Isolation and Characterization of NUC70, a Cytoplasmic, Hematopoietic Apoptotic Endonuclease*

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Endonucleolytic DNA fragmentation is the common end point and the prevailing indicator of apoptosis. We have identified a 70-kDa endonuclease (NUC70) that is activated in drug-induced apoptosis of human hematopoietic cells. We purified NUC70 to homogeneity and generated a rabbit polyclonal antibody to distinguish it from previously identified nucleases. Biochemical characterization of isolated NUC70 demonstrates that it is Ca²⁺/Mg²⁺-dependent and active over a pH range of 6–8. When incubated with isolated HeLa nuclei, NUC70 was capable of generating internucleosomal DNA fragmentation. This endonucleolytic activity was inhibited by Zn²⁺, aurintricarboxylic acid, N-ethylmaleimide, spermine, and iodoacetamide. Western immunoblots using the anti-NUC70 antibody and DNA-SDS-polyacrylamide gel electrophoresis assays indicate that NUC70 expression and activity is restricted to human hematopoietic cells. No such activity was detected in human epithelial cell lines or murine hematopoietic cells. We also observed no difference in levels of NUC70 expression between apoptotic and nonapoptotic cells, suggesting that activation of NUC70 may be by posttranslational modification. We demonstrate that NUC70 activity is diminished in cells pretreated with the caspase inhibitors z-DEVD-fmk, z-VAD-fmk, and Z-CH2-Asp-DCB. Time course studies of cytoplasmic and nuclear endonuclease activities during apoptosis show that NUC70 is a cytoplasmic endonuclease that is translocated to the nucleus after the initiation of apoptosis. We confirmed this with immunostaining studies using anti-NUC70 antibody. These results demonstrate that NUC70 is an endogenous cytoplasmic endonuclease that is activated during apoptosis in a caspase-dependent mechanism.

Apoptosis is a physiological process of orderly cell death that occurs in response to a number of physiological, pathological, and cytotoxic insults. Apoptotic cells are identified morphologically based on compaction of chromatin against the nuclear membrane, condensation of the cytoplasm, and nuclear and cytoplasmic blebbing to form membrane-bound fragments that are phagocytosed by adjacent cells (1–3). The morphological changes are paralleled by activation of a number of complex biochemical effector pathways that contribute to dissolution of cellular structural elements. One common end point of apoptosis is the reduction of high molecular weight genomic DNA into smaller oligonucleosomal fragments that can be effectively packaged. Chromatin condensation has been shown to be dependent on DNA digestion. The characteristic appearance of 180–200-base pair oligonucleosomal ladders on agarose gels in cells undergoing apoptosis is in contrast to the pattern of random DNA digestion occurring in the setting of necrosis and denotes the importance of activation of specific endonucleases in apoptosis, regardless of the stimulus for cell death (4, 5).

Identification of apoptotic endonucleases has been limited by the low abundance of these proteins and by poor understanding of the complex patterns of their regulation. The first studies of apoptotic endonucleases in glucocorticoid-induced apoptosis indicated that these enzymes are likely constitutive and are activated by Ca²⁺ and inhibited by Zn²⁺ (6). Other studies in epithelial cells demonstrated Ca²⁺-independent apoptotic DNA degradation, suggesting that a family of apoptotic endonucleases exists and may differ according to tissue type (7). Thus far, candidate endonucleases have been identified with differing biochemical characteristics, including pH and cation requirements as well as pattern of tissue expression. Because only a few of these endonucleases, caspase-activated nuclease and inhibitor of caspase-activated DNase (CAD)1 NUC18, and the 27-kDa splenic endonuclease, have been purified or cloned (8, 9, 10, 11), there is little information regarding their activation and interaction with other effector components of the apoptotic cascade.

We have identified a 70-kDa apoptotic endonuclease activity in human hematopoietic cells in modified DNA-SDS-PAGE renaturation assays that is heat-stable and Ca²⁺/Mg²⁺-dependent and the activity of which is inhibited in the presence of inhibitors of caspase. We now describe the purification and biochemical characterization of NUC70 and demonstrate by immunohistochemistry that it is a cytoplasmic protein that is translocated to the nucleus early in apoptosis.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Vincentine, cycloheximide, actinomycin D, VP-16, and chemicals and protease inhibitors for buffers were obtained from Sigma. Proteinase K, DNase, and RNase were obtained from Life Technologies. The caspase inhibitors z-DEVD-fmk, z-VAD-cmk, and Z-CH2-Asp-DCB were obtained from Oncogene Research (Cambridge, MA). Serine protease inhibitors TPCK and TLCK were obtained from Calbiochem (La Jolla, CA). SDS-PAGE reagents and prestained molecular weight markers were obtained from Bio-Rad (Hercules, CA), and gels were made according to the manufacturer’s instructions, with the modifications mentioned below.

