denatured extract, served as controls. The polynucleotide chains were then digested with a cocktail of enzymes to generate mononucleotides, and two nuclease-resistant trithymidylate species containing a cyclobutane dimer at the 3'-terminus, one with an intact (d-TpT'dT) and, assuming the presence of IDP in the cell-free extract, the other with a ruptured internal phosphodiester linkage (d-TpT > dT). Finally, the digestion products were resolved by reverse-phase HPLC and quantified. The resulting HPLC-

Figure 1 IDP activity in cell-free extracts of cultured human fibroblasts as detected by an enzymic digestion/HPLC assay [15]

UV-irradiated poly(dA)·poly(dT) co-polymer served as a substrate. (A) negative control (no protein present). (B) incubation with cell-free extract (2 µg protein). (C) incubation with heat-denatured cell-free extract (2 µg inactivated protein). With the exception of dT (fractions 42–45), the retention times of the authentic digestion product markers are indicated by arrows: thymine glycol (dTG), trithymidylate containing an intact cyclobutyl pyrimidine dimer (d-TpT<pp>T), and trithymidylate containing a modified dimer (d-TpT< > dT). See the Experimental section for experimental details.

generated radioactivity profiles of the reaction products are illustrated in Figure 1. As expected, omission of the crude cellular extract from the reaction mixture yields a profile in which a significant portion [11.3%; see Figure 1(A), fractions 36–40] of the radioactivity elutes in the position of the intact dimer-containing trimer. This result indicates that the UV treatment (2 kJ/m²) introduces, on average, one cyclobutane dimer every 26 thymine residues. This incidence is in good agreement with earlier data [21]. Also consistent with the previous data is the presence (in fractions 31–34) of a self-irradiation product of ³H-labelled dT, presumably a thymine glycol species [22–24], which constitutes 0.9% of the total counts. Of further interest, it appears that approx. 1% of the radiolabel resides in fractions 52–55, which corresponds to the expected position of the modified dimer-containing trimer d-TpT > dT [21]. This background level is also seen in unirradiated co-polymer samples (results not shown), and does not represent d-TpT > dT molecules since intradimer phosphodiester linkages are refractory to the enzyme digestion cocktail, including snake venom phosphodiesterase [21]. Interestingly, incubation of the UV-damaged substrate with an active cellular extract at pH 5.0 results in a shift of 5% of the total radiolabel from the retention time of the d-TpT<pp>T species to that of the d-TpT< > dT species (Figure 1B), implying that under these experimental conditions approx. 40% of the dimers contain a ruptured internal phosphodiester bond. By contrast, no modified dimer-containing trithymidylate molecules are detected when the cellular extract is heat-denatured prior to incubation with the UV-treated co-polymer (Figure 1C), indicating that the responsible factor is heat-labile and hence likely proteinaceous in nature. In sharp contrast with the results obtained when the reaction is performed at pH 5.0, little if any IDP activity is detected when the incubation is conducted at pH 8.0 (results not shown). In short, these combined data provide direct evidence that human fibroblasts do indeed contain an IDP activity with an acidic pH optimum.

Co-purification of IDP and AN activities in a single protein

Using human liver as a source, we next set out to purify the protein(s) responsible for the IDP activity detected in whole-cell

Table 1 Purification of AN/IDP from human liver

Details of the purification scheme are given in the Experimental section.

Step	Fraction	Total protein (mg)*	Intradimer phosphodiesterase				Acid nuclease			
			Total activity (units)†	Specific activity (units/mg)	Yield (%)	Purification (fold)§	Total activity (units)‡	Specific activity (units/mg)	Yield (%)	Purification (fold)
1	Tissue extract	2413	55 499	23	100	1	468 122	194	100	1
2	Acetone precipitate	923	50 765	55	91	2.4	396 890	430	85	2.2
3	QMA-accel	812	53 592	66	97	1.2 (2.9)	426 300	525	91	1.2 (2.7)
4	CM-accel	64.3	28 350	441	51	6.6 (19.0)	218 250	2910	47	5.6 (15)
5	ConA-Sepharose	0.456	10 597	23 240	19	53 (1010)	92 066	20 190	20	69 (1041)
6	Sephadex G-75	0.192	8762	45 635	16	2.0 (1984)	91 440	476 250	20	2.4 (2455)
7	Oligo(dT)-cellulose	0.100	8324	83 324	15	1.8 (3623)	86 868	868 680	19	1.8 (4478)

* Protein concentration determined by Bio-Rad protein assay kit using BSA as a standard.

