Ca^{2+}/Mg^{2+}-Dependent Endonuclease from Human Spleen: Purification, Properties, and Role in Apoptosis†

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ABSTRACT: A major event in apoptosis is the digestion of chromatin into oligonucleosomal fragments. However, the enzymes responsible for the DNA degradation have not been well characterized. Here we report the purification of an endonuclease from human spleen cell nuclei that is likely to be responsible for DNA digestion in apoptosis. Enzyme activity was measured by a sensitive fluorometric assay, which assesses the conversion of plasmid DNA from a supercoiled to an open form. The endonuclease was extracted from isolated nuclei with NaCl between 100 and 350 mM and was further purified by chromatography on columns of phosphocellulose, Superdex 75, and chelating Sepharose (Zn^{2+} form). By gel filtration, the apparent molecular mass was 22–26 kDa; on SDS–polyacrylamide gel electrophoresis, the purified enzyme showed a single 27-kDa band. The enzyme required both Mg^{2+} (optimum, 5 mM) and Ca^{2+} (optimum, 2 mM) for activity. It was inhibited by Zn^{2+} (100% inhibition at 50 μM) and by high (>10 mM) concentrations of Ca^{2+}. Aurintricarboxylic acid, spermine, *p*-(hydroxymercuri)benzoate, and *N*-ethylmaleimide were also endonuclease inhibitors. No inhibition was observed with iodoacetamide, G-actin, or nucleoside 3′,5′-bisphosphates. An optimum pH of 8.0 was found. When added to human CCRF-CEM lymphoblast nuclei, that do not contain the endonuclease, the purified splenic enzyme digested the chromatin into the mono- and oligonucleosomal fragments that are characteristic of apoptosis. On the basis of this result, and the observation that the activators and inhibitors of the purified endonuclease closely parallel those that affect apoptosis, it seems likely that this enzyme is involved in the apoptotic degradation of DNA in human lymphocytes.

Apoptosis (Kerr et al., 1972) is a physiological process by which cells are deleted from living tissues during normal embryogenesis, differentiation, and metamorphosis (Wyllie et al., 1980; Gerschenson & Rotello, 1992). The role of apoptosis in lymphocytes is especially important and has been studied in great detail [reviewed in Cohen et al. (1992)]. It is considered to participate in the deletion of immature autoreactive lymphocytes during development (Smith et al., 1989; Murphy et al., 1990), in lymphocyte depletion induced by HIV infection (Terai et al., 1991; Groux et al., 1992), and in cell death effected by cytotoxic T-lymphocytes (CTL) (Duke et al., 1983; Ucker, 1987). Apoptosis in lymphocytes may also be induced by pathological stimuli, such as ionizing radiation (Berger, 1985; Carson et al., 1986b), oxidants (Carson et al., 1986a), and various chemotherapeutic agents (Dive & Hickman, 1991). The involvement of apoptosis in cancer pathogenesis has also been suggested. The product of the oncogene *bcl-2* is able to block apoptosis (Hockenberry et al., 1990). Restoring wild-type protein p53 expression in a myeloid leukemic cell line that normally lacks it induces apoptosis (Yonish-Rouach et al., 1991).

Apoptotic cells were first described by their morphological features: compaction of nuclear chromatin against the nuclear membrane, condensation of the cytoplasm with well-preserved organelles, and nuclear and cytoplasmic budding to form membrane-bounded fragments, known as apoptotic bodies, that are rapidly phagocytosed by adjacent cells (Wyllie et al., 1980). These morphological changes are paralleled at the biochemical level by the digestion of chromatin at the internucleosomal linker regions (Wyllie, 1980; Arends et al., 1990). The resulting DNA fragments contain one or several 180–200 base pair (bp)[1] units that are visible as a typical ladder pattern after separation by aragose gel electrophoresis. The characteristic ladder pattern of DNA digestion in apoptosis contrasts with the smear observed in necrotic cells (probably due to the action of lysosomal proteases and nucleases).

The biochemical pathways that lead to cell death by apoptosis are probably complex, and depend upon cell type and activation state. However, it has been suggested that the last steps may be similar, irrespective of the initial stimulus (Ucker, 1987). The most detailed studies have been done in rodent thymocytes, in which apoptosis is rapidly induced by glucocorticoids (Wyllie, 1980). In this model, apoptosis is Ca^{2+}-dependent, is Zn^{2+}-inhibitable, and requires active synthesis of proteins and RNA (Cohen & Duke, 1984; Wyllie et al., 1984). The putative endonuclease responsible for the DNA degradation seems to be a constitutive enzyme, as nuclei isolated from normal thymocytes were able to autodigest their chromatin in the presence of Ca^{2+}, giving the characteristic ladder pattern (Cohen & Duke, 1984).

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[1] Abbreviations: ATA, aurintricarboxylic acid; bp, base pair(s); BSA, bovine serum albumin; Ca/Mg-DNase, Ca^{2+}/Mg^{2+}-dependent endonuclease; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)propanesulfonic acid; DTT, dithiothreitol; EB, ethidium bromide; MES, 2-(*N*-morpholino)ethanesulfonic acid; pdGp, deoxyguanosine 3′,5′-bisphosphate; PMSF, phenylmethanesulfonyl fluoride; pTp, thymidine 3′,5′-bisphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Wyllie et al. (1984) showed that the calcium ionophore A23187 is able to induce apoptosis in thymocytes. McConkey et al. (1989b) demonstrated that an early and sustained increase in the cytosolic Ca^{2+} concentration takes place after incubation of thymocytes with glucocorticoids, and that apoptosis can be prevented by Ca^{2+}-chelators and by calmodulin inhibitors. However, in some nonlymphoid cell lines, chromatin degradation seems to be Ca^{2+}-independent (Bansal et al., 1990; Alnemri & Litwack, 1990), or even prevented by Ca^{2+} and Ca^{2+} ionophores (Rodríguez-Tarduchy et al., 1992). These data suggest that the endonucleases that cause DNA degradation during apoptosis may differ according to cell type. To determine what regulates DNA digestion during apoptosis in lymphocytes, it is necessary to characterize the endonucleases in these cells.