1 The abbreviations used are: CAD, caspase-activated DNase; PAGE, polyacrylamide gel electrophoresis; TPCK, 1-tosylamido-2-phenyl-ethyl chloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline.
Characterization of NUC70

Cell Lines and Culture Conditions—Human leukemia cell lines Nalm-6 (pre-B leukemia), Molt-4 (acute lymphoblastic leukemia), CEM (acute lymphoblastic leukemia), Hut-102 (Sezary leukemia), K562 (chronic myelogenous leukemia), and Jurkat (T-cell leukemia); murine cell lines S49/thymoma) and P88 (lymphoid neoplasms); and human epithelial cell lines A549 (lung adenocarcinoma), MCF7 (breast adenocarcinoma), CAOS, and HT-29 (colon carcinoma) were used in this study. Cells were obtained from ATCC (Manassas, VA). All cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (10,000 units/liter), and streptomycin (100 mg/liter) at 37 °C in 5% CO2. Cell culture media, sera, and anti-biotics were obtained from Life Technologies. Human CML-3 stem cells and nonmural cells were obtained from patients on protocols approved by the Institutional Review Board.

For large scale purification of NUC70, Nalm-6 cells were grown in 500 ml of culture medium in roller bottles.

Assessment of Apoptosis by DNA Fragmentation—Cells seeded in 75-mm flasks at a semiconfluent density of 5 × 105 cells/ml. Cells were incubated in the presence or absence 10 μM vincristine. At different time points, cells were harvested by centrifugation (1000 × g for 5 min) and lysed in DNA lysis buffer (0.5% Triton X-100, 25 mM Tris-Cl, pH 7.4, 25 mM EDTA) for 30 min at 4 °C. Samples were divided into two aliquots. The first aliquot was centrifuged at 12,000 × g for 30 min. DNA concentration in the supernatant (fragmented DNA) and in the pellet (chromatin) was determined using the diphenylamine colorimetric assay for DNA as described by Gendelman et al. (12). Apoptosis was quantified by computing for % DNA fragmentation (fragmented DNA/fragmented DNA + pellet DNA).

The second aliquot was processed for DNA extraction using 100 μg/ml proteinase K, 150 mM NaCl, and 0.2% (w/v) SDS and incubated at 50 °C for 2 h. Nucleic acid was extracted twice with phenol/chloroform and treated with 1 μg/ml DNase-free RNase A for 1 h at 37 °C. DNA samples (10 μg/lane) were loaded on a 1.8% agarose gel (with 0.5 μg/ml ethidium bromide) and separated by electrophoresis using 1 × TBE buffer (89 mM Tris-Cl, 2 mM EDTA, pH 8.0, 89 mM boric acid). Gels were run at 50 V for 5 h, viewed on a UV transilluminator, and photographed.

In experiments to determine the effects of protease inhibitors, cells were pretreated with 50 μM inhibitors (TPCK, TLCK, z-DEVD-fmk, z-VAD-fmk, and z-Ch2-Asp-DCB) for 1 h prior to induction of apoptosis with 10 μM vincristine and then processed.

Preparation of Cell Lysates and Nuclear Extracts—Total cell lysates were prepared by resuspending 106 cells in protein extraction buffer (0.5% (w/v) SDS, 50 mM Tris-Cl, pH 7.4, 1 mM PMSF, 2 mM EDTA, 0.5 μg/ml leupeptin, and 1 mM B-mercaptoethanol) at 4 °C, vortexed, and centrifuged at 10,000 × g for 30 min at 4 °C to remove debris. Supernatant was collected and stored at −70 °C.

Nuclear extracts were prepared by the method of Caron-Leslie et al. (13). Briefly, 107 cells were subjected to hypotonic shock using ice-cold 1.5 mM MgCl2, 0.25% (v/v) Nonidet P-40, 1 mM PMSF, 2 mM EDTA, 0.5 μg/ml leupeptin, and 1 mM B-mercaptoethanol at 4 °C, vortexed, and centrifuged at 10,000 × g for 30 min at 4 °C to remove debris. Supernatant was collected and stored at −70 °C.

Nuclear extracts were prepared by the method of Bradford (14) using bovine serum albumin as standard.

