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Native Recombinant Cyclophilins A, B, and C Degrade DNA Independently of Peptidylprolyl *cis-trans*-Isomerase Activity

POTENTIAL ROLES OF CYCLOPHILINS IN APOPTOSIS*

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Previous work in our laboratory (Montague, J., Gaido, M., Frye, C., and Cidlowski, J. (1994) *J. Biol. Chem.* 269, 18877–18880) has shown that human recombinant cyclophilins A, B, and C have sequence homology with the apoptotic nuclease NUC18 and that denatured cyclophilins can degrade DNA. We have now evaluated the nucleolytic activity of recombinant cyclophilins under native conditions. We show that nuclease activity inherent to cyclophilins is distinct from *cis-trans*-peptidylprolyl isomerase activity and is similar to that described for apoptotic nucleases. Cyclophilin nucleolytic activity is stimulated by Ca^{2+} and/or Mg^{2+}, with a combination of the two being optimal for cyclophilins A and B. Mg^{2+} alone is sufficient for cyclophilin C nuclease activity. pH optimums are in the range of pH 7.5–9.5. Cyclophilins can degrade both single-stranded and double-stranded DNA. Additionally, cyclophilins produce 3′-OH termini in linear double-stranded substrates, suggesting the cuts produced are similar to those of apoptotic cells. Cyclophilins also display endonucleolytic activity, demonstrated by their ability to degrade supercoiled DNA. In the absence of ions, cyclophilins bind linearized DNA. When added to nuclei from nonapoptotic cells, cyclophilin C induces 50-kilobase pair DNA fragmentation but not internucleosomal fragmentation. Together, these data suggest that cyclophilins are involved in degradation of the genome during apoptosis.

Cyclophilins (Cyps)[1] comprise a highly conserved, ubiquitous family of proteins first identified as the intracellular receptors for the immunosuppressant drug cyclosporin A (CsA) (1). Cyps range in size from 15 to 40 kDa (1–3). This large family of proteins has been isolated from a diverse range of organisms, including *Neurospora* (4), *Escherichia coli* (5, 6), yeast (7), plants, and higher eukaryotes (7, 8). Cyps have been identified in every tissue studied in humans (7, 8) and have been localized to the cytoplasm (1), mitochondria (9), nucleus (8, 10), and endoplasmic reticulum (11) in various mammalian cells and the cytoplasm and periplasm (5, 12) in *E. coli*. Three well characterized Cyps, CypA (18 kDa) (1, 13), CypB (21 kDa) (11), and CypC (22.8 kDa) (14), are studied and discussed in this paper. The widespread nature of Cyps suggests they may play an important role in cellular metabolism.

Cyps have been shown to act as peptidylprolyl *cis-trans*-isomerases whose activity can be blocked by CsA. As isomerases, Cyps have been proposed to aid in protein folding. The folding rates of human carbonic anhydrase II (15), RNase T1, (16), and collagen (17) have been shown to be increased by Cyp *in vitro*. However, whether this isomerase activity is physiologically significant is still not clear. Cyps have also been proposed to act as chaperones (18, 19), chemotactic agents (20), or stress response proteins (21, 22). Additionally, CypA has been shown to bind to the p55 Gag protein of human immunodeficiency virus (23) and appears to be necessary for successful human immunodeficiency virus infection (24, 25).

Cyps also play a role in the immune system by binding to the immunosuppressant drug CsA, although the exact nature of this role is also unclear. When the Cyp·CsA complex is formed, it can bind to and inhibit calcineurin (protein phosphatase 2B) (26). The inhibition of calcineurin activity causes a decrease in the nuclear levels of the transcription factor NF-AT, preventing expression of the cytokine interleukin-2 and immune cell activation (27, 28).

We recently reported a novel nuclease activity associated with the Cyp proteins (29). The notion that Cyps might have nucleolytic capabilities was suggested by a surprising sequence and structural similarity between NUC18 (an 18-kDa nuclease isolated from apoptotic rat thymocytes) and the Cyp family of proteins. Apoptosis, or programmed cell death, is a type of cell death that is highly ordered and follows a series of distinct steps that eventually lead to the non-immune-responsive death of the cell. One characteristic of apoptosis is the specific degradation of the cell's nucleic acids. There are two DNA fragmentation patterns observed in apoptosis; one involves the production of fragments of DNA that are 180–200 base pairs in length (or multiples thereof), and the other involves the production of larger DNA fragments that are approximately 50 kb in length (30, 31). While the characteristics of these apoptotic nuclease activities have been extensively described (32–35), the actual enzymes responsible have not been identified.

