# Identification of a Novel 97 kDa Endonuclease Capable of Internucleosomal DNA Cleavage

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ABSTRACT: The two steps of DNA digestion seen in apoptotic cells were recreated in nuclei isolated from 5123tc rat hepatoma cells. The initial DNA cleavage, into high molecular weight fragments (300−50 kb), was stimulated by magnesium ions alone, whereas the second step required both calcium and magnesium ions and produced the ladder of oligonucleosomes. Endonucleolytic activities involved in both steps of DNA cleavage could be separated under appropriate conditions since the magnesium-modulated activity was tightly bound to the chromatin whereas the calcium/magnesium-dependent internucleosomal cleaving activity was easily extractable with a low ionic strength buffer. This calcium/magnesium-dependent activity was attributed to a novel 97 kDa endonuclease which was also activated by manganese and cobalt and inhibited by millimolar concentrations of zinc, consistent with the properties ascribed to the apoptotic endonuclease. Furthermore, this activity became resistant to extraction with a low salt buffer in nuclei of apoptotic cells. Isoelectrofocusing revealed that the p97 protein existed in multiple forms of different isoelectric points (p*I* range 4.6−5.0), indicative of its postranslational modification. The p97 enzyme was present constitutively in a variety of cultured cells and rat tissues. It was active over a broad range of pH (6−9), but it was inactivated by reducing agents. *In vitro,* it displayed both endo- and exonucleolytic activities, and it was capable of both single- and double-stranded DNA cleavage. Rabbit polyclonal anti-p97 antibodies were generated and used to further distinguish this protein from other known cellular nucleases, namely, DNases I and II.

Although DNA cleavage into both loop-size and oligonucleosomal fragments has become the hallmark of apoptotic cell death, the molecular mechanisms of nuclear disintegration are still not fully understood. The phenomenon has been extensively studied both *in vivo* and *in vitro* in a variety of cell systems, and, as a result, a number of cellular endonucleases, namely, DNase I, DNase II, and Nuc18, as well as several other endo- and exonucleases have been implicated. However, a consensus has not been reached thus far as to the identity of the apoptotic endonuclease (Wyllie et al., 1980; Caron-Leslie et al., 1991; Gaido & Cidlowski, 1991; Barry & Eastman, 1992, 1993; Pietsch et al., 1993a, 1994; Fraser, 1994; Torriglia et al., 1995; Anzai et al., 1995; Gottlieb et al., 1995; Shiokawa et al., 1995; Takauji et al., 1995). There is also evidence that within any given cell type separate endonucleolytic activities are involved in the generation of high and low molecular weight DNA fragments (Filipski et al., 1990; Walker et al., 1994, 1995; Sun & Cohen, 1994). Therefore, it is not clear from these studies whether several different endonucleases are involved in apoptosis of the various cell systems or whether an as yet unidentified enzyme is responsible for chromatin fragmentation during active cell death in all cell types. Furthermore, although all apoptotic cells degrade their genome, the oligonucleosomal DNA ladder is not universally present in every apoptotic cell (Ucker et al., 1992; Oberhammer et al., 1993; Zakeri et al., 1993; Pandey et al., 1994). When DNA from such cells is analyzed by pulsed field gel electrophoresis (PFGE), a specialized technique for resolving DNA molecules in the range of kilo- to megabases, there is considerable high molecular weight (HMW) DNA degradation producing fragments of 50−300 kb (Walker et al., 1991; Oberhammer et al., 1993). This raises the question of whether such cells are lacking an enzymatic activity responsible for internucleosomal DNA cleavage or its activation pathway is defective. Our recent data suggest that cells maintain separate pools of endonucleolytic activity responsible for the high and low molecular weight DNA fragmentation and, depending on the cell type, one or both enzymatic pools become activated during apoptosis (Walker et al., 1994; Pandey et al., 1994).

In the present study, we have characterized a novel $Ca^{2+}$/$Mg^{2+}$-dependent endonuclease of approximately 97 kDa capable of internucleosomal DNA cleavage which may be responsible for the secondary stages of chromatin degradation in apoptosis. We also demonstrated that following removal of this enzyme a $Mg^{2+}$-dependent HMW DNA cleaving activity remains more tightly associated with chromatin and its activity is sufficient to degrade DNA to 50−300 kb fragments and bring about apoptotic nuclear changes. Furthermore, we have generated rabbit polyclonal anti-p97 antibodies and used them to further distinguish the p97 protein from other known cellular nucleases, namely, DNases I and II.

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## EXPERIMENTAL PROCEDURES

*Cell Lines and Tissues.* Rat 5123tc hepatoma cells derived from the original malignant hepatoma produced in a female Buffalo rat fed the slow-acting carcinogen *N*-2-fluorenylphthalamic acid (Morris, 1965) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL). Human breast carcinoma MCF7 cells (ATCC HTB 22) were grown in MEM supplemented with 10% FBS and 100 nM bovine insulin. Rat adrenal pheochromocytoma PC12 cells (ATCC CRL 1721) were grown in RPMI medium supplemented with 5% FBS and 10% horse serum (Gibco BRL). Human prostatic carcinoma DU145 cells (ATCC HTB 81) were propagated in Eagle's MEM supplemented with 10% FBS. Rat epithelial T51B cells, established by Swierenga et al. (1978), were grown in MEM supplemented with 10% FBS.

Liver, thymus, kidney, and brain tissues were obtained from 5 week old 150−200 g male Sprague-Dawley rats bred in this Institute.

*Isolation and Digestion of Nuclei.* Cultured cells (~$8 \times 10^6$/sample) were harvested by mechanical dislodging and washed twice in isolation buffer (0.25 M sucrose, 15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 2.0 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 5.0 mM DTT, and 0.2 mM PMSF). Cells were then suspended in 10 mL of isolation buffer containing 0.3% Triton X-100 and incubated on ice for 10 min. Nuclei were recovered by centrifugation at 2500 rpm at 4 °C for 5 min. Isolated nuclei (from ~$2 \times 10^6$ cells) were resuspended in 100 $\mu$L of a reaction mixture consisting of 10 mM Tris-HCl, pH 7.4, 60 mM KCl (TK buffer) containing either 2 mM $CaCl_2$ and 5 mM $MgCl_2$, or an appropriate chelating agent (2 mM EGTA or 5 mM EDTA) and were incubated for 1 h at room temperature. In some experiments, the nuclear pellet was washed twice with 0.5 mL of TK buffer prior to the incubation with $CaCl_2$ and $MgCl_2$. This nuclear wash fraction was collected, freeze-dried, redissolved in appropriate buffers, and either used in the reconstitution experiments or analyzed on DNA-substrate gels. In other experiments, the washed nuclei were also extracted with 0.42 M NaCl (in 20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 0.5 mM PMSF, and 25% glycerol) according to Dignam et al. (1983) to obtain the nuclear salt extracts.