SDS-PAGE and Gel Nuclease Assay—Total cell lysates were normalized for protein concentration and nuclease assay was assayed by the method of McGrew and Green (15). Cell lysates were analyzed by SDS-PAGE gels (10%) loaded with 50 μg/ml high polymer calf thymus DNA (Sigma) according to the method of Laemmli using the Bio-Rad Mini-Protean II system. After electrophoresis, SDS was removed by washing the gels in renaturation buffer (40 mM Tris-Cl, pH 9.0, 2 mM EDTA, 0.02% (w/v) sodium azide with DE-52 (Whatman). DE-52 was prepared by equilibrating 10 g of preswollen DE-52 in 200 ml Tris-Cl, pH 9.0, for 1 h at room temperature and washed three times with the same buffer. The resulting resin was suspended in the renaturation buffer at a concentration of 0.1 g/ml.

Gels were washed with two changes of the renaturation buffer for 30 min on a moving platform and then incubated in renaturation buffer without DE-52 for 60 min. After a brief rinse with distilled water, gels were placed in activation buffer (40 mM Tris-Cl, pH 7.4, 0.02% sodium azide, 5 mM MgCl2, 5 mM CaCl2), and incubated for 12 h at 37 °C to allow DNA hydrolysis. In experiments using different ion and pH conditions, gels were cut into strips and treated in the same manner with appropriate modifications of the activation buffer. The reaction was stopped by washing the gels in 40 mM Tris-Cl, pH 7.4, 1 mM EDTA. The gels were stained with ethidium bromide (0.5 μg/ml) and visualized and photographed. In a further purification step, the gels were eluted in SDS-PAGE loading buffer and separated using preparative electrophoresis (Model 491 Prep Cell, Bio-Rad) attached to a peristaltic pump (Model P1, Amersham Pharmacia Biotech) and fraction collector (Waters, Bedford, MA). Fractions with activity as determined by cleavage of intact HeLa nuclei were pooled; simultaneously, fractions were concentrated and SDS was dialyzed off using equilibration buffer (20 mM Tris-Cl, pH 7.4, 5 mM EDTA, 1 mM PMSF) on Amicon (Beverly, MA) microconcentrators with a 30-kDa cut-off. The consolidated sample was loaded onto a SP-Sephadex C25 (Amersham Pharmacia Biotech) column equilibrated with the equilibration buffer and eluted with a NaCl (0.1–0.5 M) step gradient. Fractions with endonuclease activity were pooled and reconstituted in equilibration buffer with 50 mM NaCl. The sample was then loaded onto a double-stranded DNA-agarose column (Amersham Pharmacia Biotech) and eluted in a manner similar to that described above.

Endonuclease activity was detected in the fractions by concentrating 50 μl on a Speed-Vac Plus lyophilizer (Savant Instruments Inc. Farmingdale, NY) and analyzed on DNA-SDS-PAGE gel as described previously. Fractions containing activity were quantified by measuring the ability to generate DNA laddering with isolated nuclei (6). One unit of enzyme activity was defined as fragmentation of 25% of the DNA from intact HeLa nuclei in 5 min in the presence of 5 mM Ca2+ and 5 mM Mg2+ at 37 °C. Fragmented DNA was quantified using the diphenylamine DNA colorimetric assay (12) and expressed as a percentage of the total nuclear DNA. Specific activity was expressed as activity units per mg of protein. Protein concentration was determined by the Bradford method (14).

Biochemical characterization was performed using 4 units of enzyme incubated at different pH, ions and recognized nuclease inhibitors. Relative activity is expressed by quantifying experimental activity as a percentage of the standard nuclei assay described above.

Antibody Production—About 1 mg of purified NUC70 was obtained from the double-stranded DNA purification scheme, bypassing the ion-exchange procedure (SP-Sephadex column). Fractions from the double-stranded DNA-cellulose column were lyophilized using Speed-Vac Plus. Polyclonal antibody (rabbit antisera) production was subcontracted to Zymed Laboratories Inc. (South San Francisco, CA) for their proprietary accelerated procedure (PolyQuick).

Western Blotting—Cell lysates from apoptotic and nonapoptotic Nalm-6 cells were analyzed by Western blot analysis on 10% SDS-PAGE gels and electrotransferred onto polyvinylidene difluoride (Millipore, Bedford, MA) for 1 h at 4 °C using 100 V. Membranes were blocked with 5% nonfat milk, PBST (0.1% Tween-20, 10 mM phosphate buffer, pH 7.4, 2.7 mM potassium chloride, 137 mM sodium chloride) for 1 h followed by incubation with anti-NUC70 antibody (dilution, 1:5000) for another 1 h at room temperature. The blots were washed three times with PBST and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) and then developed using chemiluminescence (NEN Life Science Products). Images were developed on Kodak X-AR film.