In this manuscript, we have evaluated and characterized the nuclease activity of Cyps under native conditions and assessed their potential role in apoptosis. We demonstrate that the nuclease activity from Cyps under native conditions is distinct from the isomerase activity and that there are many similarities between the characteristics of nuclease activity from purified Cyps and those reported for apoptotic nuclease activity. In addition, CypC is shown to produce 50-kb fragments in chromatin, further suggesting a nucleolytic role for Cyps in apoptosis.

EXPERIMENTAL PROCEDURES

Materials—Purified recombinant CypA, -B, and -C were generously provided by Sandoz Laboratories in Basel, Switzerland (36). They re-

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[1] The abbreviations used are: Cyp, cyclophilin; CypA, -B, and -C, cyclophilins A, B, and C, respectively; CsA, cyclosporin A; TBE, Tris/borate/EDTA; TE, Tris/EDTA; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

veal no contaminants according to the limits of the silver stain analysis of SDS-polyacrylamide gels (29). The sources for other materials and enzymes are provided throughout.

Thymocyte Preparation—Thymocytes were prepared from thymi isolated from adrenalectomized male Sprague Dawley rats. Control thymocytes were isolated from untreated rats, and apoptotic thymocytes were prepared from thymi removed at different times following injection of the rat with 5 mg/kg of body weight dexamethasone (Steraloids). After removal from the rat, the thymus gland was placed into ice-cold phosphate-buffered saline, cut into small pieces, and homogenized with 10–15 passes of a loose-fitting Dounce homogenizer. The sample was then poured through a Nitex mesh filter, and the cells were collected by centrifugation at 4 °C (5 min, 4,000 rpm). The cells were resuspended in fresh phosphate-buffered saline and passed through another Nitesh mesh filter. After a second centrifugation, the cells were resuspended in 5 ml of phosphate-buffered saline and counted with a Coulter counter. 1×10^8 cells were removed and spun down in a microcentrifuge tube. Resuspension of the cells in 200 μl of 10 mM $MgCl_2$, 0.25% Nonidet P-40 buffer caused lysis of the plasma membrane and release of the nuclei. The cells were incubated in this solution for 5 min on ice, and then the nuclei were centrifuged out of solution (5 min, 4,000 rpm). The cytoplasmic supernatant was transferred to a clean tube, and the nuclei were resuspended in an equal volume of the $MgCl_2$/Nonidet P-40 solution. The extracts were prepared by the addition of an equal volume of a loading buffer containing SDS and boiled 5 min.

Western Blot Analyses—Equal volumes of the cytoplasmic and nuclear preparation of control and apoptotic thymocytes were electrophoresed through a 15% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose (BA-85) in a TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA)/methanol buffer over 2 h at 80 V. Protein was detected following the ECL (Amersham Corp.) detection procedure. Antibodies directed against purified recombinant CypA were prepared in rabbits and purified according to the protocol of Weigel *et al.* (37) using the purified recombinant CypA affinity column.

Preparation of Substrate DNA—HB101 cells (Life Technologies, Inc.) containing pUC18 plasmid (2690 base pairs) were grown at 37 °C in Luria broth to an OD of 0.8 at 595 nm. Chloramphenicol was added to a final concentration of 170 mg/liter, and incubation was continued overnight. The cells were harvested, and the plasmid was purified via standard cesium chloride gradient techniques. Plasmid DNA was linearized (where stated) with the restriction endonuclease *Sma*I or *Xba*I (New England Biolabs). Single-stranded DNA was prepared by boiling the double-stranded DNA and immediately cooling on ice.

Solution Nuclease Assay—The solution nuclease assay was used to determine optimal digestion conditions and enzyme kinetics. CypA, -B, or -C was incubated with a nucleic acid substrate (supercoiled plasmid, linear double-stranded DNA, and linear single-stranded DNA) at 37 °C in a 20 mM Tris solution with appropriate ions and pH, as described in Table I. In general, 1 μg of Cyp and 1 μg of DNA (linearized pUC18) were combined in a total volume of 10 μl, resulting in a final concentration of 5.6 μM CypA, 4.8 μM CypB, 4.4 μM CypC, and 56 nM pUC18 (30 mM phosphodiester bonds); any differences are noted in the figure legends. A combination of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ was used for the kinetic assays. After incubation, loading buffer was added, and the samples were heated to 65 °C for 10 min. The DNA was electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining.

