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An apoptotic endonuclease activated either by decreasing pH or by increasing calcium

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SUMMARY

DNA fragmentation in isolated nuclei from the murine IL3-dependent bone marrow cell line BAF3 could be stimulated either by decreasing pH below 6.5 or by adding μM calcium at neutral pH. An endonuclease which could also be stimulated either by a decrease in pH, to 6.5, or by the presence of μM calcium at neutral pH, was purified 10^4-fold from nuclei of BAF3 cells. Digestion of DNA with the purified enzyme resulted in 5′-terminal hydroxyl and 3′-terminal phosphate ends. These characteristics are distinct from those described for other mammalian endonucleases. The possible role of this enzyme in genome digestion during apoptosis is discussed.

Key words: Apoptosis, Endonuclease, pH, Calcium

INTRODUCTION

The first biochemical characterisation of apoptosis demonstrated that the DNA of dying cells was fragmented into oligonucleosome-length fragments following activation of an endogenous endonuclease (Wyllie, 1980). Sixteen years later, there remains much controversy as to the role of DNA fragmentation in apoptosis. It is clear that cells can be induced to die in the absence of detectable oligonucleosome 'ladders' (Cohen et al., 1992). Larger fragments of DNA, around 50 kilobases, have been detected in dying cells when 'ladders' were not observed (Brown et al., 1993; Oberhammer et al., 1993). Therefore it has been proposed that this 50 kb fragmentation is more general and that different enzymes may catalyse the two forms of digestion (Brown et al., 1993). However, the relative insensitivity of detection of both types of fragmentation makes any absolute conclusion difficult. The simplest model probably remains that an endonuclease is activated during apoptosis in most cell types. This enzyme will then cleave chromatin according to DNA accessibilty. Indeed, active genes with open chromatin configuration have been shown to be digested first (Vanderbilt et al., 1982). In cell types, or particular conditions, where the endonuclease is highly active, 'ladders' representing almost complete chromatin digestion will be most readily detected.

Chromatin digestion is probably important in the clearance of apoptotic cell debris in vivo. However, it is clear that cell death, with some features of apoptosis, can occur in the absence of DNA digestion. Thus, enucleated cells 'die' when exposed to kinase inhibitors (Jacobson et al., 1994) or Fas antibodies (SchulzeOsthoff et al., 1994). Cytotoxic T cells can kill targets in the absence of genome digestion (Ucker et al., 1992). Therefore, endonuclease activation is not required for apoptosis. Several endonucleases have been proposed to be the enzyme which digests DNA during apoptosis. Those which have been best characterised are a Ca^{2+}/Mg^{2+}-dependent enzyme from lymphocytes (Hewish and Burgoyne, 1973; Shiokawa et al., 1994; Nikonova et al., 1993), DNAse I (Peitsch et al., 1993), DNAse II (Liao, 1985; Barry and Eastman, 1993) and cyclophilin (Montague et al., 1994).

The murine IL-3 dependent cell line BAF3 dies by apoptosis upon IL-3 removal (Rodriguez-Tarduchy et al., 1990); this death is more rapid if cells are exposed to DNA damaging agents (Collins et al., 1992). The onset of DNA fragmentation occurs at a time when apoptosis becomes irreversible (Collins et al., 1992) and de novo protein synthesis is not required for endonuclease activation (Rodriguez-Tarduchy et al., 1990). Aurintricarboxylic acid can block DNA fragmentation and delay death of BAF3 cells (Ascaso et al., 1994). These observations suggest that DNA fragmentation occurs in BAF3 cells close to the time of commitment to apoptosis. We therefore investigated the mechanism of endonuclease activation in these cells. Here, we report the purification and characterisation of a novel endonuclease stimulated either by a pH decrease or by μM calcium.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were obtained from Gibco Europe. [^{3}H]thymidine (20 Ci/mmol) was from Amersham International. DNAseI was from Sigma. Blue Sepharose and ConA resins, pre-packed Superose 12 and Mono S columns were from Pharmacia. BAF3 cells (Palacios and Steinmetz, 1985) were maintained in DMEM with 10% FBS and 10% conditioned medium (CM) from the IL-3-producing cell line Wehi-3B.