Immunoprecipitation—Nalm-6 (107) cells were washed with PBS (10 mM phosphate buffer, pH 7.4, 2.7 mM potassium chloride, 137 mM sodium chloride) and lysed with the addition of 1ml of immunoprecipitation buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) under nondenaturing conditions and incubation for 1 h with constant agitation on a vortex mixer. Insoluble material was removed by centrifugation at 14,000 × g. Supernatant was collected and precleared with 10 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Samples were centrifuged (14,000 × g) for 10 min at 4 °C, and the resulting supernatants were precleared with 10 μg of anti-NUC70 antibody on a rocking platform for 12–16 h. Immunoprecipitates were collected by centrifugation (5,000 × g) and washed three times with immunoprecipitation buffer. All procedures were performed at 4 °C. Pellets were resuspended in SDS-PAGE loading buffer and analyzed for endonuclease activity on 10% DNA-SDS-PAGE gels.
Immunostaining and Fluorescence Microscopy—Nalm-6 cells were exposed to 10 μg/ml etoposide for 8 h to induce apoptosis. Cells were immobilized onto glass slides by cytospin (Shandon, Pittsburgh, PA), fixed and permeabilized by immersion in methanol/acetone (1:1) for 10 min at 220 °C and air-dried. Slides were blocked with 1% bovine serum albumin in PBS for 10 min, incubated with anti-NUC70 antibody (dilution, 1:500) for 1 h, and probed with goat anti-rabbit IgG-fluorescein isothiocyanate (Sigma) together with 1 μg/ml 4',6-diamidino-2-phenylindole stain for 1 h. Slides were washed with PBS between buffer changes and mounted in glycerol. Immunofluorescent staining was viewed using a Nikon Optiphot 2 fluorescence microscope (Nikon Instruments, Melville, NY) equipped with mercury lamp and excitation filters for UV(365 nM) and fluorescein isothiocyanate (470–490 nM). Images were recorded using a Nikon photographic attachment (MicroFlex-UFX-DX), on Kodak ASA200 color film. Figures were scanned using Microtek ScanMaker IIXE and assembled using Adobe Photoshop.

RESULTS

A 70-kDa Endonuclease Is Exclusively Activated in Hematopoietic Cells—To identify the size and activity of putative apoptotic endonucleases, we fractionated whole cell lysates from apoptotic cells on DNA-SDS-PAGE gels under denaturing conditions using a technique adapted from McGrew and Green (15). After electrophoresis, the gels were exposed to alkaline pH to renature proteins in the presence of DEAE to remove SDS efficiently and allow restoration of enzymatic activities with minimum denaturation and degradation. Previous studies using a similar method have solely utilized the passive method of renaturation of Rosenthal and Lacks (17). Endonuclease activity was defined as negative staining in the gels treated with ethidium bromide and visualized under ultraviolet light.

Using this method on time and dose-dependent studies on apoptotic Nalm-6 cells, we detected a previously described constitutively active endonucleolytic activity that appeared as a triplet with an apparent molecular mass of 40–50 kDa in untreated cells (Fig. 1B, lane 1) and that was unchanged or only slightly increased when cells were induced to undergo apoptosis (18, 19). In addition, we identified an endonuclease activity (NUC70) that was present only in minute amounts in untreated cells (Fig. 1A, lane 1) and that increased when cells were treated with different concentrations of etoposide for 8 h (lanes 2–5).

Fig. 1. Time- and dose-dependent induction of NUC70 endonuclease. A, agarose gel electrophoresis of DNA from Nalm-6 cells treated with 10 μM vincristine for 0, 12, 24, and 48 h (lanes 2–5, respectively), or with 0, 0.5, 1, 10, and 20 μM vincristine for 24 h (lanes 6–10, respectively). Panel B shows corresponding renatured DNA-SDS-PAGE gel.

Fig. 2. Expression of NUC70 in hematopoietic and epithelial cells. Representative gels demonstrating DNA fragmentation (A and B) and endonuclease activity (C and D). Panel A represents Nalm-6 (lanes 1–4) and CEM (lanes 5–8) cells, and panel B represents A549 (lanes 1–4) and MCF-7 (lanes 5–8) cells. All cells were treated with 10 μM vincristine for 0 (lanes 1 and 5), 12 (lanes 2 and 6), 24 (lanes 3 and 7), or 48 (lanes 4 and 8) h.