† One unit defined as amount of protein which hydrolyses 1 pmol of intradimer phosphodiester linkages in 1 min at 37 °C.

‡ One unit defined as amount of protein which converts 10% of the substrate (i.e. 0.68 µM co-polymer) to acid-soluble oligonucleotides in 1 min at 37 °C.

§ The first number is the fold purification for each step; the number in parentheses is the cumulative fold purification.

Figure 2 ConA-Sepharose chromatographic elution profiles

IDP activity (○), as measured by incidence of modified dimers, and AN activity (●), as measured by amount of trichloroacetic acid (TCA)-soluble material released, were assayed as described in the Experimental section. Protein (■) was assayed using Bradford reagent.

extracts of cultured human fibroblasts. The purification scheme entailed seven steps. Briefly, after tissue extraction and acetone precipitation to isolate soluble proteins, the vast majority of the activity was recovered in the flow-through fraction from an anionic exchange (QMA-accell) column. The recovered material was then chromatographed, first upon a cationic exchange (CM-accell) column, eluting between 250–300 mM NaCl, and subsequently on an affinity (conA-Sepharose) column, eluting in 1.0 M mannose. Finally, the protein conferring IDP activity was subjected to gel filtration (Sephadex G-75) and affinity [oligo(dT)-cellulose] chromatographies. The results of a typical purification are summarized in Table 1. In most individual steps the degree of IDP purification is modest (approx. 1.2 to 6.6-fold), the notable exception being conA-Sepharose chromatography, which yields a dramatic increase (53-fold) in the purity of the protein. This increase is illustrated in the conA-Sepharose elution profile shown in Figure 2. Clearly, the active material (fractions 15–22) remains bound to the column and can be eluted with 1 M mannose, whereas the bulk protein (fractions 3–9) does not interact with conA. Also noteworthy is the final purification step in which the purified protein is absorbed on an oligo(dT)-cellulose column. The subsequent two washes in Buffer E, supplemented first with 50 mM NaCl and then with 200 mM NaCl, serve to remove minor contaminants bound to the column. The second wash is particularly effective in removing bound DNase II but not AN/IDP, as revealed by both zymography and Western blotting (results not shown). In summary, from 100 g of human liver, 0.1 mg of essentially pure protein is typically recovered with a 15% yield relative to the tissue extract. The final preparation [Fraction 7; oligo(dT)-cellulose] had a specific activity of approx. 85000 units/mg, representing an approx. 4000-fold purification from the starting material. The molecular mass of the protein in the native state was 46 kDa, as determined by gel filtration.

As alluded to earlier, it was fortuitously discovered in the course of purification of IDP that the responsible protein also exhibits a non-specific AN activity, as observed by digestion of the undamaged poly(dA)·poly(dT) co-polymer into trichloroacetic acid-soluble oligomers. It is evident in Figure 2 that the IDP and AN activities co-elute from the conA-Sepharose column. In fact, the two activities are inseparable throughout the entire purification scheme. This, in combination with almost identical activities, percent yields and purification factors in each of the seven steps (Table 1), provides overwhelming evidence that the

Figure 3 pH dependence (A) and temperature dependence (B) of AN and IDP activities residing in the bifunctional protein purified from human liver

UV-irradiated poly(dA)·poly(dT) was used as a substrate. Samples of the reaction products were analysed by the enzymic digestion/HPLC and trichloroacetic acid solubility assays when measuring IDP (○) and AN (●) activities, respectively. The pH of the reaction mixtures was maintained by employing either sodium acetate buffer (pH 4–6.5) or Tris buffer (pH 7–9). Details are given in the Experimental section.

two activities reside in one and the same protein, and hence the dual designation AN/IDP. The pure protein (Fraction 7), which is routinely obtained in a completely active state after a 5-day purification procedure, can be stored in Buffer F at -20°C for at least one month without any loss of either activity.