*DNA Sample Processing and Pulsed Field Agarose Gel Electrophoresis (PFGE).* In order to avoid DNA shearing during sample handling, cellular or nuclear samples were immobilized in low melting point (LMP) agarose plugs before deproteinization. Approximately $8 \times 10^6$ cells were harvested, washed once with nuclear buffer (15 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 60 mM KCl, and 15 mM NaCl), and pelleted at 700*g* for 5 min at 4 °C. The cell pellet was resuspended in 0.25 mL of nuclear buffer and mixed with an 0.25 mL aliquot of molten 1.5% (w/v) LMP agarose (at 37 °C) and 100 $\mu$g of Proteinase K. The mixture was transferred immediately to a 1 mL syringe to solidify. The agarose plug was removed from the syringe and incubated overnight at 37 °C in 3 mL of TEN buffer (10 mM Tris-HCl, pH 9.5, 25 mM EDTA, 1 mM EGTA, and 10 mM NaCl), 400 $\mu$g of Proteinase K, and 1% (w/v) laurylsarcosine. The plug was subsequently washed in TE buffer (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) and stored at 4 °C.

Isolated nuclei (from $2 \times 10^6$ cells) were resuspended in 100 $\mu$L of reaction mixture for DNA digestions, and, following digestion, the nuclear suspension was combined with 100 $\mu$L of the nuclear buffer, 200 $\mu$L of 1.5% (w/v) LMP agarose and 60 $\mu$g of Proteinase K. The plug was prepared and processed as described above.

Equal length slices (approximately 4 mm) of the agarose plugs were loaded on an 0.8% agarose gel in TBE buffer (0.089 M Tris, 0.089 M boric acid, and 25 mM EDTA, pH 8−8.5), the wells were sealed with 1.5% (w/v) LMP agarose, and PFGE was carried out using a Q-life Autobase Electrophoresis System (Kingston, ON) as described by Walker et al. (1993). The gels were stained with ethidium bromide and photographed on a UV transilluminator.

*Protein Separation by Gel Filtration Chromatography.* The proteins of the nuclear wash were fractionated on a Pharmacia FPLC chromatography system using a Superose 12B gel filtration column equilibrated with an elution buffer consisting of 50 mM Tris-HCl, pH 7.4, and 300 mM KCl. Fractions corresponding to $OD_{280}$ peaks were collected and analyzed for endonucleolytic activity as described below.

*Analysis of Endonucleolytic Activities.* The DNA cleaving activity of the nuclear wash and salt extracts was identified using a slightly modified DNA substrate gel assay previously described by Heeb and Gabriel (1984) and Seiliev et al. (1992). Briefly, protein samples were separated on 10% SDS−polyacrylamide gels copolymerized with 30 $\mu$g/mL sheared salmon testis DNA (either double-stranded or heat-denatured single-stranded), with or without $10^6$ cpm/mL $^{32}$P-labeled DNA. Radiolabeled DNA was prepared using [$\alpha$-$^{32}$P]dATP and [$\alpha$-$^{32}$P]dGTP (800 Ci/mmol; DuPont, Oakville, Ontario) and the Multiprime DNA labeling system (Amersham, Oakville, ON). After the separation, the proteins were renatured in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 20% 2-propanol during a 30 min incubation (with gentle rocking) at room temperature and with two changes of the buffer. The gel was transferred to a reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1% Triton X-100) and incubated at 37 °C for 3 h. The radioactive gels were dried and exposed to Kodak X-Omat film (at −70 °C, overnight). The nonradioactive gels were stained with ethidium bromide and photographed on a UV transilluminator.

Bovine pancreatic DNase I, purchased from Boehringer Mannheim (Laval, PQ), and bovine spleen DNase II, from Sigma Chemical Co. (St. Louis, MO), were also run on the gels.

*Two-Dimensional Gel Electrophoresis.* Nuclear wash fractions were dialyzed against water, freeze-dried and reconstituted in a buffer consisting of 9.2 M urea, 2% NP-40, and 2% broad range ampholytes (Biolytes pH 3/10; BioRad Laboratories, Mississagua, ON) and separated in tube gels by isoelectrofocusing using broad pH range ampholytes according to O'Farrel (1975). Subsequently, the tube gels were loaded on 10% SDS−PAGE copolymerized with 30 $\mu$g/mL salmon sperm DNA and separated in the second dimension. The gels were subjected to the renaturation of the nucleolytic activity as described above.

*In Vitro Plasmid Digestion Assay.* One microgram of plasmid DNA was incubated with fractions containing endonucleolytic activity in the presence of 50 mM Tris-HCl,

pH 7.4, 5 mM $MgCl_2$, and 1 mM $CaCl_2$ for 1 h at 37 °C. The reaction mixture was analyzed by electrophoresis on 0.8% agarose gels in 40 mM Tris−acetate buffer, pH 8.5, and 2 mM EDTA at 20 V overnight. Gels were stained with ethidium bromide and photographed on a UV transilluminator.

*Antibody Production*. Nuclear wash fractions, prepared as described above, were used for further purification of p97 endonuclease. The fractions were freeze-dried and reconstituted in water, and the proteins were separated on 10% SDS−polyacrylamide gels copolymerized with dsDNA in order to identify the band(s) of endonuclease activity. Gel strips containing a DNA cleaving activity of 97 kDa were cut out, and the p97 protein was extracted from the strips with 50 mM Tris-HCl buffer, pH 7.9, containing 200 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 0.5 mM PMSF, and 0.1 mM benzamidine. The proteins were acetone-precipitated, reconstituted in sterile PBS, and used for vaccination. White male New Zealand rabbits (1.5−2.0 kg) were immunized with 50−200 $\mu$g of protein/innoculation according to a standard protocol. Animals were bled 10 days after the last injection, and serum samples were tested for antibody titer and specificity.

*Immunoprecipitations*. Anti-p97 antibody was affinity purified by binding to and eluting from nitrocellulose-immobilized p97 protein. The antibodies were incubated with 100 $\mu$g of total protein extract from nuclear wash fractions and goat anti-rabbit IgG-coated Dynabeads (Dynal AS, Oslo, Norway) for 2 h at 4 °C. The beads were washed (3×) with a low-stringency RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100) and resuspended in gel loading buffer (60 mM Tris-HCl, pH 6.7, 10% glycerol, and 0.01% bromophenol blue) containing 4% SDS but devoid of reducing agent. The resulting supernatants were used directly used in the DNA-substrate gel assay.

*Western Blotting.* For Western blotting, proteins were separated by 10% SDS−PAGE (BioRad Minigel system, Mississauga, Ontario) and eletrotransferred onto a Hybond-C nitocellulose membrane (Amersham, Oakville, Ontario). The membranes were blocked with 5% non-fat milk powder in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl (TBS), washed in 0.1% Tween-20 in TBS, and incubated for 1 h with either anti-p97 (dilution 1:500) or anti-DNase I (1:500) antibodies, followed by incubation with appropiate secondary antibodies conjugated to alkaline phosphatase. Blots were washed and developed by colorimetric reaction using BICP/NBT substrate (KPL, Gaithersburg, MA). The polyclonal sheep anti-bovine pancreatic DNase I antibody was purchased from Bethyl Laboratories Inc. (Montgomery, TX). Alkaline phosphatase conjugated goat anti-rabbit IgG (Fc) was from Promega (Madison, WI), and donkey anti-sheep IgG (H+L) was from Jackson ImmunoResearch Laboratories (West Grove, PA), and they were both used at a dilution 1:10 000.

