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Xeroderma pigmentosum fibroblasts of the D group lack an apurinic DNA endonuclease species with a low apparent K_m

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ABSTRACT

Apurinic DNA endonuclease activity from cultured human fibroblasts was resolved into two species by phosphocellulose chromatography. The species had sedimentation coefficients of 3.3 S and 2.8 S and apparent K_m's for apurinic sites of 5 and 44 nM, respectively. The low K_m species was absent from extracts of cell lines XP5BE, XP6BE and XP7BE of xeroderma pigmentosum complementation group D.

INTRODUCTION

Studies with cultured cells have shown that many human genetic diseases which lead to a high cancer risk involve a defect in DNA repair. The best characterized of these diseases is xeroderma pigmentosum (XP). XP subjects have a high frequency of skin cancer in areas exposed to sunlight, and, in some forms of the disease, impairment of nervous functions and developmental anamolies. Cell lines derived from skin biopsies of such patients are defective in excision repair of lesions introduced into the DNA by UV-irradiation and by various types of chemicals[1]. The XP phenotype falls into at least six complementation groups[2,3]. Cells from complementation groups A through E are apparently defective in an incision step near the DNA lesion[4,5]. The large number of complementation groups indicates that the incision step is regulated in a complex manner or that there might be multiple repair systems which recognize overlapping spectra of damage. In this regard Sutherland and coworkers[6,7] reported that under certain culture conditions some XP lines have reduced levels of photoreactivating enzyme, suggesting that the XP phenotype could be due to multiple enzyme defects, or that perhaps photoreactivation and excision repair are regulated by common mechanisms.

The incision function was originally thought to be carried out exclusively by specific endonucleases which can recognize altered nucleo-

tides. However, the recent discovery of DNA N-glycosidases[8-10] suggests an alternative pathway in which damaged bases are removed from the DNA by hydrolysis of the glycosidic bond between the base and the deoxyribose moiety, then the phosphodiester bond near the apurinic/apyrimidinic site is cleaved by a specific apurinic/apyrimidinic endonuclease. Kunhlein, Penhoet and Linn[11] have analyzed this endonuclease activity in unfractionated extracts from human fibroblasts and found it to be altered in extracts of cells of the A and the D complementation group of XP. The activity in these extracts had a higher K_m for apurinic sites than in extracts from other cells. In addition, the cells from the D group had a reduced level of activity. In this report we note that the D lines appear to lack one of two separable species of the DNase.

MATERIALS AND METHODS

Growth of cells and preparation of enzyme: Cells were grown in 32 ounce prescription bottles with 50 ml of Dulbecco's modified Eagle's medium (Gibco) containing 4.5 mg/ml dextrose and 10% fetal calf serum (Irvine Scientific); no antibiotics were added. Incubation was at 37° in a CO_2 incubator. Confluent cells were "split" 1:4 after treatment with trypsin. Periodic checks for mycoplasma were made either by determining the uptake ratio of ^{3}H-uridine to uracil[12] or by staining with "supermouse"[13]. Cells were harvested from groups of 8 to 12 bottles and suspended in 40 ml of 25 mM potassium phosphate (pH 7.0) - 0.15 M NaCl - 0.015 M sodium citrate by scraping from the bottle surface. After being washed twice by centrifugation they were suspended in 2 ml of 50 mM Tris-HCl (pH 7.5) and frozen in liquid nitrogen.

For extraction, the cell suspension was thawed and sonicated three times for 15 sec at 50 watts using a Biosonic sonicator with a needle probe. The sonicate was centrifuged for 50 min at 50,000 rpm ("high speed supernatant") and stored in liquid nitrogen. Ten bottles normally yielded 2 to 7 mg of soluble protein. Extracts were thawed, made 0.4 M in NaCl, then passed through a 0.6 - 0.8 ml type 40 DEAE-cellulose column (Brown and Co.) which had been equilibrated with 0.4 M NaCl - 50 mM Tris-HCl (pH 7.5). Flow-through fractions containing apurinic DNase activity were pooled and dialyzed against 10 mM potassium phosphate (pH 7.4). The dialyzed material, containing 80% of the protein and 60 to 70% of the activity, was applied to a 3.5 ml Whatman P11 phosphocellulose column (in a plastic syringe) which had been equilibrated with the same buffer.