Characterization of the catalytic properties of AN/IDP

pH dependency

One distinct feature of the purified protein (Fraction 7) is illustrated in Figure 3(A). When UV-irradiated poly(dA)·poly(dT) is used as a substrate, the AN and IDP activities exhibit superimposable pH-dependency profiles, each having an optimal pH of 5 and declining sharply to approx. 5% maximal activity at pH 6 and to approx. 50% maximal activity at pH 4. Neither activity was detectable at pH 7 or above.

The influence of pH on the AN activity of the protein was examined further by employing, as a substrate, closed circular DNA of both double-stranded (plasmid pBR322) and single-stranded (plasmid M13) configurations. When using the former plasmid as a substrate, the endonucleolytic activity of a given protein is indicated by the sequential conversion of: form I [double-stranded, circular (DSC) DNA] to form II [double-stranded, open (DSO) DNA], form II to form III [double-stranded,

Figure 4 pH dependence of AN activity toward double-stranded (A) and single-stranded plasmid DNA (B) as revealed by analysing the plasmid digestion products on 1% agarose gels

Reactions were carried out for 10 min in standard citrate/phosphate buffer containing 1 mM β -mercaptoethanol and 10 ng of purified AN/IDP protein (Fraction 7). (A) DSO, DSL and DSC indicate, respectively, open circular, linear and covalently closed configurations of the double-stranded plasmid pBR322. (B) SSL and SSC indicate, respectively, linear and closed configurations of the single-stranded plasmid M13. Lane 1, control (untreated plasmid) at pH 5.2; lanes 2–10, reactions performed at different pH values, ranging from 3.0 to 7.0.

linear (DSL) DNA] and form III to degraded linear fragments of decreasing sizes. Once form III plasmid is generated, subsequent digestion products may also reflect the action of an accompanying putative exonuclease activity [9]. Using the plasmid assay, we were able to show that the AN activity has a broad pH dependency (Figure 4A). Its activity, which reaches a maximum between pH 4.6 and 5.0, remains robust down to pH 3.6 and up to 5.6. Even at pH values as low as 3.0 and as high as 6.6, the nuclease is active to a limited extent, as evidenced by the conversion of pBR322 plasmid from form I to form II. A similar pH-activity relationship is observed when single-stranded M13 plasmid is utilized as a substrate (Figure 4B), with optimal activity occurring at pH 4.6 (see lane 5). What is more, in this case, the nuclease exhibits detectable activity up to and including pH 7.0.

Effect of buffer composition on AN activity at neutral pH

The last observation led us to study AN activity at neutral pH in greater detail. To this end, pBR322 was incubated with a 10-fold

Figure 5 Effect of different buffer compositions on the AN activity of the purified protein toward plasmid pBR322 at pH 7.0

Con, control (no protein in citrate/phosphate buffer); CP, citrate/phosphate buffer. DSO, DSL and DSC indicate, respectively, open circular, linear and covalently closed forms of the double-stranded DNA macromolecules. All buffers were at 100 mM and contained 1 mM β -mercaptoethanol. Ten ng of purified protein were used for the assay.

higher concentration of Fraction 7 and the reaction mixture was maintained in different buffers. Under these conditions, the endonucleolytic action of the protein can be detected in citrate/phosphate, the buffer used in previous reactions (see Figure 5). This buffer, however, is the least effective of the five tested, as the digestion of the plasmid is limited to the conversion of a fraction of form I into form II DNA molecules. In contrast, three buffers, namely Bes, Bicine and Hepes, all support the nucleolytic generation of many degraded linear fragments. The fifth buffer, Tris, is even more effective, permitting the AN activity to digest the covalently closed, double-stranded plasmid to relatively short linear pieces. A similar buffer dependency is observed when the single-stranded circular M13 plasmid is employed as a substrate (results not shown).