*Microscopy.* Cells were grown on, and isolated nuclei were attached to, poly(L-lysine)-coated glass coverslips. The slides were rinsed in PBS, fixed for 5 min at room temperature with 4% paraformaldehyde (in PBS), stained for 1.5 min with Hoechst 33258 dye (1 $\mu$g/mL in PBS), and photographed under an Olympus Bmax Microscope using phase contrast and epifluorescence optics. Fixed nuclei were also immunostained with a mouse monoclonal anti-PI2 (Chaly et al., 1984) and FITC-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies.

*Other Methods*. Apoptosis was induced by serum-deprivation. Routinely, 70% confluent cells grown in complete medium were washed 3 times with serum-free medium and then maintained in serum-free conditions for up to 48 h before being analyzed further.

## RESULTS

*Physically Separable Pools of Endonucleolytic Activity Are Responsible for High Molecular Weight and Internucleosomal DNA Cleavage.* In many cell culture systems, withdrawal of growth and/or trophic factors has been shown to result in apoptotic cell death (Evan et al., 1992; Barres et al., 1992). In this study, we also employed serum deprivation to generate an apoptotic signal in 5123tc cells. The cells were maintained in the presence or absence of serum for up to 48 h, and both attached and floating cells were collected, fixed, and stained with Hoechst dye (Figure 1A−D). Cell shrinkage and cytoplasmic and nuclear condensation were observed in cells maintained in the absence of serum (compare panels C and D to panels A and B). Almost all of the floating cells showed reduced cell size and chromatin condensation near the nuclear periphery consistent with apoptosis. Furthermore, in many of these cells, the chromatin was packaged into apoptotic bodies, confirming that nuclear destruction had proceeded to completion, replicating the *in vivo* process first described by Kerr et al. (1972). When the trypan blue dye exclusion assay was used to evaluate viability, 40−50% of the cells were dead within 48 h of serum deprivation (data not shown).

Previous work from this laboratory has shown that in many cells the DNA cleavage during apoptosis leads, initially, to the formation of 50−300 kb size fragments, and eventually to oligonucleosomal DNA fragments (Walker et al., 1991, 1994; Roy et al., 1992; Oberhammer et al., 1993). To evaluate DNA cleavage patterns, agarose plugs were prepared from cells harvested after 48 h in serum-free conditions and from control untreated cells. DNA integrity was analyzed by PFGE under conditions designed to resolve fragments from 200 bp to 1 Mbp as detailed under Experimental Procedures (Figure 1E). DNA fragmentation into both 50 kb and oligonucleosomal size fragments was clearly observed in the apoptotic cells (lane 2), but not in the control cells (lane 1). Furthermore, and in agreement with previously published data, DNA cleavage, similar to the apoptotic pattern, could also be recreated in nuclei isolated from 5123tc cells and digested in the presence of calcium and magnesiun ions. The data are presented in Figure 1F−I. The 5123tc cells were disrupted by 0.3% Triton X-100, and nuclei were prepared in Ca/Mg-free conditions to avoid DNA damage during the isolation procedure. The integrity of the preparations was evaluated by phase contrast and fluorescence microscopy, and the nuclei seem to be intact and substantially free from contamination by cytoplasmic membrane fragments (Figure 1F−H). The chromatin stained uniformly with Hoechst dye (Figure 1G), and the nuclei had a well-preserved nuclear envelope (Figure 1H), as shown by immunofluorescence staining with the PI2 antigen. The isolated nuclei were then incubated at room temperature for 1 h in the presence