Quantitation of Nucleic Acid Degradation—Densitometry was used to quantitate the percentage of DNA degraded. Pictures of the ethidium bromide-stained gels were scanned into a PowerMacIntosh (8100/80) and then quantitated with the Image 1.56 ppc program (National Institutes of Health). The amount of DNA used in each assay and the time of exposure of the film were designed to ensure a densitometry reading that was linear with respect to the concentration of the DNA. A two-step process was used to calculate the percentage of DNA degraded at a specific time point. First, the area under the peak corresponding to the degraded DNA (less than 2690 base pairs) was divided by the area under the peak corresponding to the total amount of DNA in the sample and then this figure was multiplied by 100. For the relative efficacy of the activating ions, the amount of degraded DNA minus background was determined, and the percentage of DNA degraded relative to the Ca^{2+}/Mg^{2+} sample was calculated. For the relative rate of DNA degradation, the amount of DNA degraded at a specific time was determined for each Cyp as well as micrococcal nuclease. This amount was then divided by the time of degradation (in minutes) and the molarity of each enzyme so that the percentage degraded/min/mol of enzyme was determined. The amount of degradation of linear double-stranded pUC18/min/mol of CypA, -B, and -C relative to the amount degraded/min/mol of micrococcal nuclease was calculated.

End Labeling Degraded DNA—After incubation with Cyp, the DNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1) extractions followed by chloroform/isoamyl alcohol (24:1) alone. 10 units of terminal deoxynucleotidyltransferase (Life Technologies, Inc.) and 750,000 cpm of [^{32}P]dCTP (ICN) were added to the DNA and incubated for 2 h at 37 °C. Labeled DNA was isolated by centrifugation through a Sephadex G-50 (Sigma) column and was then electrophoresed through a 1% agarose gel. The gel was dried and then exposed to autoradiographic film for visualization.

Gel Shift Assays—The substrate DNA was an 18-mer oligonucleotide (CGACCAGAGTACGTGATG) that was end-labeled with terminal deoxynucleotidyltransferase (Life Technologies, Inc.) and [^{32}P]dCTP. After incubating the DNA in the labeling reaction for 0.5 h at 37 °C, the reaction was stopped with 2 μl of 10 mM EDTA and brought to 100 ml with double-distilled H_2O. The labeled DNA was separated from unincorporated nucleotides by a Sephadex G-25 (Sigma) spin column. The resulting labeled DNA was then diluted to 20,000 cpm/μl. 1 μl of this DNA was incubated in the absence or presence of 1 μg of CypB in a binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5% glycerol) for 16 h at 4 °C. The samples were then electrophoresed through a 6% polyacrylamide gel, the gels were dried, and the samples were visualized by exposure to XAR film (Kodak).

Electron Microscopy—Electron microscopy was performed as described previously (38). Briefly, the CypB/DNA samples were prepared by incubation of 1 μg of CypB with 1 μg of linearized plasmid DNA in the absence of ions. The samples were fixed with 0.6% glutaraldehyde and then passed through a BioGel A5 m (Bio-Rad) column to remove unbound proteins. The samples were mounted onto charged carbon-coated grids and dehydrated through a series of graded alcohol rinses, with a final wash of 100% alcohol. The grids were then shadowed with tungsten and examined by transmission electron microscopy.

Peptidylprolyl Isomerase Assay—The *cis-trans*-peptidylprolyl isomerase assay was performed as described previously (39). 50 μM *n*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide was added to 35 mM Tris-HCl (pH 8) in a total volume of 1 ml at 10 °C. 1 μg of Cyp and 50 μl of 100 μM chymotrypsin were quickly added to the substrate and mixed. The chymotrypsin can cleave the *p*-nitroanilide substrate only when the proline residue is in the *trans* configuration. Once cleaved, the substrate produces a yellow color that can be quantitated by its absorbance at 390 nm (OD 390). The increase in optical density over time is indicative of the number of proline residues converted to *trans* over time. The OD 390 is read every 0.3 s for 3 s on a Beckman DU 640 spectrophotometer. The slope of OD *versus* time is the rate of conversion, and the average of at least three separate assays is shown for each given condition.