Temperature dependency

An analysis of the effect of temperature on IDP activity towards UV-treated poly(dA)·poly(dT) yields a bell-shaped curve, with 35 °C being the optimal temperature and 25 °C and 60 °C as the temperatures at which the activity is reduced to half (Figure 3B). The same analysis for the AN activity produces a curve which is narrower and displaced towards higher temperatures with an optimum of 50 °C (Figure 3B). The ability of AN to function at high temperatures is even more pronounced when pBR322 plasmid is selected as a substrate. Indeed, the extent of plasmid digestion by AN increases as a direct function of temperature, such that maximal activity occurs at 60 °C, the highest temperature examined (results not shown).

Cofactor requirements

The presence of divalent cations (i.e. Mg^{2+} , Mn^{2+} and Ca^{2+}), either alone or in paired combinations, in the concentration range of 0.1–5.0 mM, has little or no effect on the AN activity of the purified protein, at either acidic (pH 5.0) or neutral pH. The

Figure 6 Influence of DNA structure on AN activity of the purified protein, using double- and single-stranded plasmids in closed and open configurations

Reactions were carried out for 10 min in citrate/phosphate buffer at pH 5.2. Plasmid row: DS and SS, double-stranded (pBR322) and single-stranded plasmid (M13), respectively. Form row: C and L, closed and open plasmid forms, respectively. Enzyme row: C, 5, 2 and 1 denote 0 (control), 5, 2 and 1 ng of the purified protein in the reaction mixture, respectively. M (lane 13), molecular size markers in indicated kbp units.

same holds true for IDP at acidic pH. However, Mg^{2+} , when added to the reaction mixture in millimolar concentrations, is able to restore partially AN activity in protein preparations which have been stored for longer than one month. This latter observation implies that Mg^{2+} may be initially bound to the purified enzyme and hence be required for optimal endonucleolytic cleavage by AN. Also, the addition of β -mercaptoethanol (1–5 mM) appears to enhance both AN and IDP activities at least two-fold, suggesting that the availability of SH groups is stimulatory for both activities. Lastly, AN activity is refractory to zinc ions (results not shown).

Mechanism of DNA digestion

To gain insight into the mode of endonucleolytic attack by AN, double-stranded, covalently closed circular pBR322 plasmid was incubated with increasing concentrations of Fraction 7 [oligo(dT)-cellulose] and the DNA digestion products were resolved by agarose gel electrophoresis (Figure 6, left hand panel). Note in lane 1 (control, no protein present) that the vast majority (> 80%) of the substrate is in the form I (DSC) configuration, with the remainder in form II (DSO). In the presence of the lowest concentration (1 ng) of protein, almost all of form I plasmid is converted into form II, whereas less than 5% of the substrate is converted into form III (DSL) (compare lanes 1 and 4). Hence AN acts as a single-strand endonuclease in the presence of closed duplex DNA. A two-fold increase in protein concentration results in the remaining form I plasmid (< 5%) being converted into nicked, circular (form II) molecules (lane 3) whereas a five-fold increase produces linear (form III) molecules as the predominant species, accompanied by the appearance of degraded linear fragments (lane 2). It is presumed that both the linearization of form II plasmid and the progressive degradation of form III plasmid primarily arises from the occurrence of juxtaposed single-strand nicks in opposite DNA strands, and not from a low incidence of double-strand breaks. Interestingly, no change is observed in the digestion pattern of pBR322 plasmid when the plasmid contains a limited number of cyclobutyl pyrimidine dimers or apurinic sites (results not shown).

Figure 7 Enzymic phosphorylation of the strand termini of plasmid pBR322 DNA following digestion with purified human liver AN/IDP or spleen DNase II

The autoradiogram depicts the ^{32}P -end-labelled products arising from incubation of the AN/IDP- or DNase II-digested DNA with either terminal deoxynucleotidyl transferase (TdT) in the presence of $[\alpha\text{-}^{32}P]\text{CTP}$ (lanes 2 and 3) or T4 polynucleotide kinase (PNK) in the presence of $[\gamma\text{-}^{32}P]\text{ATP}$ (lanes 4 and 5). Lane 1, control (no protein nor end-labelling treatment).