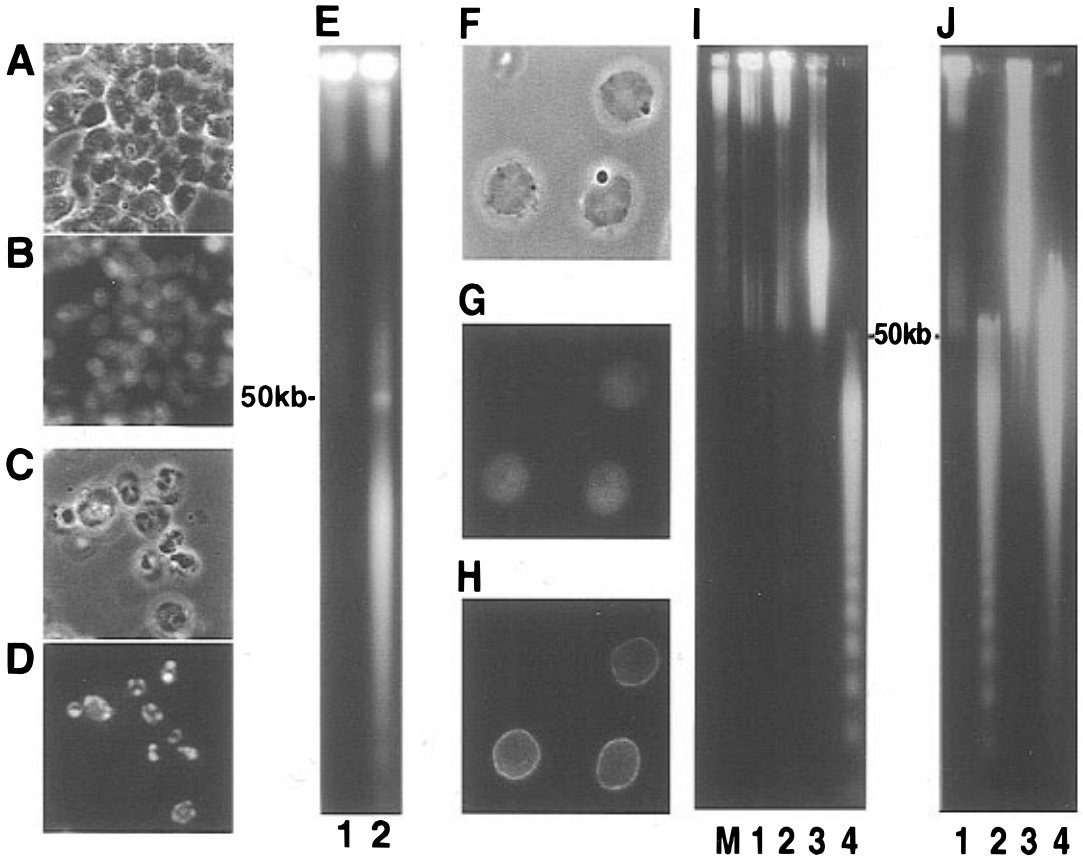


FIGURE 1: Characterization of apoptotic markers and DNA fragmentation in intact 5123tc cells and isolated nuclei. Cells were grown on poly(L-lysine)-coated glass coverslips for 48 h in the presence (A and B) or absence of serum (C and D), and isolated nuclei were attached to the coverslips (F−H). The samples were fixed with paraformaldehyde and stained with Hoechst 33258 dye (B, D, G). Nuclei were immunostained with anti-PI2 antibody and FITC-conjugated anti-mouse serum (H). The slides were photographed with an Olympus Bmax fluorescence microscope using phase contrast (A, C, F) and epifluorescence (B, D, G, and H) optics. Magnification: cells 250×, nuclei 625×. For analysis of DNA fragmentation, whole cells (E) or isolated nuclei (I and J) were embedded in LMP agarose plugs, equal amounts of DNA were loaded onto an 0.8% agarose gel, and PFGE was carried out as decribed in the Experimental Procedures. Gels were stained with ethidium bromide and photographed on a transilluminator. In panel E: control 70% confluent 5123tc cells (lane 1); and 48 h serum-starved (lane 2). The position of a 50 kb DNA size marker is indicated. In panel I: lambda DNA ladder (lane M); DNA from freshly isolated nuclei (lane 1); DNA from nuclei digested in the presence of 5 mM EDTA (lane 2), 2 mM EGTA and 5 mM $MgCl_2$ (lane 3), and 5 mM $MgCl_2$ plus 2 mM $CaCl_2$ (lane 4). In panel J: DNA from freshly isolated unwashed nuclei (lane 1); DNA from unwashed nuclei incubated with 5 mM $MgCl_2$ plus 2 mM $CaCl_2$ (lane 2); DNA from extensively washed nuclei incubated with 5 mM $MgCl_2$ plus 2 mM $CaCl_2$ (lane 3); and DNA from washed nuclei but reconstituted with the wash fraction and incubated with 5 mM $MgCl_2$ plus 2 mM $CaCl_2$ (lane 4).

or absence of calcium and magnesium ions and embedded in agarose, and DNA integrity was analyzed by PFGE (Figure 1I). Whereas there was no significant DNA damage in freshly isolated nuclei, a small amount of DNA degradation occurred during a 1 h incubation in the presence of 5 mM EDTA or 2 mM EGTA (lanes 1 and 2, respectively). When the incubation was performed in the presence of 5 mM $MgCl_2$ alone (calcium ions were chelated with 2 mM EGTA), the DNA digestion produced only HMW fragments of $>50$ kb (lane 3). The nuclei also contained an enzymatic activity capable of generating oligonucleosomal ladders that was activated when the nuclei were incubated with both 2 mM $CaCl_2$ and 5 mM $MgCl_2$ (lane 4). In agreement with the previously published data on other cell lines (Walker et al., 1995), these results indicated that in 5123tc cells the two distinct stages of HMW and oligonucleosomal DNA degradation could also be distinguished based upon their cation requirements.

The results from the above experiments indicated that the endonucleolytic activities responsible for both steps of DNA degradation were constitutively present in the isolated nuclei (Figure 1I, lane 4, and Figure 1J, lane 2). When nuclei were washed twice with a low ionic strength buffer (10 mM Tris-HCl, pH 7.4, and 60 mM KCl) and then incubated, they failed to produce the oligonucleosomal pattern, and the DNA digestion was restricted to only HMW fragments (Figure 1J, lane 3) even in the presence of both cations. The fragmentation pattern was very similar to that seen in the unwashed nuclei incubated with magnesium ions alone (Figure 1I, lane 3). Thus, the washing procedure caused either inactivation or extraction of a loosely-associated endonuclease responsible for producing internucleosomal DNA cleavage. In order to address the latter issue, reconstitution experiments were performed in which the nuclear wash fractions were collected, concentrated by freeze-drying, and added back to the washed nuclei for the subsequent digestion of DNA. As shown in Figure 1J, lane 4, in the presence of the nuclear wash fraction the $Ca^{2+}/Mg^{2+}$-mediated internucleosomal DNA degradation resumed. The reconstition experiments clearly indicated that the endonuclease activity responsible for internucleosomal digestion was loosely-associated with nuclei and could be extracted with the low ionic strength buffer. Significantly, these data established that a $Ca^{2+}/Mg^{2+}$-dependent activity was required for further degradation

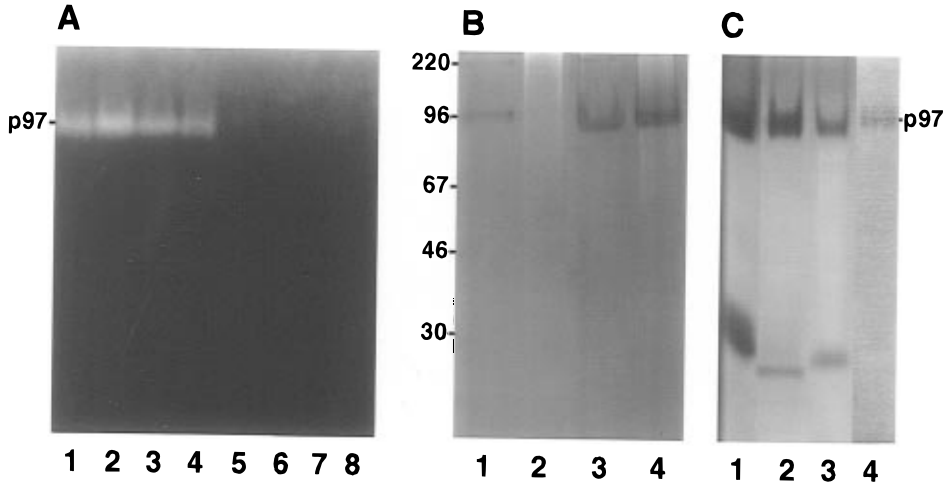


FIGURE 2: Detection of an endonucleolytic activity on DNA-substrate gels. Total proteins (approximately 20 $\mu$g/lane), from the nuclear wash fractions and salt extracts, were separated on 10% SDS−polyacrylamide gels copolymerized with either ss $^{32}$P-labeled (A) or ds unlabeled DNA (B and C). The protein samples were mixed with gel loading buffer devoid of reducing agents. After electrophoresis, the proteins were renatured and incubated with calcium and magnesium ions as described under Experimental Procedures. Gels were dried and autoradiographed for 24 h at −70 °C on Kodak X-Omat film (A), or were stained with ethidium bromide and photographed on a UV-transilluminator (B and C). In panel A: nuclear wash fractions from 5123tc (lane 1), MCF7 (lane 2), PC12 (lane 3), and DU145 (lane 4); in lanes 5−8, nuclear salt extracts from the same cells and loaded on the gel in the same order. In panel B: the nuclear wash and 0.42 M NaCl extract from control 5123tc cells (lanes 1 and 2, respectively) and the same fractions from 48 h serum-deprived 5123tc apoptotic cells (lanes 3 and 4, respectively). In panel C: nuclear washes from kidney (lane 1), liver (lane 2), brain (lane 3), and thymus (lane 4).

of HMW DNA fragments, but it was not required for their generation.

*Identification of an Extractable $Ca^{2+}/Mg^{2+}$-Dependent 97 kDa Endonuclease.* Since the nuclear wash fraction contained an enzyme activity capable of internucleosomal DNA degradation, we analyzed this fraction for the presence of a $Ca^{2+}/Mg^{2+}$-dependent endonuclease (Figure 2). The nuclear wash fractions (lanes 1−4) and 0.42 M NaCl extracts (lanes 5−8), prepared from four different cell lines (5123tc, PC12, human MCF7, and DU145 cells), were analyzed on a radiolabeled DNA substrate gel (Seiliev et al., 1992). Proteins were separated by 10% SDS−PAGE copolymerized with sheared, radiolabeled ssDNA. As shown in Figure 2A, only the nuclear wash fractions (lanes 1−4) contained an enzymatic activity capable of DNA degradation following electrophoretic separation, renaturation, and incubation in the buffer containing both calcium and magnesium ions. The loss of radioactive DNA was evident in only one area of the gel and is seen as a light band on the autoradiograph (Figure 2A). This single band was present in the nuclear wash fraction obtained from all four cell lines (lanes 1−4) but not the 0.42 M salt extracts (lanes 5−8). It was further established, based on the size of the markers, that the molecular mass of the protein responsible for this DNA degradation was approximately 97 kDa.