Measurement of DNA fragmentation by [^{3}H]thymidine release

Nuclei were prepared from [^{3}H]thymidine labelled cells (incubated overnight with 0.1 μCi/ml of [^{3}H]thymidine) as for 'enzyme purification', then resuspended at 5×10^5 per 50 μl reaction in 20 mM Tris/Hepes (pH as indicated), 20 mM KCl, 1 mM $MgCl_2$ and 1 mM EGTA (reaction buffer). $CaCl_2$ addition was calculated using the Chelator programme (R. A. Steinhardt, UCal, Berkeley, USA) to give the free Ca^{2+} concentration indicated. Final reaction pH was measured at 37°C, using a microprobe. After a 1 hour incubation at 37°C, nuclei were lysed by addition of an equal volume of 10 mM Tris-HCl, pH 7.5, 2 mM EDTA and 0.2% Triton and high molecular mass chromatin and fragmented DNA were separated by centrifugation (13,000 ***g*** for 15 minutes). The radioactivity in the 13,000 ***g*** supernatant and the pellet were determined in a scintillation counter and fragmentation was expressed as % of total DNA: (supernatant/pellet + supernatant) ×100. Each value is the mean ± s.d. of triplicates.

Nuclear extract preparation

BAF3 cells were pelleted and washed twice in phosphate buffered saline (PBS), then incubated on ice for 15 minutes in 1 ml/10^9 cells of 10 mM Tris/Hepes, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$ and 0.05% Nonidet P40 (lysis buffer) containing 10 μg/ml antipain, 10 μg/ml elastinal, 10 μg/ml leupeptin, 50 μg/ml aprotonin and 20 μg/ml pepstatin A (protease inhibitors). The lysate was then underlayered with lysis buffer containing 30% sucrose and protease inhibitors and spun at 13,000 ***g*** for 10 minutes at 4°C. The lysis buffer suspension and pelleting steps were repeated on the nuclear pellet. The nuclei were then resuspended in 1 ml Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 10 mM NaCl (TMN) containing protease inhibitors, the final NaCl concentration was adjusted to 50 mM and the nuclear suspension was incubated on ice for 1 hour. The supernatant, following a 13,000 ***g*** spin for 15 minutes at 4°C, was mixed with an equal volume of TMN containing 50% glycerol and protease inhibitors and stored at −70°C as 'nuclear extract' (Table 1). The final protein concentration was between 10 and 20 mg/ml.

Gel filtration was performed with nuclear extract on a Pharmacia SMART system using a 2 ml Superose 12 column equilibrated and eluted in 10 mM Tris/Hepes, pH 7.5, 50 mM NaCl, 0.1% Triton X-100 and protease inhibitors. Activity in the load and fractions was assayed in 20 mM Tris/Hepes, pH 6.5, 20 mM KCl, 1 mM EGTA, 1 μM free Ca^{2+} (calculated using Chelator equation). Nuclease activity gels were run with nuclear extract and DNase I (Sigma) as previously described (Shiokawa et al., 1994). Following SDS removal and renaturation, gels were incubated either under optimal conditions for DNase I, or under optimal conditions for BAF3 nuclear extract (see legend to Fig. 3A) DNA remaining was then visualised by ethidium bromide staining. Isoelectric focusing was performed with nuclear extract, slices of the gel were then incubated overnight at 4°C in 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 25% glycerol, 20 μg/ml bovine serum albumin (BSA) and protease inhibitors. Parallel slices from a second gel were incubated in 0.5 ml of 500 mM NaCl and the pH was determined with a microprobe. Eluted protein from each slice was precipitated with acetone, resuspended in 1 μl saturated urea then diluted in 50 μl of 20 mM Tris/Hepes, pH 6.5, 20 mM KCl, 1 mM EGTA, 1 μM free Ca^{2+} (calculated using Chelator equation) and incubated overnight at 4°C. Plasmid (1 μg) was added to each fraction; these were then incubated at 37°C overnight and run on a 1% agarose gel.