To characterize further the hydrolytic properties of AN/IDP, pBR322 plasmid was incubated with purified protein; the digested DNA material was then incubated with either T4 polynucleotide kinase and $[\alpha\text{-}^{32}P]\text{CTP}$ or terminal deoxynucleotidyl transferase and $[\alpha\text{-}^{32}P]\text{CTP}$, and the radiolabelled phosphorylation products were separated by gel electrophoresis and visualized by autoradiography. In a parallel (control) experiment, AN/IDP was replaced with DNase II, which generates DNA cleavage products containing 5'-OH and 3'- PO_4 termini. As is evident in Figure 7, form I plasmid DNA, upon digestion with AN/IDP, serves as a substrate for phosphorylation by polynucleotide kinase (lane 4) but not by terminal deoxynucleotidyl transferase (lane 2), implying that AN/IDP catalyses the introduction of 5'-OH and 3'- PO_4 termini at incised sites in polynucleotides. As expected, similar results are obtained when the DNA digestion products of DNase II are radiophosphorylated and analysed (lanes 3 and 5).

Substrate specificity

To ascertain the influence of DNA structure on AN activity, form I pBR322 plasmid was digested with a restriction enzyme, and the linear duplex molecules were used as a substrate. Comparison of lanes 2–4 with lanes 6–8 in Figure 6 reveals that AN is more active towards linear (form III) plasmid than closed (form I) plasmid. As a case in point, incubation with 5 ng of protein (lane 6) reduces the size of the form III molecules from approx. 4 kbp to 1–2 kbp, which is equivalent to, on average, 1–2 double-strand breaks per molecule. When the parental form I molecules are incubated under the same conditions (lane 2), the major reaction products are form II and form III molecules, implying that, on average, less than one double-strand break is introduced per molecule. Preference for the linear compared to the closed polynucleotide structure is even more pronounced when the single-stranded M13 plasmid serves as a substrate (compare lanes 10 and 12 in Figure 6). This demonstrable preference for linear as opposed to closed DNA structures implies that the degradative properties of AN may be more complex than that of a single-strand endonuclease.

Figure 8 Structural characterization of purified human (and bovine) liver AN/IDP protein as revealed by SDS/PAGE and Western blot analyses

See the Experimental section for details. Lane M, molecular-mass standards, in indicated kDa units. (A) SDS/PAGE of purified bovine liver protein. Lanes 1 and 2, 2 and 5 μg of protein, respectively. (B) SDS/PAGE of purified human liver protein. Lane 1, 1 μg of untreated protein; lane 2, 1 μg of protein digested with N-glycosidase F. (C) Western blot of bovine and human liver AN/IDP. Lanes 1 and 2, 0.4 μg and 0.2 μg of bovine protein, respectively; lane 3, 0.2 μg of human protein. The blot was incubated for 1 h with rabbit anti-AN/IDP serum diluted 1000-fold. (D) Western blot of nuclear extracts of bovine liver and spleen tissues. Lane 1, 0.4 μg of purified bovine liver AN/IDP protein; lanes 2 and 3, 30 μg of bovine liver nuclear proteins obtained by extraction with 0.3% Triton X-100 and with 0.3% Triton X-100 followed by resuspension of the nuclear pellet in 0.3 M KCl, respectively; lane 4, 30 μg of bovine spleen nuclear proteins extracted with Triton X-100; and lane 5, 1 μg of purified bovine spleen DNase II. The blot was incubated for 3 h with rabbit anti-AN/IDP serum diluted 500-fold.