The column was washed with 5 ml of 10 mM potassium phosphate (pH 7.4), then 3 ml of 50 mM potassium phosphate, and eluted with a 40 ml linear gradient between 50 and 275 mM potassium phosphate. Fractions of 1 ml were collected into plastic tubes and 10 µl of 10 mg/ml acetylated bovine serum albumin were added to stabilize the enzyme activity. For unexplained reasons recoveries of activity after phosphocellulose chromatography varied from 40 to 80% in different experiments. Apurinic DNase in the column fractions was sensitive to dialysis and freeze-thawing. During storage on ice a nonspecific endonuclease activity appears in the flow-through fractions. This appearance can be retarded by storing the fractions in 40% glycerol at -15°.

Assay of apurinic endonuclease: Assay mixtures (0.05 ml) contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM PM2 [^{3}H]DNA nucleotide, and 20 mM NaCl - 2 mM sodium citrate that were introduced with the depurinated DNA. (DNA was depurinated to contain 2.2 alkali-labile sites per molecule by incubating for 15 min at 70° in 0.1 M NaCl - 0.01 M sodium citrate - 0.01 M Tris, the final pH being adjusted to 5.2 with HCl.) After enzyme incubation for 10 min at 37°, the reactions were chilled and 0.15 ml of 0.01% sodium dodecylsulfate - 0.25 mM EDTA (pH 7.0) was added. Two hundred microliters of 0.3 M K_2HPO_4 - KOH measured as pH 12.3 with a Radiometer GK 2302 C electrode was added; after 2 min at room temperature 100 µl of 1 M KH_2PO_4 - HCl (pH 4.0) was added. This treatment denatures nicked PM2 DNA, but not covalently closed molecules. Two hundred microliters of 5 M NaCl and 5 ml of 1 M NaCl - 50 mM Tris-HCl (pH 8.2) were added successively and the solution was filtered through a Schleicher and Schuell type BA85, 0.45 micron pore size nitrocellulose filter which selectively retains denatured DNA under these conditions. The filters were washed with 5 ml 1 M NaCl - 50 mM Tris-HCl (pH 8.2) then 5 ml 0.3 M NaCl - 0.03 M sodium citrate, dried, then counted by liquid scintillation. The number of nicks introduced was calculated as described earlier[11] by assuming a Poisson distribution of the traget sites among the various DNA molecules and that each site had the same probability of becoming nicked during the enzyme reaction. A blank to correct for the nicking that occurred during the preparation of apurinic DNA and exposure to alkali amounted to 0.2 to 0.3 nicks per molecule. A unit of endonuclease activity catalyzes the production 1 pmol of nicks per min and a conductivity of 10 mM HO corresponded to 90 mM potassium phosphate (pH 7.4).

RESULTS

Separation of two enzyme forms: Upon chromatography on phosphocellulose of apurinic/apyrimidinic endonuclease from normal cultured human fibroblasts, two peaks of activity are observed: one in the flow-through and one that is eluted from the column at about 240 mM potassium phosphate (Fig. 1). The fraction eluting at high salt is virtually specific for DNA containing apurinic sites; on the other hand, the flow-through fraction is quite specific only directly after elution. Upon storage for several days, it rapidly accumulates endonuclease activity that acts upon non-depurinated DNA and interferes with and obscures the assay for apurinic DNase.

The separation of apurinic endonuclease activity into two forms did not appear to be an artifact of chromatography. The two fractions were dialyzed against 10 mM potassium phosphate (pH 7.4), then reapplied to