Because of their low abundance, the endonucleases in normal human lymphocytes have not been purified and studied kinetically. We report here the purification to homogeneity of a nuclear Ca^{2+}/Mg^{2+}-dependent endonuclease (Ca/Mg-DNase)[1] from normal human spleen that is able to digest chromatin in situ into mono- and oligonucleosomal fragments. A very sensitive assay method was used to study several biochemical properties of the purified enzyme.

MATERIALS AND METHODS

Materials. Superose 12 PG, chelating Superose FF, a prepacked column of Superdex 75 HR 10/30, molecular weight markers for gel filtration, and DNA markers were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Molecular weight markers for SDS–PAGE were from Amersham Co. (Arlington Heights, IL) and BioRad (Richmond, CA), phosphocellulose P-11 was from Whatman (Clifton, NJ), and Centriprep, Centricon, and Microcon concentrators were from Amicon (Beverly, MA). Micrococcal nuclease (EC 3.1.31.1) was obtained from Pharmacia, DNase I (EC 3.1.21.1; type IV), bovine serum albumin (BSA, acetylated), and G-actin were from Sigma (St. Louis, MO), and calf thymus DNA ("high molecular weight") was from Boehringer Mannheim GmbH (Indianapolis, IN). pBluescript II SK+ (Stratagene, La Jolla, CA) used to assay the endonuclease activity was purified from *Escherichia coli* cultured in Luria–Bertani medium (Sambrook et al., 1989) using Qiagen columns (Qiagen, Chatsworth, CA), according to the manufacturer's instructions.

Buffers. The following buffers were used during enzyme extraction and purification: buffer A, 10 mM Tris-HCl, pH 7.5, 10% glycerol, 0.2 mM EDTA, and 0.5 mM DTT; buffer B, 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 0.5 mM DTT; buffer C, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5 mM DTT; buffer D, 25 mM Tris–acetate, pH 7.5, and 1.0 M NaCl; buffer E, 25 mM Tris–acetate, pH 7.5, and 1.0 M NH_4Cl. Immediately prior to use, buffers were supplemented with 0.5 mM phenylmethanesulfonyl fluoride (PMSF; from a 50 mM stock solution in 2-propanol), 1 μg/mL antipain, and 1 μg/mL leupeptin. Antipain and leupeptin in combination have been shown previously to protect endonuclease activity in cell culture (McConkey et al., 1990).

Endonuclease Assay. Method I. Endonuclease activity was assayed by the fluorometric method of Paoletti and Le Pecq (1971), slightly modified. The method depends upon the difference in the amount of ethidium bromide (EB) that binds to a fully closed circular DNA as compared with the same DNA which contains one or more breaks. Because open circles can bind more EB, an increase in fluorescence is observed when a plasmid is opened by an endonuclease. The assay mixture contained in a volume of 0.35–1 mL (see below) 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 5 μg/mL plasmid (pBluescript II SK+), and enzyme (usually 10 μl/mL of a diluted preparation in buffer A containing 1 mg/mL acetylated BSA). The mixture was preincubated at 37 °C for approximately 10 min, and the reaction was started by addition of enzyme. At different times after addition of endonuclease, 100-μL aliquots were pipetted out and added to 1.25 mL of stop solution at room temperature (0.6 μg/mL EB, 6 mM Tris-HCl, pH 8.0, 12 mM NaCl, and 3 mM EDTA). The actual volume of the reaction mixture was fixed according to the number of time-points wanted (usually 0, 10, and 30 min for routine assays and 0, 2, 5, 10, and 20 min for kinetic studies). Fluorescence intensity was measured in a Gilson filter fluorometer (excitation 520 nm, analysis 565–600 nm) in 10 × 75 mm glass tubes. One unit is the amount of enzyme required to open 50% of the plasmid substrate present in 100 μL of assay mixture in 10 min under standard conditions. This is equivalent to 1.11×10^{10} breaks per minute. During enzyme purification, controls in which 2 mM EGTA was substituted for $CaCl_2$ were run in parallel. With purified enzyme, the assay was shown to be linear over at least a 25-fold range; similar results were obtained with DNase I (not shown).

Method II. The previous method was not applicable when testing the effect on the endonuclease of compounds that quench EB fluorescence, or that compete with EB for binding to DNA. In these cases, the endonuclease activity was assayed by measuring the amount of perchloric acid-soluble DNA fragments generated by the enzyme. The reaction mixture (250 μL) was as in method I with high molecular weight calf thymus DNA (20 μg) substituting for the plasmid; the reaction was stopped with 50 μL of ice-cold 3 N perchloric acid, left at 4 °C for 15 minutes, and centrifuged at 16000*g* for 10 min, after which the absorbance of the supernatant (acid-soluble fragments) was read at 260 nm in a 300-μL cuvette.