Characterization of the physical properties of AN/IDP

Purified preparations of AN/IDP, of both human and bovine liver origins, were analysed under denatured conditions by SDS/PAGE. The molecular mass of the two proteins is calculated to be 60.4 and 59 kDa, respectively (Figures 8A and B). The discrepancy between the molecular mass of the human protein when measured by gel filtration (46 kDa) and SDS/PAGE (60.4 kDa) may be attributable to peculiar electrostatic properties of the protein which leads to its partial unfolding and resultant unusual shape in the presence of SDS. Consistent with this notion is the observation that the electrophoretic migration of both human and bovine proteins is affected by the ratio of acrylamide to bis-acrylamide present in the polymerized gels. The apparent kDa values given above are calculated using gels with an acrylamide to bis-acrylamide ratio of 30:0.8. At a ratio of 29:1.0, for example, the apparent molecular mass of the human protein is 56 kDa. The difference in the calculated size of the latter protein under native and denaturing conditions may also be ascribable to post-translational modification. In keeping with the latter possibility, the mobility of the human and bovine

AN/IDP proteins on SDS/PAGE gels increases after digestion with N-glycosidase F, corresponding to a decreased mass of 57.5 kDa for the human protein (Figure 8B) and 57.2 kDa for its bovine counterpart (results not shown).

Finally, to provide additional evidence that the human AN activity is indeed associated with the 60 kDa polypeptide, a zymography assay was performed according to Famulski et al. [25]. Digestion of calf thymus DNA, co-polymerized with the acrylamide mixture, can be seen only at a migration position corresponding to a polypeptide of this molecular mass (results not shown).

To determine the similarity of AN/IDP to DNase II and other nucleases, antibodies were raised against the bovine protein and employed in Western blotting assays. The resulting rabbit anti-AN/IDP serum, when diluted 1000-fold, readily recognizes both the purified protein and its human equivalent after 1-h incubation (Figure 8C, lanes 1–3, and Figure 8D, lane 1). In contrast, DNase II purified from bovine spleen reacts very weakly toward anti-bovine AN/IDP antibodies, requiring for detection that both the serum (diluted only 500-fold) and protein (1 μg) be more concentrated and the immunocomplexes be permitted to form for a longer period (3 h) than in the previous experiments with purified human and bovine AN/IDP proteins. Even under these incubation conditions, only a faint band, migrating slightly below the 33 kDa marker as expected for DNase II (not visible in Figure 8D, lane 5), is detectable.

Intracellular distribution of AN/IDP

To determine the intracellular location of the human AN/IDP protein, both activities were measured in nuclear and cytosolic fractions of two human cell lines, namely EBV-immortalized lymphoblastoid lines GM 3714 and GM 1526C. Both activities are present in both intracellular locations. However, both activities are appreciably higher (3–4 times) in the nuclear in comparison with the cytosolic extracts [i.e. AN activity = $(37.3 \pm 17.2) \times 10^3$ and $(8.67 \pm 1.60) \times 10^3$ units/mg, respectively; IDP activity = 311 ± 176 and 91 ± 43 units/mg, respectively], indicating that most of the protein resides in the nucleus.

To verify the presence of AN/IDP protein in the cell nucleus, Western blot immunostaining was undertaken. To this end, purified nuclei were prepared from bovine liver, nuclear proteins were extracted, and Western blots were probed with anti-AN/IDP antibodies. As shown in Figure 8(D), lane 2, incubation of nuclei in a detergent-containing hypotonic buffer leads to the release of substantive quantities of the protein. Resuspension of the remaining nuclear pellet in a high-salt buffer solubilizes additional protein (Figure 8D, lane 3). Together, these findings indicate that AN/IDP resides in two different nuclear compartments, i.e. the soluble fraction and chromatin. However, no cross-reaction with the antibodies is seen in detergent-prepared extracts of purified bovine spleen nuclei (Figure 8D, lane 4), a finding consistent with the absence of the protein in spleen tissue.

DISCUSSION

Perhaps the most intriguing observation emerging from the present studies is the direct and unequivocal demonstration that human cells possess a nuclease activity, IDP, which can rupture the sugar-phosphate backbone between cyclobutane pyrimidine dimers in UV-injured DNA (Figure 1). This result confirms and extends our earlier, indirect evidence for the existence of such an activity (see the Introduction section). In our working hypothesis