Chromatin Degradation Assay—The HeLa nuclei assay was performed as described previously (40).[2] Briefly, HeLa cells were grown in suspension culture (1–7 $\times 10^5$ cells/ml) at 37 °C in Joklik's minimum essential medium supplemented with 2% fetal calf serum, 2% calf serum, 2 mM glutamine, 75 units/ml penicillin and 50 units/ml streptomycin sulfate. HeLa nuclei were prepared by pelleting the cells and resuspending in 10 mM $MgCl_2$, 0.25% Nonidet P-40. The nuclei were then pelleted and resuspended in 50 mM Tris (pH 7.4). Aliquots (2×10^6 nuclei) were then added to tubes containing 50 mM Tris (pH 7.4), 2 mM $MgCl_2$, 1 mM $CaCl_2$ (final concentrations in a total volume of 400 μl) in the presence or absence of 500 nM CypC. Samples were incubated for 5 h at room temperature with gentle rotation. Studies in our laboratory have shown that HeLa nuclei incubated under these conditions undergo very little spontaneous chromatin degradation (41).

For pulsed field analysis, 200 μl was removed at the end of the incubation, mixed with an equal volume of 1% Incert agarose (FMC Bioproducts) at 37 °C, transferred to a 0.5-cm^2 mold, and placed at 4 °C for 5 min to solidify. Plugs were then incubated in 100 mM EDTA, 1% (w/v) *N*-lauroylsarcosine (10 ml) at 37 °C overnight before being transferred to 1 ml of the same buffer containing proteinase K (100 μg) and reincubated for a minimum of 16 h at 50 °C. Plugs were stored at 4 °C in this buffer until used. A 2.0-mm-thick slice of the agarose plug was equilibrated in 50 ml of 0.5 $\times$ TBE buffer for >3 h before being incorporated into a 1% agarose gel. The gel was placed in a clamped homogeneous electric field pulsed field system (Bio-Rad) filled with 0.5 $\times$ TBE and allowed to equilibrate to 14 °C before electrophoresis for 19 h at 6.0 V/cm with a linear switch interval ramp from 0.5 to 45.0 s. The parameters were chosen to optimally separate DNA fragments ranging from 10 to 500 kb (42)[3] The gel was then stained with ethidium bromide

[2] Hughes, F., and Cidlowski, J. (1997) *Cell Death Diff.*, in press.
[3] E. Lai, personal communication.

and visualized by UV transillumination.

Following removal of the aliquot for pulsed field analysis, conventional electrophoresis samples were prepared from the remaining nuclei by first adding EDTA, NaCl, and sodium dodecyl sulfate to a final concentration of 25 mM, 540 mM, and 0.5%, respectively. TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA) was added to bring the final volume to 400 μl, and Proteinase K was added to 0.5 mg/ml. Samples were then incubated for 1 h at 50 °C and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) alone. DNA was precipitated by adding 10 μl of 5 M NaCl plus 1 ml of ice-cold 100% ethanol and incubating at −70 °C for at least 20 min. DNA was collected by centrifugation, dried, and resuspended by incubating the pellet overnight at 37 °C in TE buffer containing 0.33 mg/ml DNase-free RNase A. DNA concentrations were determined spectrophotometrically, and samples (15 μg) were then electrophoresed (3.25 h; 80 V) on a 1.8% agarose gel submerged in 0.5 × TPE (40 mM Tris-phosphate, 4 mM EDTA). Gels were subsequently stained with ethidium bromide and visualized by UV transillumination.

RESULTS

Recombinant CypA, -B, and -C Degrade DNA under Nondenaturing Conditions—Previously, we used a radioactive gel nuclease assay to demonstrate that three members of the Cyp family, CypA, -B, and -C, are able to degrade DNA (29); however, this assay used a protein denaturation and renaturation step prior to analysis of nuclease activity, and thus it was unclear if native Cyps have the same nucleolytic activity. To determine whether the native Cyps could degrade DNA, we employed a solution nuclease assay that evaluates the nuclease activity from the nondenatured recombinant protein. In this assay, the purified Cyp is incubated with linearized plasmid (pUC18) in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}. The production of low molecular weight DNA indicates an active nuclease in that sample. The results of the solution nuclease assay with purified CypA, -B, and -C are shown in Fig. 1. Samples with DNA only, in the absence or presence of ions (Fig. 1*A*, *Con panel*, *+EDTA*, *+Ca^{2+}/Mg^{2+} lanes*, respectively) do not contain detectable levels of degraded DNA. CypA and CypB show greater DNA degradation in the presence of Ca^{2+} and Mg^{2+} than in the absence of these ions (Fig. 1*A*, *CypA* and *CypB panels*), whereas CypC can also degrade substantial levels of DNA in the absence of any additional ions (Fig. 1*A*, *CypC panel*, *+EDTA lane*). The nuclease activity of CypC is, however, enhanced by the addition of the divalent cations (Fig. 1*A*, *CypC panel*, *+Ca^{2+}/Mg^{2+} lane*). Similarly, single-stranded DNA is best degraded by CypA and CypB in the presence of Ca^{2+} and Mg^{2+} and by Cyp C in either the absence or the presence of these ions (Fig. 1*B*). Together, these data demonstrate a nucleolytic action on different forms of DNA by native Cyps.