Nuclease purification

Nuclease activity from batches of nuclear extract was purified using a plasmid digestion assay where samples of column fractions were incubated in 50 μl of 20 mM Tris/Hepes, pH 6.5, 20 mM KCl, 1 mM EGTA, 1 μM free Ca^{2+} (calculated using Chelator equation) and 1 μg plasmid (pUC 13/18), for 15 minutes at 37°C. Digestion was detected by running the reactions on a 1% agarose gel. Triton X-100 (0.1%) was added to the nuclear extract, which was then applied to a 20 ml Blue Sepharose column, on a Pharmacia FPLC system, pre-equilibrated in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 37.5 mM NaCl, 0.1% Triton X-100 and protease inhibitors. Activity eluted between 300 and 400 mM NaCl on a linear 37.5-1,000 mM NaCl gradient. Peak fractions were applied to a 1 ml concanavalin A (ConA) agarose column on a Pharmacia SMART system equlibrated in 20 mM Tris/Hepes, pH 7.5, 500 mM NaCl, 0.1% Triton X-100 and protease inhibitors. Bound activity was eluted in 20 mM Tris/Hepes, pH 5.5, 50 mM NaCl, 500 mM α-methylmannoside, 0.1% Triton X-100 and protease inhibitors. The eluate was applied to a 0.2 ml Mono S column, on a Pharmacia SMART system, pre-equilibrated with 20 mM Tris/Hepes, pH 5.5, 50 mM NaCl, 0.1% Triton X-100 and protease inhibitors. Activity eluted between 400 and 500 mM NaCl on a linear 100-1,000 mM NaCl gradient. The peak fractions were pooled to analyse ionic requirements, individual fractions were analysed by SDS-PAGE on a 12.5% gel, followed by silver stain (Morrissey, 1981), or transfer to nitrocellulose, which was blocked in Tris buffered saline with 0.1% Tween-20 and 1% BSA then probed with 0.05% peroxidase coupled ConA (Sigma), washed in the same buffer and developed with ECL (Amersham, UK)

End labelling assay and calculation of specific activity

Samples (20 ng) of undigested or digested plasmid, or 20 ng of BAF3 cell DNA prepared as previously described (Collins et al., 1992), were boiled then incubated in 50 μl for 1 hour at 37°C with either terminal deoxynucleotide transferase (TdT) (1U, Pharmacia) in recommended buffer and 0.01 μCi [α-^{32}P]dCTP (Amersham, UK) or T4 polynucleotide kinase (T4 PNK) (5U, Pharmacia) in recommended buffer, 10 nM ATP and 0.05 μCi [γ-^{32}P]ATP. Incorporated radioactivity was determined by spotting a portion onto DE81 paper and washing 3 times in 2× SSC. Between 0.1 and 5 ng of a synthetic 20mer oligonucleotide were labelled as a control. Incorporation of radioactivity by T4 PNK was linearly proportional to the amount of oligonucleotide added within this range. The number of 5′OH ends generated in plasmid by nuclease preparations was therefore calculated from this calibration curve. One unit of nuclease was defined as that which generated 1 pM of 5′OH ends/minute from 1 μg plasmid in a 50 μl reaction after subtracting the background radioactivity incorporated in uncut plasmid.

RESULTS

We have previously observed that de novo protein synthesis is not required for onset of DNA fragmentation during apoptosis in BAF3 cells. We therefore attempted to directly stimulate DNA fragmentation in nuclei isolated from non-apoptotic BAF3 cells, to identify endogenous endonuclease(s) which might be activated during apoptosis. In these experiments pH was measured in the final reaction using a microprobe and the calcium concentration was controlled by EGTA buffering. Fig. 1A shows that DNA fragmentation could be induced either by decreasing the pH of the reaction, or by the addition of μM calcium at neutral and higher pH. Similar stimulation of DNA fragmentation by μM calcium at neutral pH has been observed in isolated rat liver nuclei (Jones et al., 1989) and stimulation by low pH has been observed in isolated CHO cell nuclei (Barry and Eastman, 1993). Maximal stimulation was observed at concentrations of calcium between 0.3 and 1.0 μM (data not shown). Fragmentation stimulated by lowering the pH was inhibited by potassium concentrations in excess of 60 mM, by the addition of magnesium in excess of 1.5 mM and by 0.5 mM zinc (data not shown). The fragmentation that was observed by monitoring release of labelled chromatin could also be detected