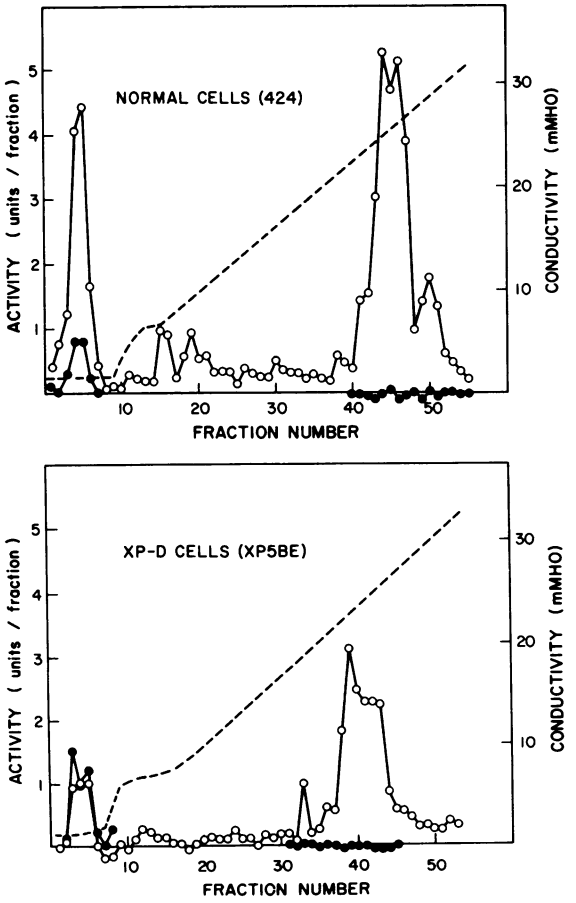

Figure 1: Chromatography upon phosphocellulose. Apurinic endonuclease from the lines indicated was prepared and chromatographed as described in Materials and Methods. (o-o) depurinated DNA substrate, (●-●) untreated DNA.

Fig. 1

phosphocellulose columns which were eluted with 10 mM, then 0.3 M buffer. With the flow-through fraction, greater than 80% of the recovered activity was again found in the flow-through, whereas with the high-salt eluate, all recovered activity was found in the 0.3 M salt eluate.

Comparison of enzyme forms: The conditions under which we depurinate DNA are reported also to deaminate cytosine and perhaps introduce other damage[14,15]. We therefore tested whether the two apurinic DNase species cleave DNA only at alkali-labile (presumably apurinic) sites (Fig. 2). PM2 DNA was depurinated to contain an average of 1.2 alkali-labile sites per molecule, and incubated with enzyme for varying lengths of time. Aliquots were also removed and treated with alkali in order to hydrolyze all remaining alkali-labile sites. With both enzyme species, the DNA which had been exposed to both alkali and enzyme was nicked to the same extent as the limit for DNA that had been exposed only to enzyme, indicating that the enzymes had introduced nicks only at alkali-labile sites. Further, the limit reached in the enzyme reaction corresponded to the number of alkali-labile sites originally present in the DNA, indicating

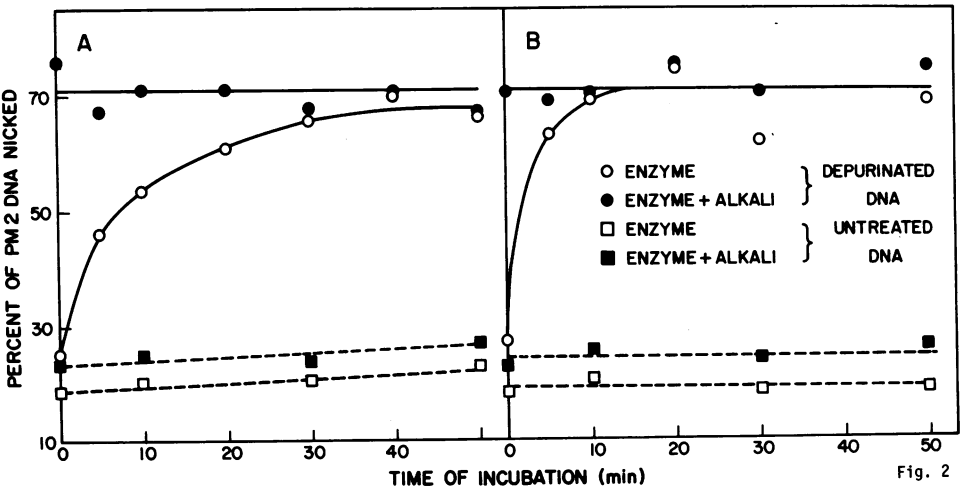

Figure 2: Specificity of the endonuclease activities. Assays were in 50 mM potassium phosphate (pH 7.8) with 0.3 units/ml of flow-through activity and 0.9 units/ml of high-salt eluate, respectively. At times indicated two 50-µl aliquots were removed; one was assayed for nicks as described in Materials and Methods, and the second was similarly processed, except that after addition of potassium phosphate (pH 12.3) it was incubated for 24 hours at room temperature in order to hydrolyze all remaining alkali-labile sites.

A. Flow-through fraction. B. High-salt eluate.

that both forms of apurinic DNase can cleave all of the alkali-labile sites in the DNA.

In the above assays double-stranded circular DNA was used as a substrate and the nicked DNA molecules were selectively denatured and separated from the covalently closed circles by filtration through nitrocellulose. Such an assay conceivably might not distinguish between nicking of a DNA circle and tight binding of an intact circle to a protein molecule. Therefore, we also measured nicking by separating the nicked from intact circles by sedimentation through a neutral sucrose density gradient. When the assays were carried out at different enzyme levels for both species of apurinic DNase, the results corresponded identically. In addition, the sedimentation analysis revealed that no non-sedimentable material was formed during enzymatic cleavage of the DNA.