Whereas there was no endonucleolytic activity in the 0.42 M salt nuclear extracts prepared from control exponentially growing cells (Figure 2A, lanes 5−8, and Figure 2B, lane 2), the same nuclear fraction prepared from 48 h serum-deprived cells contained this activity (Figure 2B, lane 4), suggestive of its possible recompartmentalization in response to the apoptotic stimulus.

In this experiment, the extracts were analyzed on a DNA-substrate gel using nonradioactive dsDNA stained with ethidium bromide. A similar activity was also found in the nuclear wash fractions (Figure 2C, lanes 1−4) prepared from rat kidney (lane 1), liver (lane 2), brain (lane 3), and thymus (lane 4) tissues. However, unlike the cultured cell extracts, tissue samples also contained a nuclease-like activity of lower molecular mass range reminiscent of DNase I. This may be explained by a contaminating presence of DNase I from blood, since the intensity of this band in liver, perfused with saline prior to extraction (lanes 2), was much lower than in those of unperfused kidney and brain samples (lanes 1 and 3).

In addition to the DNA-substrate gel described above (Figure 2), an *in vitro* plasmid DNA digestion assay was also used to demonstrate nucleolytic activity associated with the nuclear wash fraction (Figure 3). The sample was subjected to a chromatographic gel filtration separation (Figure 3A), and $OD_{280}$ peak fractions were collected (peaks 1−4). These four fractions were first analyzed on the dsDNA-substrate gel for the presence of the p97-associated activity (Figure 3B) and were then taken for the plasmid digestion assay (Figure 3C). Of the four fractions analyzed, the p97 band, the same one which was present in the unfractionated nuclear wash fractions (Figure 2, and Figure 3B, lane N), was detected only in peak 1 (Figure 3B, lane 1). However, this protein was eluted from the column in the early peak fraction (Figure 3A), suggesting that, in solution, this enzyme existed as a high molecular mass complex which was not disrupted by 0.3 M KCl present in the elution buffer. The plasmid DNA digestion assay confirmed these data since the plasmid was digested either by the sample of unfractionated nuclear wash (Figure 3C, left-hand panel, lane +p97) or by the sample of the peak 1 fraction only (Figure 3C, right-hand panel, lane +1). These results also showed that the enzyme possessed both exo- and endonuclease activities since it was capable of digestion of closed supercoiled plasmid into a smear of oligonucleotides (Figure 3C, lanes +p97 and +1).

We further characterized the cation and pH requirements of the 97 kDa endonuclease (Figure 4). Thus, the enzymatic activity on polyacrylamide-embedded dsDNA could be reconstituted in the presence of both calcium and magnesium ions (Figure 4A), but chelation of either one of them resulted

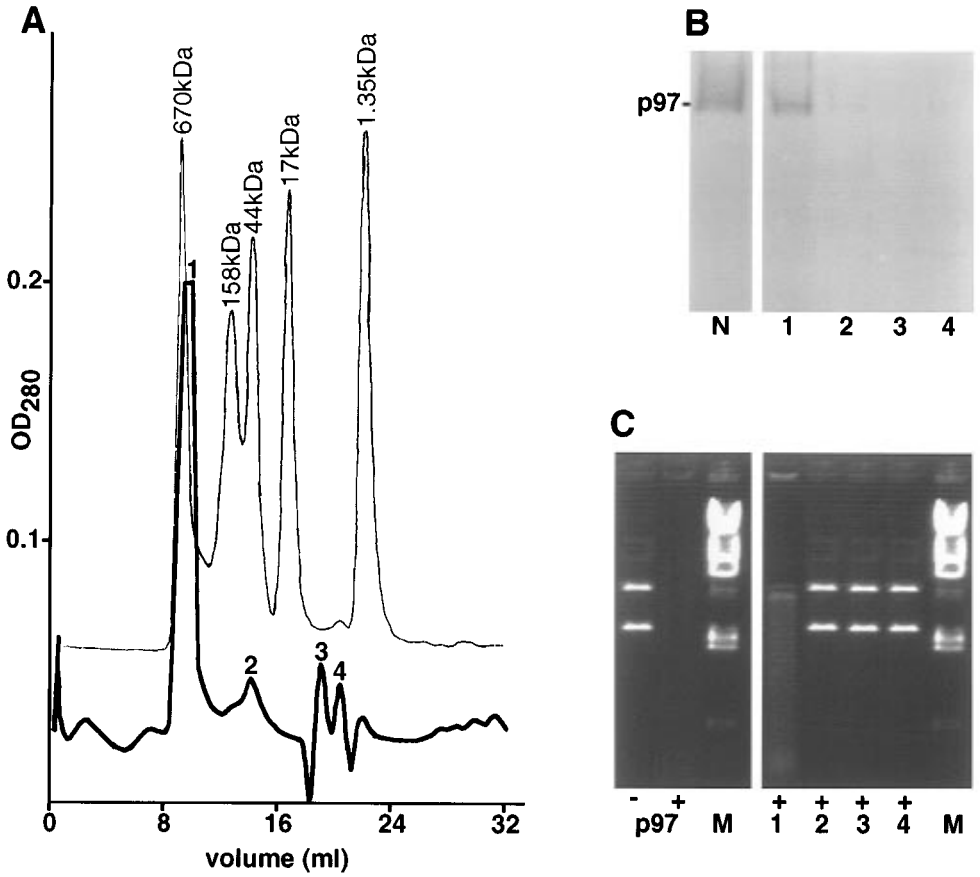


FIGURE 3: Chromatographic fractionation of the nuclear wash fraction and *in vitro* plasmid DNA digestion assay. Approximately 100 $\mu$g of proteins was separated on a Superose 12B column (A). The peak fractions identified as 1−4 were collected, dialyzed against water, freeze-dried, and analyzed on the DNA-substrate gel (B) or assayed for plasmid DNA digestion (C). In panel A: gel filtration profile of nuclear wash fraction and molecular size markers (thyroglobulin, bovine $\gamma$-globulin, chicken ovalbumin, equine myoglobulin, and vitamin B12). In panel B: a sample of unfractionated nuclear wash (lane N); samples corresponding to peaks 1−4 (lanes 1−4) . In panel C: undigested plasmid DNA (−) and plasmid digested by a sample of unfractionated nuclear wash (+); lanes 1−4, (+) plasmid digestion by column peak fractions 1−4; lane M, lambda 1 kb DNA size marker.