Chromatin Digestion in Situ. Nuclei were prepared by hypotonic shock [modified from Wira and Munck (1974)] from CCRF-CEM human T lymphoblasts (ATCC CCL 119), cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 2 mm L-glutamine (Gibco BRL, Grand Island, NY). Briefly, $\sim 5 \times 10^8$ cells were incubated in 10 mL of 1.5 mM $MgCl_2$ on ice for 15 min, and the nuclei were pelleted, resuspended in 5 mL of a 25% glycerol buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 25% glycerol; Spelsberg et al., 1974) with 0.5 mM DTT and 0.5 mM PMSF, and stored at −20 °C until needed. Chromatin digestion was done essentially as described by McConkey et al. (1990). Prior to use in the protocol, nuclei were resuspended in TKM buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, and 10 mM $MgCl_2$) at $\sim 10^7$ nuclei/mL. Assays were carried out in 400-μL aliquots to which $CaCl_2$ (1 mM) and enzyme were added; control tubes in which EGTA (5 mM) was substituted for $CaCl_2$ and/or buffer A for enzyme were run in parallel. Tubes were tumbled at 37 °C for the desired times, the reaction was stopped with 800 μL of ice-cold lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100), and the low molecular weight DNA fragments were separated from the high molecular weight chromatin by centrifugation for 15 min at 16000*g*. Pellets were resuspended in 0.2 mL of STE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM EDTA), and then 0.2 mL of STE with 1% SDS was added. To quantitate the extent of the chromatin digestion, both pellets and supernatants were incubated overnight with proteinase K

(100 μg/mL) and assayed for DNA (Burton, 1956). DNA fragmentation was expressed as the percentage of the total DNA (supernatant plus pellet) present in the 16000*g* supernatant. To assess the type of fragments generated, aliquots (700 μL) from the supernatants were made 200 mM NaCl, precipitated with 2-propanol (700 μL), washed with 70% ethanol, resuspended in 10 mM Tris-HCl, pH 8.0, and 10 mM EDTA, treated with RNase (50 μg/mL), and separated by electrophoresis in a 2% agarose gel in Tris–acetate/EDTA buffer (pH 8.0) containing 0.5 μg/mL ethidium bromide for 2.5 h at 60 V.

Nuclei Purification from human Spleen. Nuclei were purified from human spleen by a modification of the method described by Spelsberg et al. (1974). Surgically removed human spleen was brought to the laboratory as quickly as possible, washed in cold phosphate-buffered saline (PBS), sliced, and weighed after removing the outer membrane, large blood vessels, and excess buffer. Tissue was homogenized in 6 volumes of buffer B supplemented with 0.32 M sucrose in a blender with three 1-min pulses, and the homogenate was filtered through two layers of cheesecloth and centrifuged for 30 min at 10000*g*. The pellet was resuspended in 6 volumes of buffer B with 1.7 M sucrose, homogenized further in a glass–Teflon homogenizer with 4 strokes, and centrifuged as before. This last step was repeated once, and the new pellet was resuspended in 5 volumes of buffer B with 0.32 M sucrose and 0.2% Triton X-100 and centrifuged as before. The obtained nuclear pellet was resuspended directly in extraction buffer (see Figure 2).

Molecular Weight Determination. The native molecular mass was determined by gel filtration both in a prepacked Superdex 75 HR 10/30 column and in a Superose 12 column (1.6 × 50 cm; 100 mL), using the following proteins as standards (Pharmacia): bovine serum albumin (67 kDa), hen egg ovalbumin (43 kDa), bovine pancreas chymotrypsinogen A (25 kDa), and bovine pancreas ribonuclease A (13.7 kDa).

SDS–Polyacrylamide Gel Electrophoresis (PAGE). SDS–PAGE was performed as described by Laemmli (1970) in 8 × 7 cm slabs containing 14% acrylamide, stained with Coomassie blue (0.025% in 25% 2-propanol/10% acetic acid), or with silver following Morrissey's method (1981) as modified by Jones (1990). The following proteins (BioRad) were used as molecular mass markers: rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). Proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes (BioRad) in 10 mM CAPS buffer, pH 11, with 10% (v/v) methanol (Matsudaira, 1987), and the membranes were stained with Coomassie blue (0.1% in 50% methanol) and/or with colloidal gold (BioRad).

Other Methods. Protein concentrations were determined by the method of Bradford (1976) using BSA as standard. DNA concentrations were assayed by the method of Burton (1956) or, in purified plasmid samples, by the absorbance at 260 nm (Sambrook et al., 1989).

RESULTS

Enzyme Purification. In order to check that a Ca^{2+},Mg^{2+}-dependent endonuclease (Ca/Mg-DNase) activity was present in nuclear extracts prepared from human splenocytes, nuclei purified as described under Materials and Methods were extracted successively with different concentrations of NaCl. The endonuclease activity of each preparation was then assayed in the presence of Ca^{2+} and Mg^{2+} (Figure 1). The nuclear extract obtained between 100 and 350 mM NaCl contained the bulk of the activity and, more important, had the highest specific activity; this preparation (referred henceforth as "nuclear extract") was thus used as starting material for the purification of the enzyme. Endonuclease activity in all fractions was inhibited more than 95% when EGTA was substituted for $CaCl_2$.

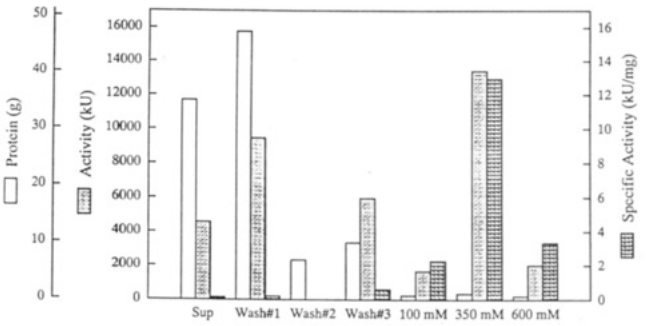

FIGURE 1: Endonuclease activity in the different fractions obtained during nuclei purification and extraction. Activity was assayed fluorometrically in the presence of Ca^{2+} and Mg^{2+} as described under Materials and Methods (method I). The 10000*g* supernatant ("Sup") and the nuclear wash eluants ("wash # 1", "wash # 2", and "wash # 3", the latter containing 0.02% Triton X-100) were prepared as described under Materials and Methods. Nuclear extracts were obtained by sequential extraction with 100, 350, and 600 mM NaCl in buffer C, and were diluted at least 100-fold prior to assay, to prevent cation inhibition. In all cases, when EGTA was substituted for $CaCl_2$, more than 90–95% of the activity was inhibited. Activity values in crude preparations could not be measured accurately because exonucleases and other endonucleases are present. The values reported are thus approximate.