When the optimal reaction conditions for the two peaks of activity were determined, they were found not to differ significantly. Each had a pH optimum near 7.5 and was optimally active in 10 mM $MgCl_2$. Each had some residual activity in the absence of divalent cation, was stimulated 2.5-fold by 10 mM KCl with $MgCl_2$ present, and was stimulated slightly by 10 mM ATP, but inhibited by higher ATP concentrations. Finally, both were inactivated in 230 mM potassium phosphate buffer at 45° with a half-life near 6 min.

Whereas substrate specificity and optimal conditions were indistinguishable for the two forms, two properties showed significant differences. First, a slight, but reproducible difference was found between their sedimentation coefficients. Two determinations with the flow-through activity indicated S^w_{20} values of 3.3 and 3.2, whereas with the high-salt eluate species, values of 2.8 and 2.7 were obtained. These values were determined by comparison with the sedimentation rates of myoglobin ($S^w_{20} = 2.04$), chymotrypsinogen ($S^w_{20} = 2.54$) and ovalbumin ($S^w_{20} = 3.55$). Both apurinic DNase activities sedimented in single symmetric peaks.

An even more striking difference was noted upon comparison of the apparent K_m for apurinic sites (Fig. 3). The value for the flow-through fraction was 4.6 ± 1.3 nM (90% confidence interval, 2.4 - 8.2 nM). Conversely, the high-salt eluate could barely be saturated with substrate, generating a K_m of 44 ± 17 nM (90% confidence interval, 22 - 174 nM).

Comparison among cell lines: In a previous study[11] the cell line XP5BE, obtained from a xeroderma pigmentosum complementation group D individual, was found to be reduced in apurinic DNase activity. When an

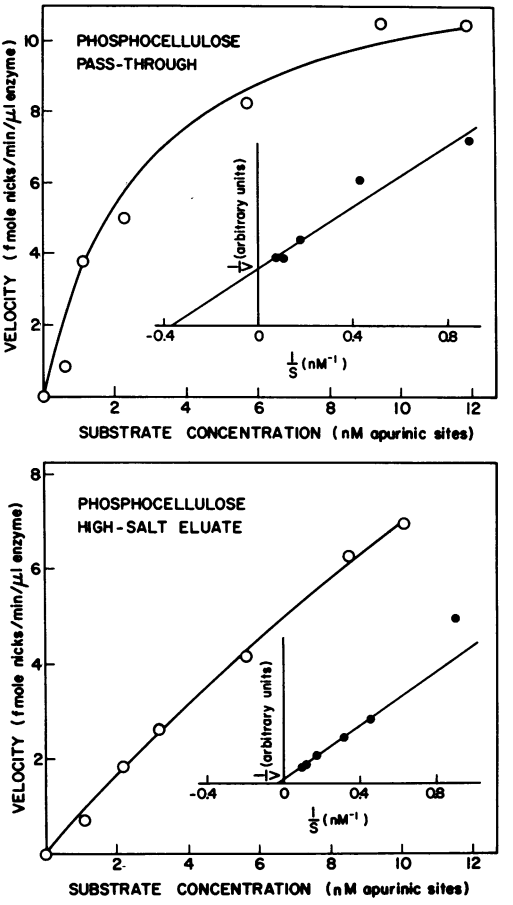

Figure 3: Apparent K_m for enzyme species. Reaction velocities were determined in 10 min reactions at enzyme levels which nicked no more than 20% of the DNA molecules. The concentration of apurinic sites was varied by adding different amounts of depurinated [^{3}H]DNA. Unlabelled DNA which had not been depurinated was added to keep the total DNA concentration constant at 100 μM nucleotide. K_m values were calculated from the experimental points by the regression method of Wilkinson[16].

Fig. 3

extract from this line was chromatographed upon phosphocellulose, only the enzyme species eluting in 240 mM phosphate was observed (Fig. 1, Table 1). Among several other cell lines examined (Table 1), including one from a subject with ataxia telangiectasia, only the other cell lines from the D complementation of xeroderma pigmentosum showed a similar absence of the flow-through species. While recoveries of activity after chromatography were variable, the absence of a flow-through species of activity was a unique and reproducible feature of the D line extracts. The absence of the low K_m species would seem to explain our previous observation that apurinic endonuclease in crude extracts from group D lines has a K_m for substrate that is several-fold higher than normal.