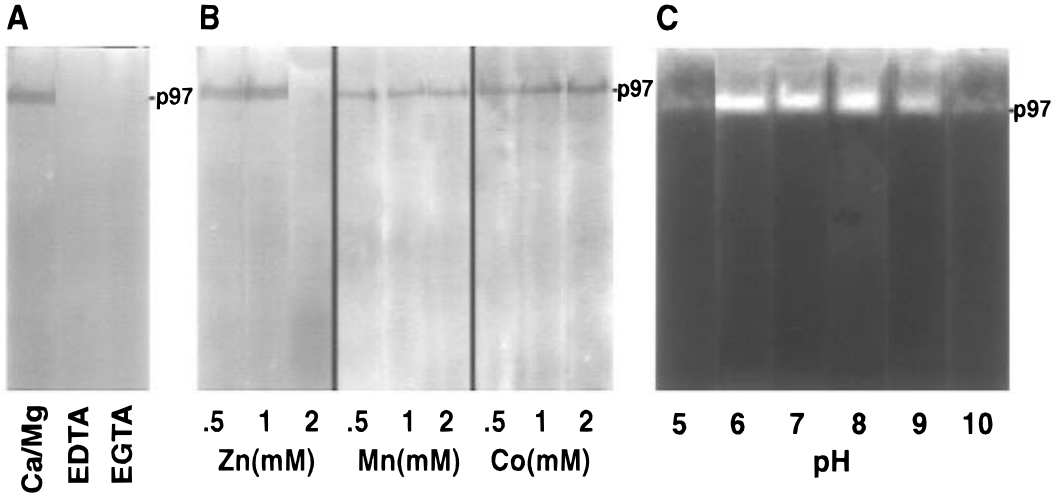


FIGURE 4: Effects of different divalent cations and pH on the activity of p97 endonuclease. Approximately 20 $\mu$g/lane of proteins from 5123tc nuclei (the wash fraction) was separated on 10% SDS−polyacrylamide gels copolymerized with either ds unlabeled (A and B) or ss $^{32}$P-labeled (C) DNA. The protein samples were mixed with gel loading buffer devoid of any reducing agents. The p97 activity was developed as decribed under Experimental Procedures. The gels were stained with ethidium bromide and photographed on a UV-transilluminater (A and B), or were dried and autoradiographed for 24 h at −70 °C on Kodak X-Omat film (C). In panel A: after the electrophoretic separation, individual lanes were cut out and separately incubated with either 5 mM $MgCl_2$ and 2 mM $CaCl_2$ (lane Ca/Mg), 5 mM EDTA (lane EDTA), or 2 mM EGTA and 5 mM $MgCl_2$ (lane EGTA). In panel B: gel strips were incubated with varying concentrations of $ZnCl_2$ (Zn 0.5−2 mM), $MnCl_2$ (Mn, 0.5−2 mM), and $CoCl_2$ (Co, 0.5−2 mM). In panel C: the gel strips were separately incubated in Tris-HCl buffer of different pH (indicated under each lane), and containing both 5 mM $MgCl_2$ and 2 mM $CaCl_2$.

in a complete lack of enzymatic activity (Figure 4A, lanes marked EDTA, EGTA). Zinc ions at concentrations up to 1 mM could substitute for calcium/magnesium and activate the enzyme, but inhibited the enzyme at 2 mM concentration

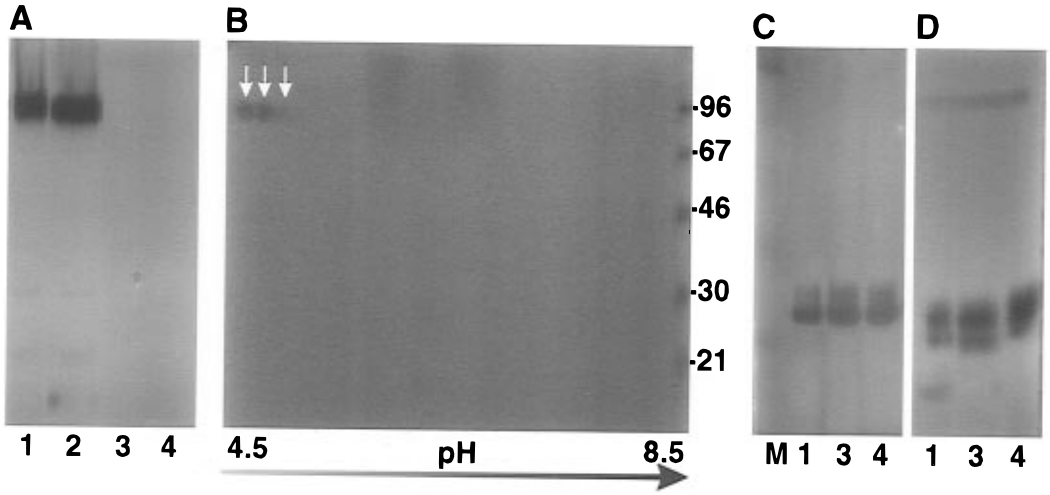


FIGURE 5: Comparison of p97, DNase I, and DNase II catalytic properties. (A) Approximately 20 $\mu$g/lane of total proteins from the 5123tc nuclear wash was separated on 10% SDS−polyacrylamide gels copolymerized with unlabeled dsDNA. Lane 1, the sample was mixed with gel loading buffer devoid of any reducing agents; lane 2, the sample was treated with 5 M urea; lane 3, the sample was mixed with gel loading buffer without reducing agents, but it was boiled for 5 min; lane 4, the sample was mixed with gel loading buffer containing 2% $\beta$-mercaptoethanol. (B) Approximately 50 $\mu$g of the same nuclear wash fraction was first subjected to isoelectrofocusing (using broad pH range ampholytes pH 3/10), followed by further separation in the second dimension on SDS−PAGE copolymerized with unlabeled dsDNA. (C) 1.0 unit/lane of DNase I and (D) 5.0 units/lane of DNase II were separated on 10% SDS−polyacrylamide gels copolymerized with unlabeled dsDNA. Lane 1, the sample were mixed with gel loading buffer devoid of any reducing agents; lane 3, the samples were mixed with gel loading buffer without reducing agents, but it was boiled for 5 min; lane 4, the samples were mixed with gel loading buffer containing 2% $\beta$-mercaptoethanol; lane M, size markers. The endonucleolytic activities were developed as decribed under Experimental Procedures, except the p97 and DNase II cleavage reactions were carried out overnight, whereas the DNase I reaction was stopped after 30 min. The gels were stained with ethidium bromide and photographed on a UV-transilluminater.

(Figure 4B). Furthermore, both manganese and cobalt ions alone, at concentrations up to 2 mM, were sufficient to support enzyme activity (Figure 4B). In addition, it was found that the enzyme was active over a broad pH range from 6.0 to 9.0 (Figure 4C).