Fig. 3. NUC70 activity in CD34+ human hematopoietic progenitor cells and fresh leukemia cells from a patient with chronic lymphocytic leukemia. DNA-SDS-PAGE gels of whole cell lysates from CD34-selected cells (A) and Ficoll-separated lymphocytes (B) exposed to 5 μM Fludarabine at different time points. Lane 1, untreated cells; lane 2, 12 h postexposure; lane 3, 24 h; lane 4, 48 h.

Fig. 4. Analysis of purified NUC70. Fractions isolated from preparative electrophoresis were further purified by SP-Sephadex and DNA cellulose columns and endonuclease activity determined by incubation with isolated HeLa nuclei. A, Coomassie Blue stain; lane 1, whole cell extract; lane 2, eluted sample from SP-Sepahex; lane 3, eluted sample from DNA-cellulose column. B, ethidium bromide-stained 2% agarose gel. Lane 1, untreated HeLa nuclei; lane 2, treated with SP-Sephadex elutriate; lane 3, with DNA cellulose elutriate.
untreated cells and that increased 10–50-fold as measured by densitometric analysis of DNA-SDS-PAGE gel (Fig. 1B) in apoptotic cells. This increase in the 70-kDa activity was concordant with the appearance of DNA fragmentation (Fig. 1A). The low level of basal expression in the untreated cells (lane 2) likely results from spontaneous apoptosis, which occurs in 1–5% of cells in culture as detected in our diphenylamine assays. The fact that the whole cell lysates examined in these experiments were prepared under denaturing and reducing conditions indicating that the renatured NUC70 activity was heat resistant and likely represented a single protein species.

We next attempted to identify NUC70 activity in cells of different lineages. Apoptosis was induced in the epithelial cell lines MCF-7, T47D, CACO-2, HT-29, HeLa, and A549 using vincristine or VP-16 and in the hematopoietic cell lines Molt 4, CEM, Hut 102, Hut 78, K562, Jurkat, and Raji using vincristine, dexamethasone, and, for Jurkat cells, anti-CD95-antibody to induce Fas-mediated apoptosis. We found induction of NUC70 activity in all hematopoietic cell lines examined irrespective of the method used to induce apoptosis. Interestingly, no endonucleolytic activity representing a 70-kDa endonuclease was identified in any of the epithelial cell lines tested under conditions where apoptosis was induced as measured by DNA fragmentation, but the previously described 40–50-kDa endonuclease activity was identified in these cells (Fig. 2, A–D). This 40–50-kDa activity was also present in the hematopoietic cell lines. These data suggest that NUC70 activity is restricted to cells of hematopoietic lineage, whereas the 40–50-kDa endonucleases appear to be ubiquitously expressed.

We next examined normal CD34+ human hematopoietic progenitor cells obtained from CD34+ selected peripheral blood stem cell collections and lymphocytes from Ficoll-separated peripheral blood from a patient with chronic lymphocytic leukemia. We found induction of NUC70 activity and a 50-kDa endonuclease when apoptosis was induced by exposure to 5 μM Fludarabine (Fig. 3). At 24 h, 40- and 90-kDa endonuclease activities were also observed in CD34+ cells. When we examined murine hematopoietic cell lines, including P388, S49, and 2E8, we detected a 40-kDa endonucleolytic activity and several smaller sized endonucleases but were unable to detect NUC70 activity when apoptosis was induced, suggesting that the murine analog of NUC70 may have a different molecular mass.

| Purification step | Protein | Total activity | Specific activity | Purification fold |
|------------------|---------|----------------|------------------|------------------|
| 1. Total cell lysate | 3.970 mg | 3,970 units/mg | 1,000 | 1 |
| 2. Preparative electrophoresis | 4.6 mg | 1244.5 units/mg | 270.55 | 270.55 |
| 3. SP-Sephadex | 0.050 mg | 687.6 units/mg | 13,752 | 13,752 |
| 4. DNA-agarose | 0.009 mg | 467.5 units/mg | 50,053 | 50,053 |

**Table I.** Purification scheme.
Because it has been proposed that mycoplasma endonucleases may interfere with endonuclease renaturation assays (20, 21), we assayed for mycoplasma infection in our cells by reverse transcription-polymerase chain reaction (ATCC) and treated cells with ciprofloxacin (Bayer) for 2 weeks (22). The treatment did not affect the kinetics of NUC70 activation during apoptosis, and transfer of conditioned media from NUC70 positive Nalm-6 cells onto NUC70 negative cell lines (MCF-7 and A549) did not result in expression of NUC70. In cell lines that were positive for mycoplasma, we detected varying increases in the 40–50-kDa endonucleases described above (data not shown). Nalm-6 cells from which NUC70 was isolated and purified were mycoplasma negative by reverse transcription-polymerase chain reaction.