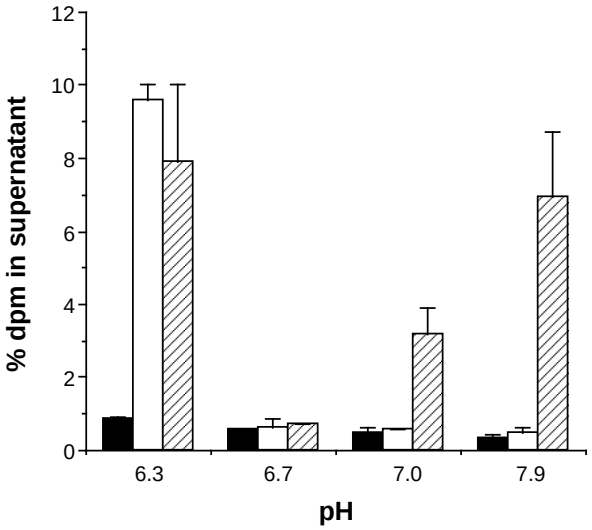

Fig. 1. Regulation of DNA fragmentation in isolated nuclei from BAF3 cells. Release of labelled chromatin from isolated nuclei kept on ice (t=0, ■), or incubated for 60 minutes at 37°C in the absence (□), or presence (▨), of 1 μM free Ca^{2+} as indicated. The incubations were at the pH indicated.

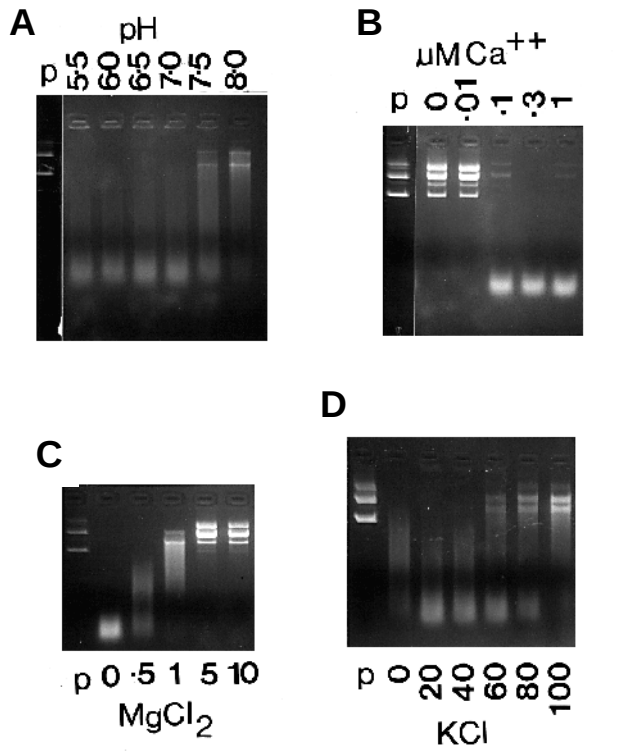

Fig. 2. Requirements for nuclease activity in BAF3 cell nuclear extract. (A) BAF3 cell nuclear extract (approximately 1 μg protein) was incubated for 15 minutes at 37°C with 1 μg plasmid, in 20 mM Tris-Hepes at the pH shown, 20 mM KCl and 1 mM EGTA. Untreated plasmid (p) and nuclease treated samples were then run on a 1% agarose gel. The 3 predominant bands in the untreated plasmid lane represent form I (closed circle, fastest migrating), form II (nicked circle) and form III (linear, slowest migrating); after nuclease digestion degraded linear fragments are seen. (B) Incubations were in 20 mM Tris-Hepes, pH 7.1, with 20 mM KCl, 1 mM EGTA and the concentration of free Ca^{2+} shown, calculated using the Chelator programme. (C) Incubations were in 20 mM Tris/Hepes, pH 6.5, 20 mM KCl, 1 mM EGTA and the concentration of $MgCl_2$ shown. (D) Incubations were in 20 mM Tris/Hepes, pH 6.5, 1 mM EGTA and the concentration of KCl shown.

as the release of oligonucleosomal length DNA fragments (data not shown). This rapid induction of fragmentation in isolated nuclei demonstrates that an enzyme(s) capable of rapid DNA fragmentation is associated with the nuclei of non-apoptotic BAF3 cells.

In order to identify the activatable endonuclease(s) responsible for chromatin fragmentation in BAF3 cell nuclei, extract of BAF3 cell nuclear proteins was analysed using plasmid digestion to assay for endonuclease activity. Crude extract contained activity that was activated by acidic pH, or by 0.1-1 μM calcium at neutral pH, and was inhibited by magnesium and potassium in an identical fashion to fragmentation in whole nuclei (Fig. 2). Nuclease activity, at pH 6.5 or pH 7.0 in the presence of 1 μM calcium, was inhibited by 0.5 mM zinc and by 10 μM auritricarboxylic acid (ATA) but not by G-actin under conditions where activity of partially purified DNAse I was completely inhibited (data not shown). We have previously demonstrated that ATA will inhibit DNA fragmentation in intact BAF3 cells (Ascaso et al., 1994).