The p97 endonuclease is distinct from both DNase I and DNase II. The data described above indicated that the p97 endonuclease shared some biochemical properties with DNase I, particularly the cationic requirements and pH profile (Figure 4B). In order to rule out the possibility that the nuclease activity of apparent molecular mass of 97 kDa resulted from a stable association of known enzymes, i.e., DNase I or DNase II, with some other cellular protein(s), we analyzed all three activities on dsDNA-substrate gels under different denaturing conditions (Figure 5A,C,D) and the p97-associated activity after the separation in the two dimensions (Figure 5B). As shown in Figure 5A, the p97 endonuclease activity was detected in samples loaded on the gel in the absence of any reducing agent (lane 1), and neither its activity nor its apparent molecular mass was affected by the presence of 5 M urea (lane 2). However, the activity was completely abolished in samples boiled prior to gel loading (lane 3) and in samples treated with 1% $\beta$-mercaptoethanol (lane 4). The molecular mass of the endonuclease did not change after isoelectrofocusing in the first dimension and SDS−PAGE separation in the second dimension (Figure 5B). It was, however, resolved into discrete spots (indicated by arrows) of different isoelectric points ranging from 4.6 to 5.0. On the other hand, the activities of both DNase I (Figure 5C) and DNase II (Figure 5D) remained unaffected by boiling (Figure 5C,D, lane 3) and by the reducing agent (Figure 5B,C, lane 4). These data showed that neither the activity of p97 endonuclease nor its molecular mass was altered by the urea treatment, supporting the notion that the activity was associated with a single 97 kDa polypetide. Furthermore, unlike DNase I and DNase II, after denaturation the p97 endonuclease could not refold back to an active conformation and intramolecular disulfide bridges are essential for the catalytic function of the enzyme.

*Characterization of the p97-Specific Antibodies.* We have purified the p97 DNA-cleaving protein from 5123tc cells and generated rabbit polyclonal antibodies according to the protocol described under Experimental Procedures. The serum was affinity-purified and tested for its ability to immunoprecipitate the endonuclease activity. As shown in Figure 6A for immunoprecipitates analyzed on dsDNA substrate gels, the affinity-purified serum from the immunized rabbit (lane 1) but not preimmune serum (lane 2) brought down the p97 DNA degrading activity. Furthermore, on a Western blot (Figure 6B), this antibody specifically reacted with a protein doublet of molecular mass 95−97 kDa (lane 1). Under identical conditions, there was no cross-reactivity with either DNase I (lane 2) or DNase II (lane 3). Similarly, a commercially available anti-DNase I antibody labeled only the DNase I protein (Figure 6C, lane 1) but not the p97 endonuclease (lane 2). We have also analyzed the p97 protein level in different cell and tissue extracts by Western blotting (Figure 6D). The same total amounts of protein extract (50 $\mu$g/lane) prepared from 5123tc (lane 1), MCF7 (lane 2), and T51B (lane 3) cells and liver (lane 4) and thymus (lane 5) tissue were separated on 10% SDS−PAGE under reducing conditions, transferred to a nitrocellulose membrane, and immunoblotted with the affinity-purified anti-p97 serum. The same molecular mass protein doublet (95/97 kDa), identical to that shown in lane 1 of panel B, was present in all samples, including apoptosis-sensitive rat thymocytes (lane 5), but its highest level was detected in liver (lane 4). Therefore, the p97 endonuclease seemed to be constitutively present in a variety of mammalian cells. The experiments described in Figure 6B,D provided

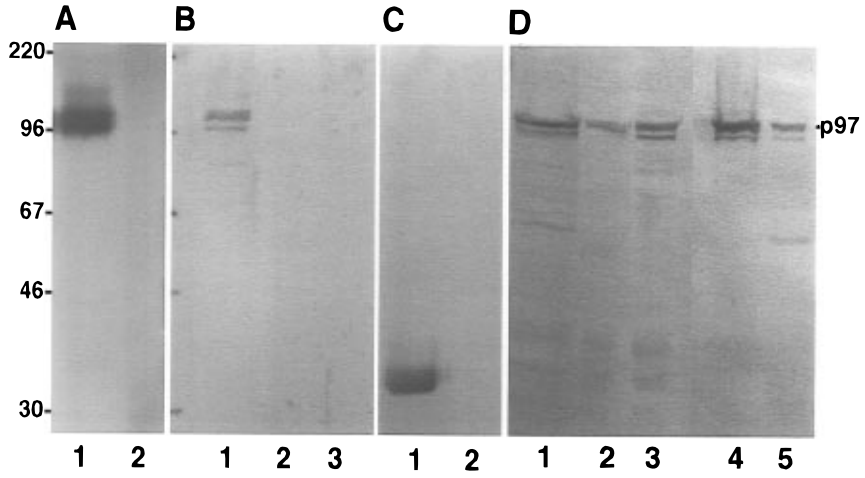


FIGURE 6: Characterization of anti-p97 serum. (A) Immunoprecipitation of the endonucleolytic activity with anti-p97 serum. The immunoprecipitates were separated on a 10% SDS−polyacrylamide gel copolymerized with ds unlabeled DNA, and the endonucleolytic activity was developed as decribed under Experimental Procedures. The gel was stained with ethidium bromide and photographed on a UV-transilluminater. (B−D) Western blots of proteins separated on 10% SDS−PAGE, electrotransferred onto a nitrocellulose membrane, and blotted with either affinity-purified anti-p97 serum (B and D) or anti-DNase I (C), followed by detection with alkaline phosphatase-conjugated secondary antibody. (A) Immunoprecipitates with affinity-purified p97 antibody (lane 1) or preimmune serum (lane 2) from approximately 100 $\mu$g of total proteins from the 5123tc nuclear wash. (B) 50 $\mu$g of total proteins from the 5123tc nuclear wash (lane 1), 2 $\mu$g of DNase I (lane 2), and 5 $\mu$g of DNase II (lane 3). (C) 2 $\mu$g of DNase I (lane 1) and 50 $\mu$g/lane of the 5123tc nuclear wash fraction (lane 2). (D) 50 $\mu$g/lane of total proteins from the nuclear wash fraction of 5123tc (lane 1), MCF7 (lane 2), T51B cells (lane 3), liver tissue (lane 4), and thymus (lane 5).

additional evidence that a single p97 polypeptide was, indeed, responsible for the DNA cleaving activity since, for the analysis on Western blots, the protein extracts were boiled in a buffer containing mercaptoethanol and were separated under denaturing conditions without any shift in the position of the band.

## DISCUSSION

We have identified a $Ca^{2+}/Mg^{2+}$-dependent endonuclease in the nuclei of 5123tc hepatoma cells. Based upon its molecular mass (97kDa), sensitivity to reducing agents, and catalytic properties, we believe this to be a novel enzyme. The enzyme required both $Ca^{2+}$ and $Mg^{2+}$ ions to degrade DNA on substrate gels, and it could also be activated by $Mn^{2+}$ or $Co^{2+}$ ions. Moreover, $Zn^{2+}$ activated the enzyme at low concentrations, but inhibited it at higher concentrations. Since these cation requirements were similar to those described for DNase I (Melgar & Goldthwaite, 1968; Campbell & Jackson, 1980) and since DNase I has a high specific activity, we took several steps to assure ourselves that we were not dealing either with preparations contaminated with DNase I or with a complex between DNase I and another protein. To this end, we used chromatographic gel filtration, isoelectrofocusing, and treatments with SDS and urea to denature any such complexes without seeing any reduction in size of the p97 nuclease band. Moreover, on substrate gels, the size of the protein places it well away from the 30−50 kDa region where several endonucleases, including DNase I, would run. Indeed, in tissue extracts an endonuclease activity in the 30 kDa molecular mass range is seen. This band was not further analyzed, but may well represent contaminating DNase I from serum, but it does not interfere with the analysis of p97. In cell extracts, we did not see any bands in the 30−50 kDa range, and we did not see any bands of activity in the 18−20 kDa (NUC18) range in any of the extracts (Gaido & Cidlowski, 1991). Finally, the sensitivity of p97 to boiling and reducing agents distinguished it from both DNase I and DNase II. Indeed, the latter property may explain why this enzyme has not been seen before since most activity gels are run under reducing conditions. The enzyme was capable of degrading ssDNA as well as dsDNA and was active over a fairly broad pH range including values as low as pH 6, which is within the range of values seen in apoptotic cells as discussed below. It could also convert supercoiled plasmid DNA into a smear of oligonucleotides consitent with the properties of both endo- and exonucleases.