Isolation of NUC70—We based our initial strategy for isolating NUC70 on its size under reducing conditions that indicate that it is a single protein species. With the aid of preparative electrophoresis apparatus (Bio-Rad model 491), we were able to dramatically fractionate a whole cell lysate of 10^9 apoptotic Nalm-6 cells, separating the 70-kDa endonuclease from other cellular endonucleases, including the previously described 40–50-kDa endonuclease triplet, by assaying fractions on a DNA-SDS-PAGE gel. Measurement of protein yield by Bradford assay (14) indicated that this step allowed us to obtain an approximately 1000-fold purification of NUC70. Unfortunately, we were unable to determine the amount of enzyme activity in the initial sample due to the nonspecific DNase content.

To further isolate NUC70 from proteins of similar size, we separated the pooled fractions containing NUC70 activity by ion-exchange chromatography (SP-Sephadex column) and affinity chromatography (DNA-agarose column). Our early attempts at purifying NUC70 indicated that NUC70 does not bind to DEAE column under neutral pH conditions. This distinguishes NUC70 from other Ca\(^{2+}\)/Mg\(^{2+}\) endonucleases (11, 16). NUC70 activity elutes with 400 mM NaCl from SP-Sephadex column and with 200 mM NaCl from the DNA-agarose column. Coomassie Blue staining of SDS-PAGE gels of the fractions from these columns (Fig. 4A) showed a single band at 70 kDa, and incubation of these fractions with isolated HeLa nuclei generated apoptotic DNA laddering (Fig. 4B).

Our purification scheme is summarized in Table I. We estimate a 185-fold purification from the second step. Because we could not measure endonuclease activity in our first step, this is likely an underestimate, and according to measurements of protein content by Bradford assay, we may have a more than 1000-fold purification and an at least 100,000-fold enhancement of endonuclease activity. Because the difference between steps 3 and 4 is only 3-fold, we maximized our recovery by omitting step 3 in subsequent purifications. This increased recovery provided sufficient material for the biochemical characterization of NUC70 and for antibody generation.

Biochemical Properties of NUC70—We further characterized isolated NUC70 by determining its optimum cation requirements and pH using the HeLa nuclei assay. As shown in Fig. 5A, NUC70 activity is optimum in the presence of both Ca\(^{2+}\) (2 mM) and Mg\(^{2+}\) (5 mM). However, these cations at higher concentrations inhibit NUC70 activity (Ca\(^{2+}\), IC\(_{50}\) = 6.5 mM; Mg\(^{2+}\), IC\(_{50}\) = 9 mM). Experiments in which Ca\(^{2+}\) or Mg\(^{2+}\) was used alone showed diminished NUC70 activity. Mn\(^{2+}\) and Co\(^{2+}\) did not stimulate NUC70 activity (Fig. 5B); and Zn\(^{2+}\) inhibited even at low concentrations (IC\(_{50}\) = 0.25 mM).

Assessment of NUC70 at different pH conditions indicated that NUC70 functions over a broad pH range. Optimal activity was seen at pH 7.0–7.2; however, 50% activity was observed at pH 5.7–7.0 and pH 7.5–8.0 (Fig. 5C).

We next examined the effect of identified nuclease inhibitors (16) on NUC70 activity. Endonuclease activity was inhibited by aurantricarboxylic acid (IC\(_{50}\) = 0.3 mM), spermine (IC\(_{50}\) = 2 mM), N-ethylmaleimide (IC\(_{50}\) = 0.6 mM), and iodoacetamide (IC\(_{50}\) = 0.5 mM) (Fig. 4D). G-actin, an inhibitor DNase I, did not affect NUC70 endonuclease activity.

Characterization of Polyclonal Antibody to NUC70—Enzyme-linked immunosorbent assay analysis of the antiserum raised against NUC70 showed that dilutions up to 10,000-fold could identify the 70-kDa endonuclease protein in hematopoietic cells and on Western blots at a dilution of 1:5,000–1:10,000, suggesting that our polyclonal antibody preparation has high avidity. Further, anti-NUC70 antibody was capable of immunoprecipitating the 70-kDa endonuclease activity, as shown in Fig. 6.