An 'in gel' assay of nuclear extract compared to DNase I (Fig. 3A) demonstrated that a single protein migrating at approximately 45 kDa possessed nuclease activity. Activity could only be detected when the gel was incubated under appropriate conditions so lack of ethidium bromide staining could not be attributed to a DNA binding protein; the mobility of the enzyme decreased slightly when the gel was run under reducing conditions (Fig. 3A). The relative inactivity of the BAF3 cell nuclear extract in the 'in gel' assay prevented a detailed characterisation of its activation and inhibition requirements by this method. When nuclear extract was run on an isoelectric focusing gel, nuclease activity was eluted at a single pI of approximately 6.5 (Fig. 3B). After gel filtration of nuclear extract a single peak of activity which eluted at approximately 200 kDa was detected (Fig. 3C). The peak activity was activated by acidic pH, or by μM calcium at neutral pH, and was inhibited by magnesium and potassium at pH 6.5 or at pH 7.0 in the presence of 1 μm Ca^{2+} (data not shown). The large size at which activity eluted was not altered by running the column at pH 5.5, or in the presence of 1 μm Ca^{2+}, or in the presence of 100 or 500 mM NaCl (data not shown). This suggests that the 45 kDa nuclease detected by 'in gel' assay exists as a multimer or part of a complex.

Nuclease activity was then purified using plasmid digestion as an assay. At each purification step, detailed in Table 1 and Materials and Methods, a single peak of endonuclease activity was detected. After 3 chromatography steps an approximately10^4-fold purification of activity from low salt nuclear extract was achieved (Table 1) which corresponds to a 5×10^5-fold purification from intact cells. It is of interest that activity was retained on ConA and specifically eluted by sugar. The fraction corresponding to the peak activity from the final MonoS ion exchange column (Fig. 4) contained a single protein of approximately 45 kDa, detected by ConA western blot (Fig. 4). Protein in this fraction was below the limit of