The details of the purification scheme for Ca/Mg-DNase are given in the legend to Figure 2. The enzyme eluted from the phosphocellulose column as a single peak with 700 mM NaCl (Figure 2A) and from the Superdex 75 column as a symmetrical peak with an apparent molecular weight of 26 × 10^3 (Figure 2B), and was adsorbed to the Zn^{2+}-chelating column in Tris–acetate buffer, being eluted with a gradient of NH_4Cl (Figure 2C).

The results of the purification are summarized in Table I. The specific activity after the last purification step could not be accurately calculated, insofar as the amount of protein was close to the limit of sensitivity of the Bradford method. It was also impossible to calculate the absolute purification, since the crude nuclear extracts contained a mixture of exonucleases and endonucleases. However, we estimate that the enzyme was purified more than 1000-fold, compared to the 100–350 mM NaCl nuclear extract used as starting material. Because the nuclear extract contains less than 1% of the total cell protein (Figure 1), the overall purification was much greater. The enzyme after the Zn^{2+}-chelating column (in Tris–acetate buffer with NH_4Cl) was very unstable, and 90% of the activity was lost in 48 h at 4 °C. The activity could nevertheless be stabilized by desalting quickly against buffer A. By desalting and addition of 1 mg/mL BSA, the enzyme could be stored several weeks at 4 °C with almost no further loss of activity.

The preparation from step 3 of purification (Table I), although showing several bands of protein when analyzed by SDS–PAGE (Figure 3A), was markedly enriched in a protein of apparent molecular weight 17 × 10^3, that was identified from the sequence of its *N*-terminus as the high mobility group protein HMG-17 (data not shown). This protein was effectively removed by the Zn^{2+}-chelating column, and after step 4, the preparation showed only one specific silver staining band of apparent molecular weight 27 × 10^3 on SDS–PAGE