Western Blot Analysis Using Polyclonal Anti-NUC70 Antibody—Immunoblots of untreated and apoptotic Nalm-6 cells demonstrate that the polyclonal anti-NUC70 antibody recognizes both the inactive and active forms of the protein, as shown in Fig. 7B. A replica immunoblot showing PARP cleavage confirms that the cells underwent apoptosis (Fig. 7A). NUC70 expression was noted in all hematopoietic cell lines tested but not in epithelial cell lines, confirming our earlier observation that NUC70 endonuclease activity was confined to cells of hematopoietic lineage (Fig. 8). Anti-NUC70 antibody detects a 50-kDa protein in epithelial cell lines and minimally cross-reacts with a similar protein in murine cell lines. These cross-reacting species may be homologues of NUC70.

Translocation of NUC70 from the Cytoplasm to the Nucleus during Apoptosis—In previous studies, apoptotic endonucleases were identified primarily in nuclear extracts, even though some of these endonucleases, such as DNase II and DNase I, have extranuclear locations. Our initial studies used whole cell...
extracts. To identify the cellular localization of NUC70 activity, we prepared nuclear and cytoplasmic extracts from the Nalm-6 cells using standard techniques and measured endonuclease activity in the presence of Ca\(^{2+}\)/Mg\(^{2+}\). As shown in Fig. 9A, NUC70 activity was initially confined to the cytoplasmic fraction. After induction of apoptosis, the NUC70 activity in the nuclear extracts increased significantly by 6 h along with cytoplasmic activity. By 24 h after induction of apoptosis, the activity in the cytoplasm decreased with a concordant marked increase in nuclear activity. We further extended our cellular localization studies with anti-NUC70 antibody immunostain on Nalm-6 cells exposed to 10 \(\mu\)g/ml etoposide for 8 h to induce apoptosis. Using a DAPI counterstain (Fig. 9B, panel 2) to identify apoptotic nuclei, immunoreactivity in the nonapoptotic cells was found to be perinuclear and associated with string-like cytoskeletal and vesicular structures in the cytoplasm. These suggest that, like DNase I, NUC70 is localized to the endoplasmic reticulum (string-like structures), the Golgi apparatus (heavy perinuclear stain), and perhaps secretory vesicles (Fig. 9B, panel 1).

In apoptotic cells, in which cytoplasmic shrinkage was evident, anti-NUC70 antibody stained the apoptotic nucleus heavily but appeared to be excluded from condensed DNA, as op-

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**FIG. 8.** Expression of NUC70 in cell lines by Western immunoblot analysis of whole cell lysates. Lane 1, Nalm-6; lane 2, CEM; lane 3, Molt-4; lane 4, K562; lane 5, A549; lane 6, MCF-7; lane 7, P388; and lane 8, S49.

**FIG. 9.** Cellular localization of NUC70. A, densitometric analysis of DNA-SDS-PAGE gels of fractionated Nalm-6 cells after exposure to 10 \(\mu\)M vincristine for varying times: cytoplasmic and nuclear fractions. B, fluorescence microscopy of anti-NUC70 and anti-rabbit IgG-fluorescein isothiocyanate conjugate immunostained Nalm-6 cells exposed to 10 \(\mu\)g/ml etoposide for 8 h to induce apoptosis. Panel 1, anti-NUC70 antibody; panel 2, counterstain with DAPI.
When Nalm-6 cells were pretreated with the caspase inhibitors, NUC70 activity in the presence of inhibitors of caspase-mediated events occurring early in apoptosis, we examine whether NUC70 activity, like p40 CAD, is modulated by these events. Interestingly, the endonucleolytic activity of the 40–50-kDa nucleases did not vary significantly in these inhibition experiments. NUC70 was activated in a caspase-dependent manner in vincristine-induced apoptosis, because Coomassie Blue stains and Western blot for NUC70 showed no increase in 70-kDa protein species, whereas there was a 50-fold increase in activity after induction of apoptosis. The induction of activity occurred within 6 h after drug treatment of the cells even in the presence of cycloheximide. Our immunofluorescence staining experiments demonstrate that NUC70 is localized in the cytoplasmic compartments and translocates into the nucleus during apoptosis. NUC70 was activated in a caspase-dependent manner in that its activity was abrogated in the presence of caspase inhibitors. It is unclear whether caspases directly cleave NUC70 or whether its activity is regulated by an endogenous inhibitor or activator, the activity of is modulated by a caspase-mediated event. In our immunoprecipitation studies, the 70-kDa protein is a distinct species and not a complex. NUC70, which is purified from apoptotic cells, was activated and capable of cleaving DNA in intact HeLa nuclei without co-incubation with cytoplasmic factors. Because the amount of NUC70 protein did not change significantly after induction of apoptosis as observed on our Western immunoblots, the activation is likely by posttranslational modification.