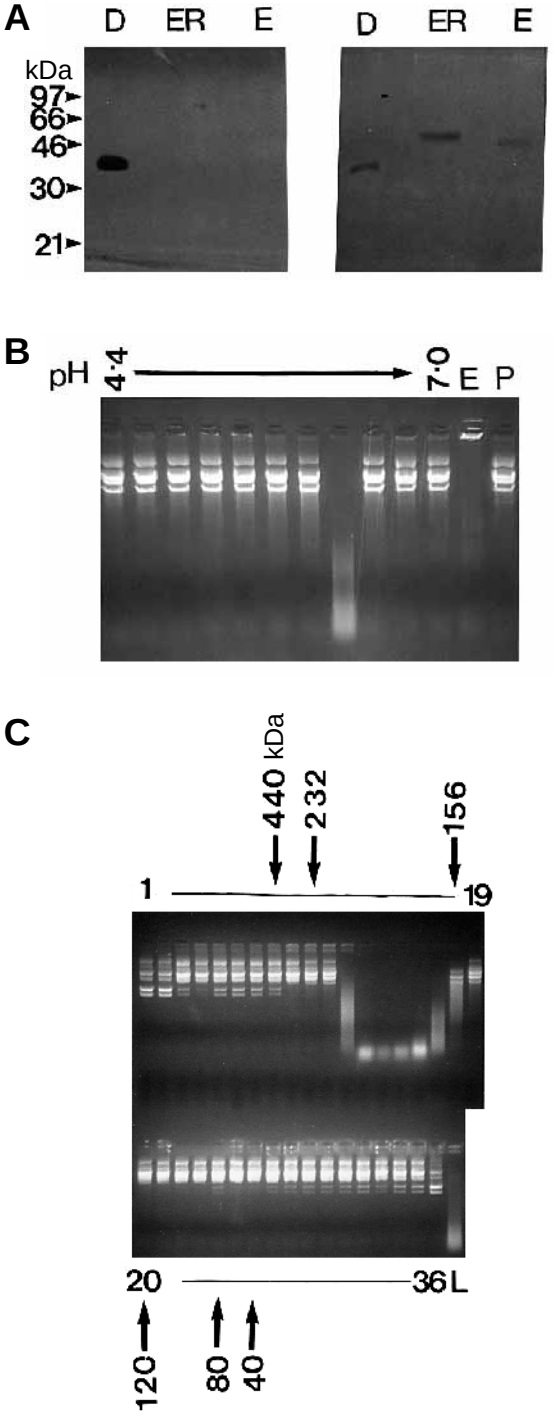

Fig. 3. Characterisation of nuclease activity in BAF3 cell nuclear extract. (A) In gel nuclease activity assay of nuclear extract (10 mg protein partially purified on Blue Sepharose, Table 1) after 12.5% SDS-PAGE under non-reducing (E) or reducing (ER) conditions. DNAse I (D) run under reducing conditions was included as a control. After removal of SDS the left-hand gel was incubated overnight in 10 mM Tris-HCl, pH 7.8, 3 mM $MgCl_2$ (optimal for DNase I) and the right-hand gel in 20 mM Tris-Hepes, pH 6.5, 20 mM KCl, 1 mM EGTA, 1 μM free Ca^{2+} (optimal for BAF3 nuclear extract) prior to DNA visualisation. (B) Nuclease activity isolated from an isoelectric focusing gel (see Materials and Methods) run using 1 mg nuclear extract concentrated by binding to glass beads. Precipitated renatured extract (10 μg) (E) and plasmid incubated in renaturation buffer (P) are included as controls. (C) Gel filtration of 0.5 mg nuclear extract on a Superose 12 column; activity of the load (L) and eluted fractions was assayed as described in Materials and Methods. The column was calibrated with native protein markers (Bio-Rad), the elution positions of which are shown.

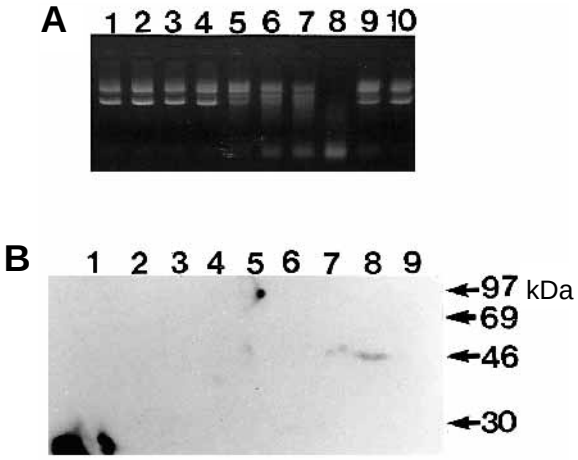

Fig. 4. Endonuclease purification from BAF3 cell nuclear extract. (A) Activity of individual fractions from the final MonoS column (Table 1) was assayed by incubating 1 μl of 50 μl in 20 mM Tris/Hepes, pH 6.5, 20 mM KCl, 1 mM EGTA, 1 μM free Ca^{2+} for 15 minutes at 37°C. (B) Samples (10 μl) of the same fractions were run on 12.5% reducing SDS-PAGE which was blotted and stained with peroxidase conjugated ConA, as described in Materials and Methods.

Table 1. Purification of nuclease activity

	Specific activity (pM ends/min per μg)	Purification fold total	Units* total
Nuclear extract	1.8×10^{-3}		136.0
Blue Sepharose	0.085	47	76.5
Con A agarose	1.35	770	12.5
Mono S†	>12	>6,700	11.5

*1 unit generates 1 pM 5′hydroxyl ends/minute from 1 μg supercoiled plasmid DNA in a 50 μl reaction.

†Protein below limit of silver stain detection, estimated as <10 ng.

detection by silver staining (data not shown). Glycosylation with mannose, detected by ConA, only occurs on proteins which have been processed through the endoplasmic reticulum (ER). Detection of mannose on the nuclease suggests that it is associated with the ER or nuclear membrane in isolated nuclei and nuclear extract.

The activation requirements of the purified endonuclease were identical to those for induction of fragmentation in whole nuclei, or DNA digestion in crude extract. Fig. 5 shows that the purified enzyme could be fully activated in the absence of calcium by incubation at pH 6.5 and below. Activation at neutral pH could be obtained by addition of calcium. The enzyme did not require magnesium and was inhibited by magnesium concentrations above 1 mM and by potassium concentrations above 60 mM (Fig. 5). The identical profiles of cation stimulation and inhibition that were observed in intact nuclei and with purified endonuclease suggest that chromatin digestion in nuclei is regulated by direct control of the endonu-