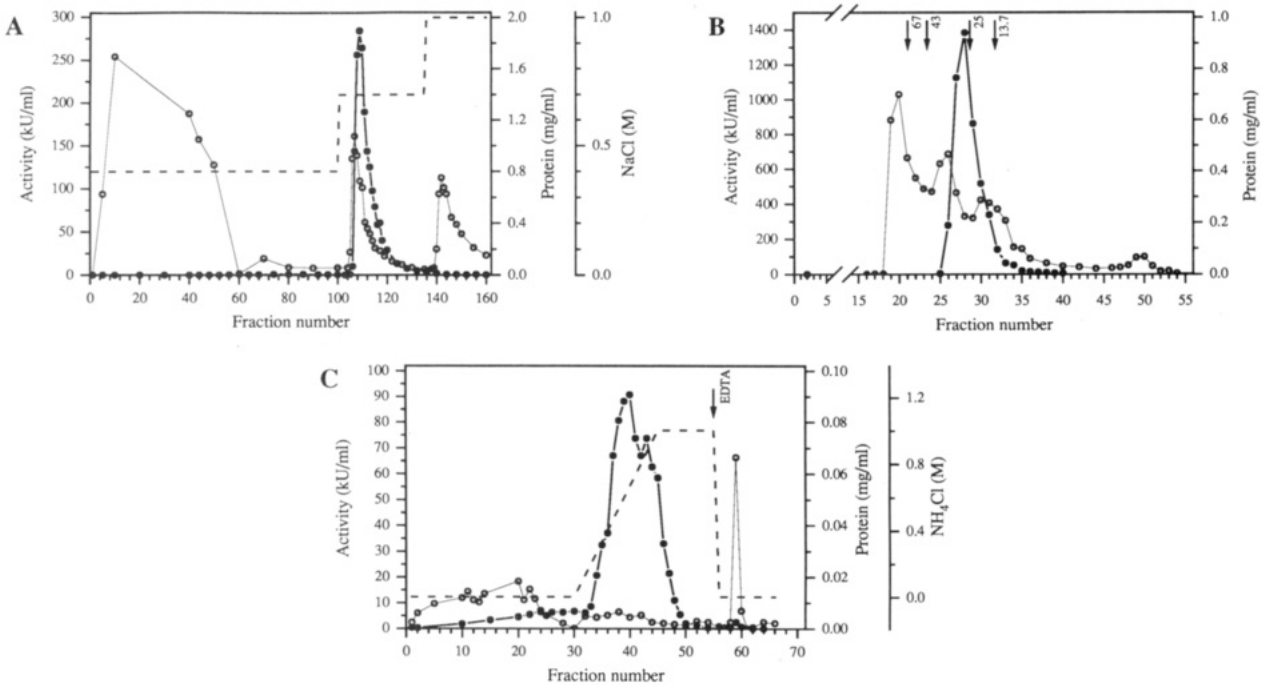

FIGURE 2: Ca^{2+}/Mg^{2+}-dependent endonuclease purification. The endonuclease was purified from nuclei prepared from 660 g of a human spleen. All procedures were carried out at 4 °C. Protease inhibitors were added to all buffers immediately prior to use at a final concentration of 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/mL antipain, and 1 μg/mL leupeptin. *Nuclear extract* (step 1). The nuclear pellets were resuspended in 0.6 mL/g of tissue of buffer C, with 100 mM NaCl, extracted for several hours in a gyratory platform at 4 °C, and centrifuged for 30 min at 3000*g*. The pellet was resuspended in buffer C with 350 mM NaCl (0.4 mL/g of tissue), extracted as before, and centrifuged 30 min at 3000*g* and again 30 min at 37000*g*. The supernatant (100–350 mM NaCl nuclear extract) was used as starting material for the purification. *Phosphocellulose column* (step 2, panel A). The nuclear extract (251 mL) was applied at a flow rate of 60 mL/h to a phosphocellulose column (2.5 × 8.1 cm; 40 mL) equilibrated in buffer A, with 0.4 M NaCl. The column was eluted with a stepwise gradient of 0.4, 0.7, and 1.0 M NaCl in buffer A, and fractions of 6 mL were collected. Fractions 108–117 were pooled and concentrated in a Centriprep-10 to approximately 1 mL. *Superdex 75 column* (step 3, panel B). Material from step 2 was applied to a prepacked Superdex 75 HR 10/30 column (1 × 30 cm; 24 mL) in several 200-μL aliquots. The column was eluted with buffer A supplemented with 100 mM NaCl at a flow rate of 24 mL/min, and the fractions (0.4 mL, each) from successive runs were collected into the same tubes. Fractions with maximum activity (27–29) were pooled and diluted 10 times with buffer D. *Chelating Superose column* (step 4, panel C). Prior to use, the chelating Superose column (0.5 × 6 cm; 1.2 mL) was equilibrated in buffer D, charged with 3 mL of 10 mM $ZnCl_2$, and reequilibrated with the same buffer. The sample was applied at 30 mL/h; the column was washed with 10 mL of buffer D and then was eluted with a 15-mL linear gradient from buffer D to buffer E. Finally, it was washed with 10 mM EDTA in buffer D. Fractions of 1 mL were collected, and those corresponding to the peak of activity (37–45) were pooled, dialyzed against buffer A, and used to characterize the enzyme. The symbols are as follows: endonuclease activity (●); protein (○); salt concentration (- - -). The arrows in panel B indicate the elution volume of protein markers run under identical conditions, with masses given in kilodaltons. The arrow in panel C shows the point of application of the 10 mM EDTA wash.

Table I: Purification of the Ca^{2+}/Mg^{2+}-Dependent Endonuclease from Human Nuclei[a]

step	protein (mg)	act. (kilounits)	sp act. (kilounits/mg)	yield (%)
(1) nuclear extract	1036	13400	12.9	100
(2) phosphocellulose	21.2	10440	500	78
(3) Superdex 75	0.38	1285	3400	10
(4) Zn^{2+}-chelating Superose	(0.04)	596	(15000)	4

[a] The purification started with 660 g of human spleen. Activity was determined as described under Materials and Methods (method I), and protein was assayed by the method of Bradford. Kilounits of enzyme are as defined under Materials and Methods. Values in parentheses are approximate (see text).

(Figure 3B,C); other minor bands seen in Figure 3C were present in all lanes of the gel, including those in which marker proteins were loaded, and are probably staining artifacts (not shown).

Chromatin Digestion in Situ. To study the effect of the purified endonuclease on intact chromatin, nuclei isolated from CCRF-CEM cells were used as substrate. According to previous reports, CCRF-CEM nuclei do not autodigest their chromatin when incubated with Ca^{2+} and Mg^{2+} as do, for instance, rodent thymocyte nuclei (Alnemri & Litwack, 1989).

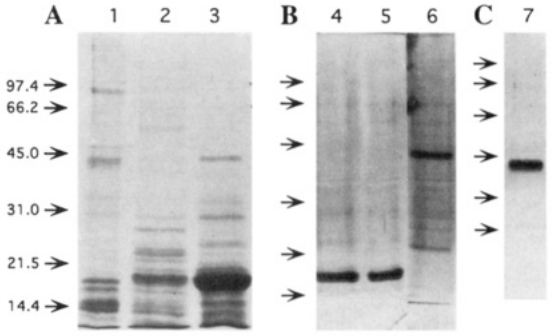

FIGURE 3: SDS–PAGE analysis of the Ca^{2+}/Mg^{2+}-dependent endonuclease activity at different stages of purification. (A, lanes 1–3) Material from the nuclear extract (40 μg of protein; 500 units of enzyme), the phosphocellulose step (3.6 μg; 1800 units), and the Superdex column (3.6 μg; 12 200 units). (B, lanes 4–6) Aliquots from Zn^{2+}-chelating column fractions 10 and 20 (flowthrough) and fraction 59 (EDTA wash). (C, lane 7) Material from step 4 of purification (Zn^{2+}-chelating column; Table I). Electrophoresis was performed by the method of Laemmli in slabs containing 14% acrylamide, and the gel was stained with Coomassie blue (panel A) or silver (panels B and C). Arrows indicate the positions of protein markers run in parallel, with masses given in kilodaltons.

Incubation of CCRF-CEM nuclei with the endonuclease resulted in DNA degradation that was Ca^{2+}- and Mg^{2+}-