to explain these two previous findings, we proposed that a cyclobutane dimer, when located in the path of an advancing DNA replisome or RNA transcription complex, initially undergoes cleavage of its internal phosphodiester linkage, and at some later time the modified dimer is released by the NER system [26]. It was further surmised that the intradimer scission event may serve to reduce conformational stress caused by the presence of the cyclobutane bridge, thus reinstating hydrogen bonding to nearby base pairs and, by doing so, permitting the *de novo* DNA synthesis and/or RNA transcription machinery to bypass non-coding dimers in a UV-damaged template [15,26]. The properties of the protein purified here are not exactly those predicted of a *bona fide* 'dimer bypass' enzyme. On the one hand, the protein is endowed with a second, non-specific DNA backbone-incising activity, AN (Table 1). At first glance this associated activity would appear to deprive the protein of the ability to act selectively on photolesions in UV-damaged DNA, as must be the case for the nuclease responsible for generating the modified dimer sites that accumulate in high-molecular-mass (> 300 kbp) genomic DNA of post-UV incubated XP A and D cells [14]. In addition, the reaction mechanism of the AN activity creates 5'-OH and 3'-PO₄ termini, which are generally not suitable substrates for the DNA-modifying enzymes mediating many DNA transactions, including *de novo* synthesis, recombination and strand ligation [1–3,27]. Lastly, IDP and AN activities are both optimal at pH 5.0, a pH dependency rarely exhibited by DNA replication or transcription nucleases [27,28]. On the other hand, the existence of a protein with hydrolytic properties consistent with a dimer-bypass function is attractive *a priori*, given that intact dimers are known to constitute complete blocks to both DNA replication and RNA transcription [29], and yet dimers persist in genomic DNA well after both DNA transactions have resumed in UV-treated human cells [15,16,30]. Furthermore, AN/IDP is the sole protein with intradimer backbone-nicking activity detected in the seven-step purification protocol used in the present study, and is the only known protein with such activity in XP group D cells (see below). Finally, the purified protein does exhibit substantive AN (and presumably IDP) activity at neutral pH under suitable buffer conditions (Figure 5), and it is not inconceivable that the regulatory control or mechanism of action of one or both activities residing in the protein may be different *in vivo* than after the protein has been purified 4000-fold and assayed on naked DNA. Consistent with the latter notion is our recent observation that liver extracts separated by SDS/PAGE under non-reducing conditions contain about 30 % of the AN/IDP protein in a high-molecular-mass complex (> 200 kDa) (results not shown). Promising as these findings may be, definite evidence to identify AN/IDP as the protein catalysing the intradimer backbone-nicking reaction in UV-irradiated human (normal and XP) cells will require further experimentation.

It would appear that the efficiency of the AN activity, when measured at neutral pH, depends strongly on the chemical composition of the buffering agent. An acid-based (i.e. citrate/phosphate, Bes or Hepes) or amphoteric (i.e. Bicine) buffer promotes the activity of AN to a much lesser extent than a very positively charged (i.e. tertiary base-derived Tris) buffer (Figure 5). This observation most likely implies that either the protein itself or the DNA substrate contains negative charges and their neutralization enhances the AN, and possibly IDP, activities. Acidic pH, which affords optimal conditions for both activities (Figures 3 and 4), undoubtedly causes protonation and the resultant neutralization of any negatively charged residues.

Depending upon the complexity of their defining characteristics, proteins can sometimes be classified in more than one way. Such is the case for AN/IDP. Based on its physical properties,

AN/IDP can be categorized as a glycoprotein, given the presence of mannose constituents and carbohydrate-peptide linkages as inferred from the tight binding of the protein to conA (Figure 2) and its sensitivity to N-glycosidase F (Figure 8B), respectively. This pattern of glycosylation is suggestive of a nucleus or nuclear membrane localization for the protein [31] and this (N-) type of post-translational glycosylation has been postulated to be a common feature of proteins with a role in cell metabolism or signal transduction [32,33]. Interestingly, AN/IDP is indeed found predominantly in nuclei of human liver tissue, cultured human skin fibroblast strains and cell lines of lymphoblastoid and tumour (HeLa and HL-60) derivation (Figure 8D and [25]). Furthermore, AN/IDP appears to be present in two different nuclear compartments. One is detergent-sensitive and comprises the nuclear membrane and/or karyolymph, whereas the second is apparently bound to chromatin and can be removed by a high salt-containing buffer (Figure 8D).