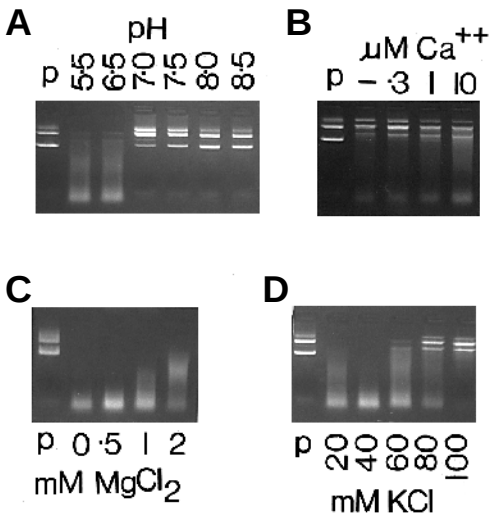

Fig. 5. Activity requirements of the purified nuclease. (A) The pooled peak fractions from the final Mono S column (Table 1, Fig. 4) were incubated for 15 minutes at 37°C with 1 μg plasmid, in 20 mM Tris/Hepes at the pH shown, 20 mM KCl and 1 mM EGTA. Untreated plasmid (p) and nuclease treated samples were then run on a 1% agarose gel. (B) Incubations were in 20 mM Tris/Hepes, pH 7.1, with 20 mM KCl, 1 mM EGTA and the concentration of free Ca^{2+} shown, calculated using the Chelator programme. (C) Incubations were in 20 mM Tris/Hepes, pH 6.5, 20 mM KCl, 1 mM EGTA and the concentration of $MgCl_2$ shown. (D) Incubations were in 20 mM Tris/Hepes, pH 6.5, 1 mM EGTA and the concentration of KCl shown.

clease. Furthermore, the activators and inhibitors must be acting on a single enzyme or tightly associated enzyme complex. The purified enzyme generated 5′OH ends after digestion of plasmid as determined by labelling with T4 PNK (Fig. 6) which was not enhanced by pretreatment of the DNA with calf intestinal phosphatase (data not shown). The 3′ends of digested DNA could not be labelled by TdT (Fig. 6). Fragmented DNA prepared from BAF3 cells undergoing apoptosis showed an identical pattern of labelling (Fig. 6). This differs from chromatin isolated from thymocytes undergoing apoptosis, where 5′PO_4 and 3′OH termini were detected (Shiokawa et al., 1994).

DISCUSSION

The endonuclease that we describe is distinct from those previously reported to be involved in apoptosis. One of the best characterised is an activity found in spleen, thymus and liver which requires magnesium and non-physiological millimolar calcium and is active at neutral pH (Hewish and Burgoyne, 1973; Ribeiro and Carson, 1993; Stratling et al., 1984). Several such calcium-dependent enzymes have been purified to apparent homogeneity from thymocytes (Nikonova et al., 1993; Shiokawa et al., 1994), one has been identified as cyclophilin (Montague et al., 1994). The BAF3 endonuclease is clearly distinct from these enzymes in its activation requirements. The pancreatic enzyme DNAse1 was reported to be present in lympoid cells and proposed to be involved in DNA degradation during apoptosis (Peitsch et al., 1993). BAF3 cells express a low level of DNAse1 mRNA (data not shown). However, we cannot detect nuclease activity with the characteristics of DNAse1 in nuclei of control or apoptotic BAF3 cells. Such activity can be defined as that inhibitable by depolymerised actin, which forms a specific complex with DNAse1. The enzyme DNAse II was reported to be present mainly in lysosomes (Liao et al., 1989); it is activated at acidic pH and has been purified to apparent homogeneity (Liao, 1985). It also generates 5′OH and 3′PO_4 DNA termini (Murai et al., 1980). Barry and Eastman (1993) recently described isolation of a DNAse II-like enzyme from CHO cell nuclei. DNAse II shares with the BAF3 enzyme the property of activation at low pH and it remains possible that the two are related as DNAse II has not been molecularily defined. However, calcium activation of DNAse II has not been reported, it is not inhibited by magnesium or potassium, its molecular mass on gel filtration is around 40 kDa (Liao, 1985; Barry and Eastman, 1993) and two inactive subunits of 35 kDa and 10 kDa are detected by SDS-PAGE (Liao, 1985). An enzyme from thymocytes activated by decreasing pH is active after SDS-PAGE, but has a molecular mass of 30 kDa and generates 5′PO_4 and 3′OH ends (Shiokawa et al., 1994).