The p97 enzyme was found to be expressed in all four cell lines of human and animal origin tested (5123tc, PC12, DU145, and MCF7) and in the four rat tissues tested (liver, kidney, brain, and thymus). In normal 5123tc cells, it was easily extracted from nuclei in buffers of low ionic strength, leaving no residual $Ca^{2+}/Mg^{2+}$-dependent activity in the nucleus. Moreover, the endonucleases remaining in the nucleus were not capable of internucleosomal DNA cleavage, even when incubated *in vitro* with $Ca^{2+}$ and $Mg^{2+}$ ions. On the other hand, in cells undergoing apoptosis in response to the deprivation of growth factors, its association with the nuclear structure became tighter and no longer breakable by the low salt buffer (Figure 2B). Since 5123tc cells degraded their DNA to oligonucleosomal fragments when they undergo apoptosis (Figure 1) and since the p97 endonuclease was the only enzyme in these cells capable of catalyzing such a reaction, it was possible that this enzyme became activated and/or recompartmentalized during cell death and it was responsible for chromatin digestion. The fact that the enzyme displayed multiple isoelectric points (after the 2D separation) revealed the existence of a posttranslational modification mechanism which might play a role in the control of its activity.

Although ***the*** apoptotic endonuclease has not been definitively identified, a number of properties that candidate enzymes must fulfill have been described. For example, the enzyme responsible for internucleosomal DNA fragmentation

requires both $Mg^{2+}$ and $Ca^{2+}$ ions (Nikonova et al., 1982, 1993; Cohen & Duke, 1984; Wyllie et al., 1984), leaving recent reports (Barry & Eastman, 1992; Zhivotovsky et al., 1993; Treves et al., 1994) which indicated a lack of involvement of calcium ions in apoptosis (at least in some cell types) difficult to reconcile. It appears to be constitutively expressed since it becomes activated in the absence of gene expression or protein synthesis in some model systems. The enzyme must be able to generate single-strand breaks (ssbs) since many ssbs are found in the linker regions of chromatin in apoptotic cells (Appleby & Modak, 1977; Gromkowski et al., 1986; Ucker et al., 1992; Pietsch et al., 1993b; Tomei et al., 1993; Arruti et al., 1995). Moreover, it must have access to the internucleosomal sites on DNA which requires an enzyme that is either nucleoplasmic or loosely-associated with chromatin. Since acidification of the cell has been observed during apoptosis (Eastman 1994), the enzyme may be required to be active in the pH range of 6.0−6.5. Finally, as part of a highly conserved process, the endonuclease would be expected to be ubiquitously expressed.

In a recent analysis of the ionic requirements for HMW and internucleosomal DNA fragmentation, it became evident that an enzyme with the properties of the DNase I family of endonucleases was the most likely candidate (Walker et al., 1995). However, the tissue distribution of the parotid form of the enzyme (the only one for which antibodies are available) has been considered to be too restrictive for it to play a general role in such a ubiquitous process as apoptosis. The observations that the 97 kDa enzyme identified in this study, and also found in a number of cells undergoing apoptosis (Pandey et al., unpublished observations), had many of the ionic properties of a "DNase I-like" enzyme provide a possible solution for this dilemma.

The 97 kDa enzyme produced DNA ladders with sharp bands as opposed to pancreatic DNase I which can produce a smear because of intranucleosomal as well as internucleosomal DNA fragmentation. Moreover, it was active at pH 6 which is as low or lower than that likely to be achieved in apoptotic cells. The p97 endonuclease is, therefore, a more likely candidate for being involved in apoptosis since it satisfies all of these criteria.

The relationship between the endonuclease activities responsible for the two stages of DNA fragmentation (HMW and internucleosomal) is still not clear. The evidence for the existence of two discrete stages has come from three sets of studies. First, some cells produce only HMW fragments and do not undergo internucleosomal DNA fragmentation. Second, in cells that do undergo internucleosomal DNA fragmentation, the latter stage can be inhibited with either serine protease inhibitors (Weaver et al., 1993) or zinc ions (Zhivotovsky et al., 1993; Sun et al., 1994). Third, in isolated nuclei, the two stages can be distinguished based on their cation requirements (e.g., Figure 1). The present study provided a fourth piece of evidence, namely, that they are in physically separable pools in the nucleus. The activity responsible for HMW fragment production appears to be tightly bound to chromatin, presumably at matrix attachment sites since domain-sized fragments are released by this enzyme (Walker et al., 1995; Lagarkova et al., 1995). The activity responsible for internucleosmal DNA fragmentation must have free access to the linker regions and would presumably be nucleoplasmic and easily extractable. However, it cannot be ruled out that a single endonuclease protein, such as p97, could be responsible for all stages of fragmentation. To satisfy the above criteria, this would require that a pool of the enzyme be modified or compartmentalized in such a way as to remain bound to chromatin, since it is neither washed out nor does it have free access to the linker regions of bulk chromatin. To distinguish between these possibilities, the activity responsible for HMW DNA fragmentation must be identified.

The reason why some cells do not completely degrade their DNA is not due to the lack of a $Ca^{2+}/Mg^{2+}$-dependent endonuclease. However, a number of reasons may explain their failure to undergo internucleosomal DNA fragmentation. First, there may be an insufficient level of Ca ions to activate the nuclease. We show here that calcium ions are only required for the final stages of DNA degradation in those cell types that produce DNA ladders, but that sufficient HMW fragmentation for apoptosis can occur in its absence [or at least without any elevation above basal levels which are estimated to be approximately 100 nM (O'Malley, 1994)]. In those cells that do alter $Ca^{2+}$ levels during apoptosis, the $Ca^{2+}/Mg^{2+}$-dependent enzyme will be activated and DNA is further fragmented to oligonucleosomes (Walker et al., 1995). Second, this stage of chromatin degradation has been shown to be dependent upon proteolysis (Weaver et al., 1993) which may not occur in some cells. Third, there may be structural alterations in chromatin that leave these sites inaccessible to nuclease attack, or the enzyme may fail to redistribute within the nucleus. These possibilities are currently being tested. Finally, since the MCF7 cells undergo type II apoptosis, in which chromatin condensation and presumably DNA fragmentation is a late event (Zakeri et al., 1995), it is possible that the cells do not carry DNA fragmentation to completion.

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