NUC70 is most closely related to the recently identified CAD and DNase I. The 40-kDa CAD endonuclease is complexed with inhibitor of CAD in the cytoplasm and is activated during apoptosis when inhibitor of CAD is cleaved by caspase-3, thereby releasing CAD to be translocated into the nucleus (9). Similarly, its human homologue, caspase-activated nuclease, is complexed with DNA fragmentation factor, a 45-kDa inhibitor that is cleaved by a caspase-mediated event (8). DNase I is also associated in its inactive form with a cytoplasmic inhibitor, G-actin. Caspase digestion of G-actin has been demonstrated as a mechanism of activating DNase I (31).

It is unlikely that NUC70 is a homologue of CAD or DNase I, given its biochemical properties. CAD, a 40-kDa protein, is Mg$^{2+}$-dependent, whereas NUC70 requires both Ca$^{2+}$ and Mg$^{2+}$ for activity. DNase I has a mass of 31 kDa and exhibits activity only with Ca$^{2+}$/Mg$^{2+}$ but also with Mg$^{2+}$/Mn$^{2+}$ (32). Nuc70 is inactive in the presence of Mn$^{2+}$, Zn$^{2+}$, or Co$^{2+}$. G-actin inhibits DNase I but not NUC70 activity. NUC70, however, can be inhibited by other previously identified nuclease inhibitors, such as aurintricarboxylic acid, N-ethylmaleimide, spermine, and iodoacetamide (33).

Because it is active at neutral pH, NUC70 is unlike the 31-kDa acidic cytoplasmic endonuclease DNase II, which is active at pH < 6.5 (25), or the 35-kDa acidic endonuclease (34).
Both endonucleases have no cation requirements for activity. Intracellular acidification has been shown to occur in drug-induced apoptosis of human leukemia cell lines HL-60 and Jurkat (35), and this alteration in pH activates DNase II and possibly other acidic endonucleases, such as a recently reported 45-kDa Ca<sup>2+</sup>-dependent endonuclease (36). Our results show that NUC70 is active at pH 6–8 but has only 60–70% activity at pH 6–6.5. Other Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonucleases that can function in an acidic milieu include DNase γ (37) and the p97 endonuclease (38).

Other 70-kDa endonuclease activities have been identified, but none has been characterized. Kabatava et al. (39) have reported a 65–70-kDa Mg<sup>2+</sup>- or Mn<sup>2+</sup>-dependent cytosolic endonuclease that is capable of internucleosomal DNA fragmentation from human myeloid leukemia cells (39). This enzyme, however, was not isolated from apoptotic cells, and its activity during the progression of apoptosis remains to be elucidated. Another Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent 70-kDa endonuclease is up-regulated in the nucleus of cervical carcinoma cells induced to undergo apoptosis by irradiation (40). The nature of this endonuclease is less well understood as it was observed under native conditions, and its ability to digest genomic DNA was not demonstrated.

Our studies confirm that NUC70 activity is unlikely to be related to mycoplasma contamination. Experiments using ciprofloxacin treatment of NUC70 expressing cell lines demonstrated that NUC70 activity was not altered. Incubation of NUC70 negative epithelial cell lines with conditioned media from NUC70 positive hematopoietic cells did not result in the appearance of NUC70 activity in these cells. Furthermore, we detected NUC70 activity in freshly isolated CD34<sup>+</sup> hematopoietic progenitor cells and freshly harvested lymphocytes from a patient with chronic lymphocytic leukemia. According to Bendjennat et al. (21), the likelihood of mycoplasma infection in these primary cells is less than 2%.

The significance of multiple endonucleases that can digest genomic DNA during apoptosis is intriguing. We and others have previously attributed this diversity to the need for tissue-specific death effector pathways. Our results show that NUC70 previously attributed this diversity to the need for tissue-specific death effectors. Our results show that NUC70 expression is restricted to human hematopoietic cells. Nonspecific death effector pathways. Our results show that NUC70, which is active at pH 6–8 but has only 60–70% activity at pH 6–6.5. Other Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonucleases that can function in an acidic milieu include DNase γ (37) and the p97 endonuclease (38).

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