Endonuclease activity in haematopoietic progenitor cell lines such as BAF3 has not previously been investigated. Indeed, with the exception of the study of Barry and Eastman (1993), previous purifications have used tissue as starting material. In these cases, it is hard to be sure that the most

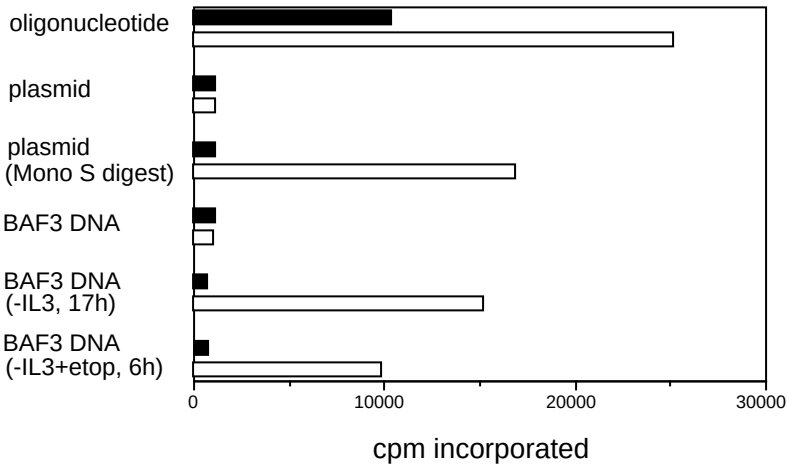

Fig. 6. Labelling of DNA termini generated by the purified nuclease or during apoptosis. Plasmid, plasmid digested with purified (Mono S peak) nuclease, DNA fragmented during apoptosis and a control oligonucleotide were labelled with TdT (■) or T4 PNK (□) as described in Materials and Methods. The values shown are the mean of triplicate determinations.

abundant nuclease detected is that involved in apoptosis of particular cells. We do not detect a nuclease with the characteristics of the BAF3 cell enzyme in similar nuclear extracts from the IL-2 dependent cell lines CTLL-2 and HT2, whereas it can be detected in extracts from the IL-3 dependent cell line FDCP-MIX (data not shown). All these cells undergo apoptosis with DNA 'laddering' on factor removal, which suggests that different endonucleases are involved.

The dual-regulated endonuclease that we have characterised is the first to be purified which can be stimulated by physiological changes in calcium. Ionophores which increase intracellular calcium induce production of IL-4, a survival factor for BAF3 cells (Rodriguez-Tarduchy et al., 1992). However, treatment with A23187 in the presence of cyclosporin A, which blocks IL-4 production stimulates apoptosis (Rodriguez-Tarduchy et al., 1992). A late redistribution of calcium has been observed following IL-3 removal from other factor-dependent cell lines (Baffy et al., 1993) and it is possible that this plays a part in nuclease activation. Likewise, intracellular acidification has been observed during apoptosis in the absence of survival factors (Gottlieb et al., 1995; Li and Eastman 1995; Rebollo et al., 1995) or in cells treated with etoposide (Barry et al., 1993). Lowering intracellular pH induces DNA fragmentation in CHO cells which contain an acid activatable nuclease (Barry and Eastman, 1993) and amiloride, an inhibitor of the Na/H^+ antiport has been reported to induce apoptosis in haemopoietic cells (Rajotte et al., 1992; Caceres-Cortes et al., 1994).

However, changes in intracellular pH or Ca^{2+} are unlikely to be all that is required to directly stimulate activity of the endonuclease that we have characterised in intact cells. Members of the ICE-like protease family are central in the regulation of apoptosis in mammalian cells. Inhibition of such proteases inhibits DNA fragmentation in a variety of cells undergoing apoptosis (Martin and Green, 1995). Experiments with isolated nuclei have suggested that an ICE-like protease can stimulate endonuclease activity (Lazebnik et al., 1993, 1994). A role of such proteases in our experiments with isolated nuclei seems unlikely as the activation of chromatin fragmentation shows an identical pattern of ionic requirements to the purified nuclease. However, the glycosylation of the nuclease with mannose implies that in intact cells it will be found within the ER or nuclear membrane. Therefore, regulators of the end stages of apoptosis such as ICE-like proteases may regulate the accessibility of chromatin to the nuclease while pH and calcium changes regulate its activity.

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