Unquestionably, the most salient functional property of AN/IDP is the dependence of both its activities on an acidic pH for optimal efficiency. Thus, the protein can be regarded as the newest member of the rapidly expanding class of acidic nucleases in mammalian cells [9]. This family of low pH-dependent nucleases, totalling no less than eight at present [9,25], can be conveniently divided into two subclasses on the basis of their size: low (27–37 kDa) and high (47–70 kDa) molecular mass ([25] and references therein). Many family members are active over a wide range of acidic and neutral pH values and some display complicated cation requirements depending on the pH [9]. At least two of the proteins appear to have multifunctional properties. Both murine bone marrow BAF3 and hepatoma 5123tc cell lines contain an endonuclease that is active at neutral pH, providing Ca²⁺ and Mg²⁺ are both present, and also at acidic pH, where the activity is cation-independent [34,35]. In view of its native molecular mass of 46 kDa, AN/IDP belongs to the high-molecular-mass subclass of acidic nucleases. However, the protein described here would appear to be distinct from all previously characterized members of this subclass, including the 40–45 kDa low pH-dependent nuclease isolated from a mouse mammary tumour cell line by Hwang et al. [36] and the 45 kDa nuclease reported by Collins et al. [34]. The former acidic nuclease, in contrast to AN/IDP, produces DNA fragments with 3'-OH and 5'-PO₄ termini groups and its activity is inhibited by zinc ions, whereas the latter nuclease, unlike AN/IDP, is dual-regulated by cation (at neutral pH) and acidic pH dependencies ([34], and see above). Differences in structural and functional properties notwithstanding, of interest here is the recent documentation of the possible physiological role of several low pH-dependent nucleases. Two archetypal acidic nucleases, DNase II [12] and L-DNase II [37], which are encoded by different genes and are expressed in different tissues and cell types [12,37], have been shown to participate in apoptosis [12,37,38]. It has also been demonstrated that other low pH-dependent nucleases are activated during the progression of apoptosis, depending on both the cell type and the nature of the apoptotic triggering agent [25,39]. Moreover, we have recently reported that not only does AN/IDP reside in copious quantities in HL-60 cells, but upon exposure of these cells to various acidotic/apoptotic-inducing agents, the nuclease is activated dramatically and in excellent temporal correlation with the progression of the apoptotic cell death pathway [25].

As mentioned earlier, the regulatory control and mechanism of action of the AN and IDP activities are such that the purified protein exhibits no obvious specificity toward plasmid DNA containing either cyclobutyl pyrimidine dimers or apurinic sites. This observation excludes the possibility that AN/IDP belongs

to the group of so-called UV endonucleases [17,40], at least one member of which, ribosomal protein S3, is imbued with the ability to execute the intradimer-backbone cleavage reaction [41]. Parenthetically, it is noteworthy that this latter UV endonuclease is presumably not responsible for the appearance of modified dimer sites in the DNA of post-UV incubated cells, as this activity is either missing from or altered in XP D cells [42]. The AN activity of the protein does, however, display a certain substrate preference with respect to its superstructure. Linear forms of plasmid DNA are digested faster than their closed circular counterparts and single-stranded DNA is preferred over the double helical configuration (Figure 6). Given the propensity of the AN activity for unwound, single-stranded polynucleotides and the tendency of the glycoprotein to be located in or near the nuclear membrane, it is tempting to speculate that one of the primary physiological roles of AN/IDP is to catalyse DNA fragmentation during apoptosis, most likely in the initial stage which occurs at matrix-attachment sites and leads to the release of chromosome loops and concomitant degradation of DNA into large fragments of 50–300 kbp [9,43]. Consistent with the latter prediction is the recent discovery of the apparent requirement of an acidic endonuclease capable of generating the 5'-OH and 3'-PO₄ end groups found in higher-order 50- and 10-kbp DNA fragments after transient cerebral ischaemia in adult rats [44]. This postulated apoptosis-associated function of AN/IDP and the putative involvement of the IDP activity in the metabolic processing of UV-induced DNA damage are both under active investigation.

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