In our previous study we also observed relatively high K_m values in crude extracts from A group lines. However, these lines have the flow-

TABLE I Distribution of Endonuclease after Phosphocellulose Chromatography

Cell line	Genotype	Units applied to column	Units in flow-thru	Units in high salt eluate
424	normal	590	48	323
CRL 1262	normal	243	28	128
CRL 1343 (Se Pan)	ataxia telangiectasia	410	22	105
XP12BE (CRL 1223)	XP-A group	452	42	186
XP25RO (CRL 1261)	XP-A group	201	30	86
XP7BE (CRL 1200)	XP-D group	265	~2	137
424	normal	283	22	62
XP5BE (CRL 1160)	XP-D group	108	< 0.2	38
XP6BE (CRL 1157)	XP-D group	152	< 0.3	54

All procedures were as described in Materials and Methods, except that the flow-through fractions of the first four entries were assayed at pH 8.0 in order to suppress non-specific nuclease activity. The "high-salt species" is the activity which eluted between 180 and 270 mM potassium phosphate. The activities of the flow-through fractions were corrected for nonspecific activity of 2 units or less.

The nomenclature for XP patients is described by Cleaver and Bootsma[1] and the assignment to complementation groups by Kraemer *et al.*[2,3]. Cell line 424 is a foreskin fibroblast cell line obtained from the Navy Biological Labs, Oakland, California; the other cell lines are from skin biopsies and were obtained from the American Type Culture Collection (ATCC). CRL 1262 is from a patient with osteogenesis imperfecta and is referred to as "normal" since this disease is believed not to affect DNA repair. All cells were between passage 10 and 25, and the fractions applied to the phosphocellulose columns had between 2 and 6 mg protein.

through species of activity (Table 1) and this activity has a normal K_m (4.2 nM apurinic sites). In addition, the K_m for substrate observed in crude extracts from normal cells is at least several-fold lower than that of either of the fractionated activities. It is therefore probable that additional factors can be involved in the apurinic endonuclease activity.

CONCLUSIONS

In normal cells the flow-through species of enzyme would appear to be present at a lower level than the retarded species of enzyme (Table 1).

However, under intracellular conditions, with relatively low concentrations of apurinic/apyrimidinic sites[17], the reaction velocities become proportional to V_{max}/K_m, so that the flow-through species that is absent in the xeroderma pigmentosum D lines might easily be the dominant activity. An assessment of the relative importance of the two species requires a determination of their actual intracellular concentration and distribution; however, it is clear that the loss of a significant fraction of the endonuclease would increase the life expectancy of an apurinic/apyrimidinic site proportionately and hence increase the possibility of secondary reactions to form crosslinks, replication errors, etc., which could impart permanent genetic damage to the cell.

As originally recognized by Cleaver[18], a deficiency in most XP lines, including those of the D group, is a reduced level of DNA repair induced by UV irradiation. Whether the apurinic DNase has any role in this repair is unclear: the enzyme does not act upon UV irradiated DNA *in vitro*[11], and no DNA N-glycosidase has been reported that converts UV lesions into sites for the DNase (though the search for such an enzyme would be worthwhile). However, currently there is no explanation for the large number of complementation groups of xeroderma pigmentosum as well as for the variations of UV-sensitivity among the groups. In addition there is no absolute method of measuring repair proficiency in these cells. Thus, the D-lines show as much as 50% the normal level of UV-induced DNA synthesis[2], but only a few percent of the normal host-cell reactivation of UV-irradiated adeno virus[19].

While XP-lines show little abnormal sensitivity to alkylating agents in terms of killing or repair replication, they do show defective repair of some alkylation damage[20], and display abnormally large numbers of sister chromatid exchanges induced by these agents[21]. In addition, by the use use of alkylating agents to measure repair proficiency of apurinic sites, one may be measuring only the excision repair of alkylated nucleotides, rather than the repair of depurinated regions. As an alternative measure of apurinic DNA repair proficiency, we have examined host-cell reactivation of acid-depurinated SV40 DNA (R. Kdruna, S. Linn, E.E. Penhoet, unpublished). Contrary to our expectations, the XP-D lines are much more effective than normal in reactivation of the depurinated DNA, though they are very defective in reactivation of UV-irradiated DNA. Clearly, until more is understood about individual repair pathways, their regulation, their fidelity, and how to measure their relative importance

in situ, we cannot easily correlate enzyme defects and disease symptoms. We can only speculate that at least some of the XP groups could be defective either in a regulatory function, or a peptide associated with several repair enzymes or complexes.

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FOOTNOTES

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