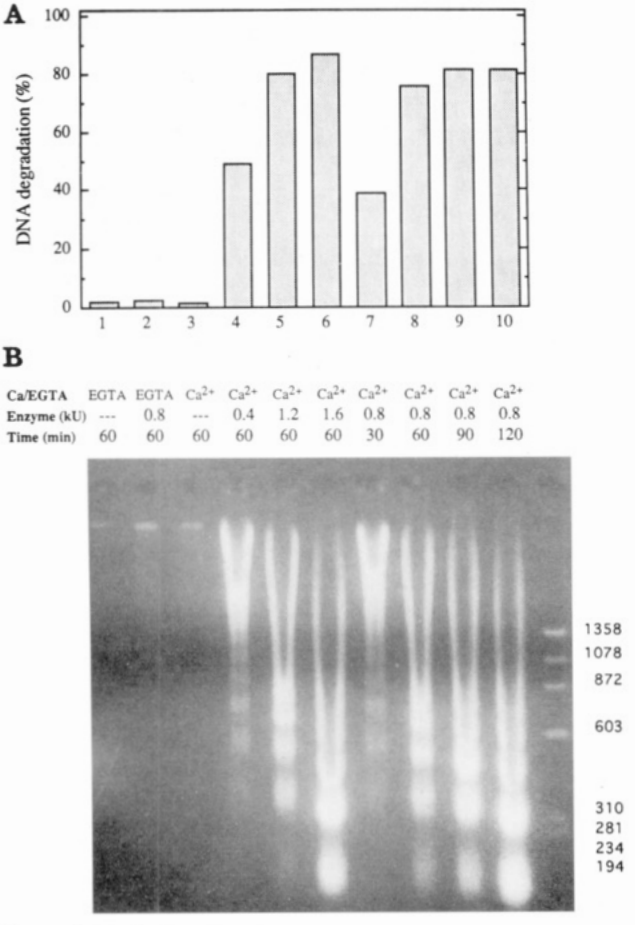

FIGURE 4: *In situ* digestion of chromatin by the Ca^{2+}/Mg^{2+}-dependent endonuclease. (A) CCRF-CEM lymphoblast nuclei (5×10^6; 35 μg of DNA) were incubated in TKM buffer in the presence of 1 mM $CaCl_2$ with different amounts of endonuclease (columns 4–6, 415, 1240, and 1660 units, respectively) for 60 min, or with 830 units of enzyme for different periods of time (columns 7–10, 30, 60, 90, and 120 min, respectively.) Columns 1–3, controls with EGTA (5 mM) substituting for Ca^{2+} in the absence (column 1) or the presence (column 2) of enzyme, and without enzyme (column 3). DNA fragmentation is expressed as the percentage of the total DNA (pellet plus supernatant) that is present in the 16000*g* supernatant. (B) DNA fragments were separated by electrophoresis in a 2% aragose gel. Lanes 1–10 correspond to columns 1–10 of (A). Lane 11, $\phi X174$ plasmid cut with *Hae*III used as standard, with fragment size, expressed in base pairs, shown to the right.

dependent (Figure 4A). When low molecular weight DNA was isolated and analyzed by electrophoresis, the oligonucleosome fragments characteristic of apoptosis were visualized (Figure 4B). Incubation with greater amounts of enzyme, or for longer periods, resulted not only in a higher percentage of DNA fragments (Figure 4A, columns 4–6 and 7–10) but also in a smaller average size of the fragments (Figure 4B). A similar ladder pattern can be obtained by treating isolated nuclei with micrococcal nuclease, but not with mammalian DNase I (Vanderbilt et al., 1982). In order to produce a ladder pattern, DNase I must be preincubated with nuclear extracts (Peitsch et al., 1993).

Molecular Weight. The native molecular weight was determined by gel filtration both in a Superose 12 column (1.6 × 50 cm; 100 mL) (not shown) and in a prepacked Superdex 75 HR 10/30 column. The endonuclease eluted after 65 mL ($K_{av} = 0.44$) and 11 mL ($K_{av} = 0.24$), respectively, corresponding to an apparent molecular mass of 22–26 kDa (see Figure 3B), consistent with the 27-kDa band observed after SDS–PAGE.

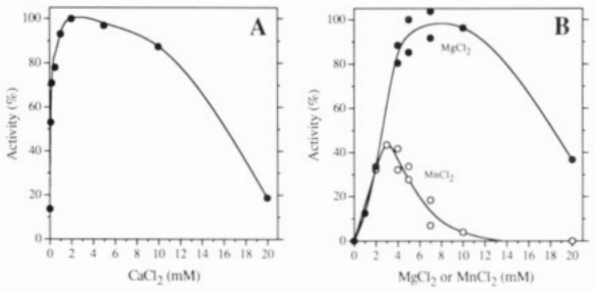

FIGURE 5: Effect of Ca^{2+} (panel A) and of Mg^{2+} and Mn^{2+} (panel B) on the endonuclease activity. The experiment shown in panel A was done in the presence of 5 mM $MgCl_2$, and those described in panel B were performed in the presence of 2 mM $CaCl_2$. Activity is expressed as a percentage of that obtained in standard conditions (see method I under Materials and Methods). The small amount of activity detected in the absence of exogenously added Ca^{2+} (in panel A) was completely inhibited by EGTA (not shown).

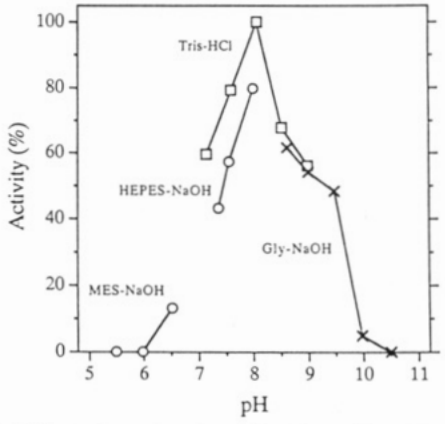

FIGURE 6: Effect of varying the pH on the endonuclease activity. Activity was assayed as described under Materials and Methods (method I), substituting the indicated buffer for Tris-HCl, pH 8.0.

Cation Requirements. The enzyme required both Mg^{2+} and Ca^{2+} for full activity. In the presence of 5 mM $MgCl_2$, maximum velocity was obtained with 1–5 mM $CaCl_2$ (Figure 5A). The residual activity observed in the absence of exogenously added Ca^{2+} was inhibited more than 99% by EGTA. In the presence of 2 mM $CaCl_2$, the maximum velocity was observed at 5–10 mM $MgCl_2$ (Figure 5B). Among several other divalent cations tested (Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}, all assayed as chloride salts at 5 mM), only Mn^{2+} was able to partially substitute for Mg^{2+} (Figure 5B), although the activity with $MnCl_2$ was only 30% of that with $MgCl_2$. High concentrations of these divalent cations inhibited the enzyme activity: more then 80% inhibition was obtained with 20 mM $CaCl_2$ or with 10 mM $MnCl_2$; 20 mM $MgCl_2$ gave an inhibition of 60%.

Optimum pH. The effect of pH on enzyme activity was monitored with MES–NaOH, HEPES–NaOH, and glycine–NaOH at pH values between 5.5 and 10.0 (Figure 6). The optimum pH of the reaction was 8.0. Similar results were obtained using Tris-HCl at pH values between 7.0 and 9.0. Unexpectedly, no activity was observed with CHES–NaOH at pH values between 8.5 and 10.0.

Inhibitors. Zinc is known to inhibit apoptosis in many (Cohen & Duke, 1984; Migliorati et al., 1993) although not all (Capri et al., 1992) models studied. $ZnCl_2$ blocked endonuclease activity at micromolar concentrations: 50% and 100% inhibition was reached at 15 and 40 μM, respectively. Among other divalent cations (chloride form) assayed at 50 μM, Hg^{2+} inhibited the enzyme 100% and Ni^{2+} and Cu^{2+}

Table II: Effect of Several Compounds on the Ca^{2+}/Mg^{2+}-Dependent Endonuclease

compd[a]	concn	inhibn (%)	reported inhibitor of
p-(hydroxymercuri)benzoate	1 mM	75	Ca/Mg-DNase (pig liver) (100 % at 1 mM)
	5 mM	100	
N-ethylmaleimide	0.02 mM	38	Ca/Mg-DNase (bull semen) (100% at 0.1 mM)
	0.1 mM	55	
	10 mM	62	
iodoacetamide	1 mM	<10	Ca/Mg-DNase (pig liver) (83% at 1 mM)
G-actin	100 μg/mL	0	DNase I
thymidine 3′,5′-bisphosphate	1 mM	0	micrococcal nuclease Ca/Mg-DNase (chicken erythrocytes)
deoxyguanosine 3′,5′-bisphosphate	1 mM	0	micrococcal nuclease
aurintricarboxylic acid[b]	1 mM	100	several DNases and RNases[c]
polyamines[b,d]			Ca/Mg-DNase (bull semen, rat liver)
spermine	1 mM	92	(100% at 1 mM)
spermidine	1 mM	<10	(50%, 1 mM; 100%, 5 mM)
putrescine	1 mM	<10	

[a] Enzyme was assayed as described under Materials and Methods (method I, except where indicated), adding the potential inhibitor to the assay mixture at the indicated concentration(s). [b] Assayed by method II (see Materials and Methods). [c] Aurintricarboxylic acid was shown to inhibit rat thymocyte apoptosis *in vitro* (100% at 0.5 mM). [d] Spermine and to a less extent spermidine have been reported to inhibit rat thymocyte apoptosis *in vitro* (100% and 10% at 0.9 mM, respectively).

around 40%. No inhibition was observed with Co^{2+}, Sr^{2+}, or Ba^{2+}.

Compounds described in the literature as inhibitors of other endonucleases were tested on the purified human Ca/Mg-DNase (Table II). The enzyme was inhibited by *p*-(hydroxymercuri)benzoate (100% at 5 mM) and *N*-ethylmaleimide (60% at 0.1–10 mM), previously described as inhibitors of a Ca/Mg-DNase purified from pig liver and bull semen, respectively (Strätling et al., 1984; Hashida et al., 1982). The enzyme was not inhibited by iodoacetamide (1 mM), a specific inhibitor of the pig liver Ca/Mg-DNase (Strätling et al., 1984), nor by G-actin (100 μg/mL), an inhibitor of DNase I (Lacks, 1981). Thymidine 3′,5′-bisphosphate (pTp), an inhibitor of micrococcal nuclease and of a Ca/Mg-DNase present in chicken erythrocyte nuclei (Sulkowski & Laskowski, 1968; Kalinski et al., 1980), and deoxyguanosine 3′,5′-bisphosphate (pdGp), a weaker inhibitor of micrococcal nuclease (Sulkowski & Laskowski, 1968), both failed to inhibit the purified human enzyme under conditions in which micrococcal nuclease was effectively inhibited completely by pTp, and partially by pdGp. Aurintricarboxylic acid (ATA), a DNase and RNase inhibitor that has also been shown to inhibit apoptosis (Hallick et al., 1977; McConkey et al., 1989a) due to its ability to bind to DNA, was assayed by method II (see Materials and Methods) and found to completely inhibit the enzyme at 1 mM. Spermine, a compound shown to inhibit both apoptosis in rat thymocytes and DNA autodigestion in thymocyte and DNA autodigestion in thymocyte nuclei (Brüne et al., 1991; Vanderbilt et al., 1982) (but not the other polyamines spermidine and putrescine, all assayed by method II), inhibited around 90% at 1 mM.

DISCUSSION

We have purified from normal human lymphoid tissue an endonuclease that absolutely requires both Ca^{2+} and Mg^{2+}, and that is able to hydrolyze native chromatin into oligonucleosomal fragments. These two major properties will be used to discuss the enzyme's function, and to compare it with other endonucleases described previously. The enzymes specific for damaged DNA [reviewed in Lindahl (1990)] showed a completely different substrate specificity from the human Ca/Mg-DNase, and will not be discussed here.

The dependence of the endonuclease on both Ca^{2+} and Mg^{2+} distinguishes it from most previously reported mammalian endonuclease activities that require only one cation, usually Mg^{2+} (Wang & Furth, 1977; Wang et al., 1978; Fischman et al., 1979; Nakayama et al., 1981; McKenna et al., 1981; Desiderio & Baltimore, 1984; Kataoka et al., 1984; Tomkinson & Linn, 1986; Cummings et al., 1987; Hibino et al., 1988, 1989; Cote et al., 1989; Gottlieb & Muzyczka, 1990). For several of these latter enzymes, Ca^{2+} was actually an inhibitor of the reaction (Wang et al., 1978; Nakayama et al., 1981; Hibino et al., 1988). In one case, it could substitute for Mg^{2+} at low concentrations, but was an inhibitor at higher concentration (Gottliet & Muazyczka, 1990). An enzyme described by Hibino et al. (1989) as a Ca^{2+}/Mg^{2+}-dependent endonuclease was actually active in the presence of either Ca^{2+} or Mg^{2+} alone, and the activity did not increase significantly when both cations were added.

The existence of an endonuclease that strictly requires both Ca^{2+} and MG^{2+} for full activity was first suggested by Burgoyne et al. (1970, Hewish & Burgoyne, 1973). The characteristic ability of the enzyme to cleave chromatin into 180–200 bp fragments aided in the characterization of the nucleosome as a structural unit of chromatin (Burgoyne & Hewish, 1978). Similar enzymes have been characterized in rat liver (Ishida et al., 1974; Yoshihara et al., 1974), calf thymus (Nakamura et al., 1981), bull seminal plasma (Hashida et al., 1982), porcine liver (Strätling et al., 1984), and more recently rat thymocytes (Gaido & Cidlowski, 1991). An endonuclease activity that was Ca^{2+}-dependent, and probably also Mg^{2+}-dependent, was extracted, but not further purified, from NIH 3T3 cell nuclei (Ucker et al., 1992). We have been able to extract and partially purify an endonuclease, with properties identical to those of the spleen enzyme reported here, from normal human thymus and placenta, but not from CCRF-CEM lymphoblasts or other malignant T lymphoblastic cell lines (Ribeiro and Carson, unpublished results). The results indicate that the enzyme is constitutively expressed in only a limited number of normal cell types, and is apparently absent in malignant lymphocytes.

Since very few groups have purified the Ca^{2+}/Mg^{2+}-dependent endonuclease to homogeneity, information on the molecular mass of the protein is limited. Hashida et al. (1982) found only one band of 36 kDa in a preparation from bull seminal plasma. Gaido and Cidlowski (1991) and Ucker et al. (1992), using *in situ* assays for activity in the gel, assigned molecular masses of 18 and 40 kDa to the endonuclease, respectively. Our preparation showed only one band of 27 kDa.

There are clear-cut differences in the effects of inhibitory compounds on the endonucleases purified from different

sources. The enzyme analyzed by Hashida et al. (1982) was inhibited by 0.1 mM *N*-ethylmaleimide (100%), whereas that described by Strätling et al. (1984) was blocked by 1 mM iodoacetamide (83%) and 1 mM *p*-(hydroxymercuri)benzoate (100%). We observed a total inhibition of endonuclease activity by 5 mM *p*-(hydroxymercuri)benzoate, but no inhibition at all in the presence of 1 mM iodoacetamide. With *N*-ethylmaleimide, we were able to inhibit 60% of enzyme activity. The effects of other compounds on the human endonuclease were similar to those reported for the Ca/Mg-DNase(s) from other species, i.e., inhibition by spermine (Hashida et al., 1982), aurintricarboxylic acid (Gaido & Cidlowski, 1991), and zinc (Gaido & Cidlowski, 1991).

Recently, Peitsch et al. (1993) reported that DNase I was responsible for nuclear DNA degradation during apoptosis in rat thymocytes. This conclusion was based upon the ability of both G-actin and anti-DNase I antibody to inhibit the major endonuclease activity extracted from nuclei. However, DNase I migrates in SDS–polyacrylamide gels at an apparent molecular mass of 34 kDa, clearly distinguishing it from the 27-kDa endonuclease from human spleen. Moreover, the human spleen endonuclease, in contrast to DNase I (Lacks, 1981), is not inhibited by G-actin. DNase I is mainly a cytosolic enzyme. It is conceivable that the nuclear Ca/Mg-DNase acts early in apoptosis in lymphocytes, whereas DNase I is important in the later stages, when dissolution of the nuclear membrane permits cytosolic enzymes to gain access to chromatin. The constitutive Ca/Mg-DNase reported here may also not be responsible for DNA fragmentation in all cell types. Thus, nuclei from cell lines (e.g., CCRF-CEM) that under some conditions can undergo apoptosis are not able to autodigest their chromatin in the presence of Ca^{2+} (Alnemri & Litwack, 1989) and lack detectable Ca^{2+}/Mg^{2+}-dependent endonuclease.

Considering that compounds known to block apoptosis (zinc, spermine, and aurintricarboxylic acid) all inhibited the activity of the purified Ca/Mg-DNase, this enzyme must be considered a likely candidate for the endonuclease involved in the apoptotic degradation of DNA in normal human spleen lymphocytes. The purification of the human spleen Ca^{2+}/Mg^{2+}-dependent endonuclease will facilitate the preparation of DNA and antibody probes that can identify the enzyme in intact cells, and may aid in the identification of specific enzyme inhibitors. Such tools are critical to evaluate the role of this endonuclease in apoptosis. It is possible that the enzyme is widely distributed but is usually inactivated. Perhaps the interaction of the enzyme with another molecule, or its posttranslational modification, can lower the concentration of Ca^{2+} needed for full activity. Additional experiments will be needed to distinguish among these possibilities.

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