Determination of Optimal Conditions for Cyp Nuclease Activity—To obtain the ideal conditions for each Cyp's nuclease activity and to be able to compare Cyp nuclease activity with that of other apoptotic nucleases, we determined the optimal pH and ion requirements of CypA, -B, and -C *in vitro*. The optimal pH for each Cyp was determined by performing a series of solution nuclease assays over a pH range from 5.5 to 10.0, with increments of 0.5 pH units. As shown in Fig. 2, CypA, -B, and -C can degrade the linearized plasmid substrate over the range of pH values, with the maximum activity seen from pH 7.5 to pH 9.5. Quantitation of these assays (data not shown) revealed that each Cyp had a slightly different pH optimum, with CypA being most efficient at pH 8.0, CypB at pH 8.5, and CypC at pH 9.5.

Cation Dependence—Many of the nucleases proposed to be involved in apoptosis have been shown to be dependent on Ca^{2+} and/or Mg^{2+} for activation (32, 34, 43, 44); therefore, we were interested in determining how these two cations affected nuclease activity derived from Cyps. As shown in Fig. 1, some degradation occurs in the absence of added ions, especially with

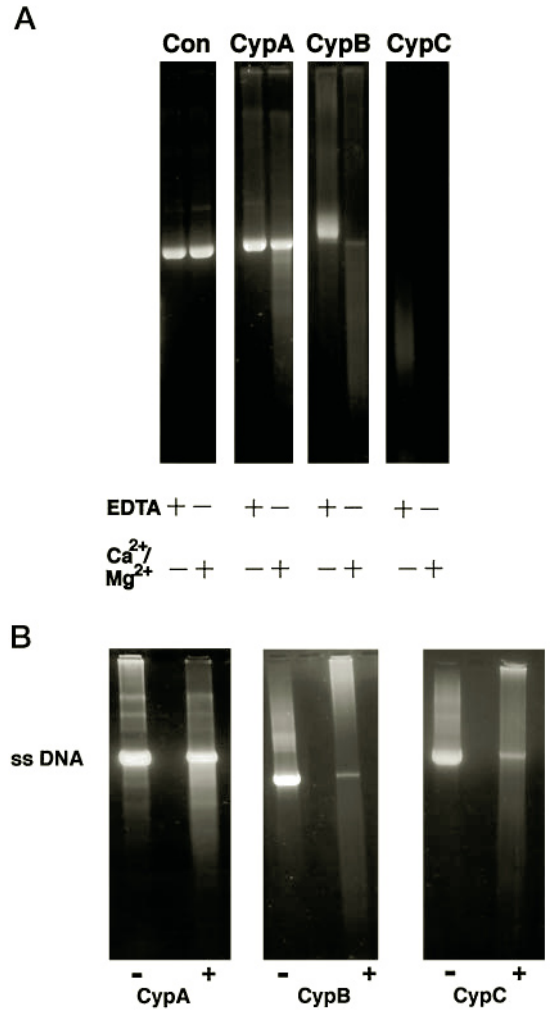


FIG. 1. **Cyclophilins A, B and C degrade linear double-stranded DNA and linear single-stranded DNA in a nondenaturing solution nuclease assay.** *Panel A* contains samples with double-stranded DNA, and *panel B* contains samples with single-stranded DNA. Each lane contains 1 μg linearized pUC18 (3 nmol of phosphodiester bonds) as substrate in either the double- or single-stranded form, prepared as described under "Experimental Procedures," plus 1 mM each $CaCl_2$ and $MgCl_2$ brought up to a final volume of 10 μl with 20 mM Tris, pH 8.0. CypA, -B, and -C are 5.6, 4.8, and 4.4 μM, respectively. The *left lane* in each set has only ions and DNA present in Tris buffer. The presence of low molecular weight DNA (*i.e.* strands that are smaller than the 2690-base pair substrate) indicate the presence of an active nuclease in that sample. Low molecular weight DNA is generated by CypA, -B, and -C with both a single-stranded and a double-stranded linear DNA substrate.

CypC; however, further experimentation with a series of solution nuclease assays performed at increasing Ca^{2+} and Mg^{2+} concentrations revealed that optimal degradation by each Cyp occurred at 1 mM of either ion (data not shown). The amount of DNA degraded in the presence of either ion alone was determined as the percentage degraded relative to the amount degraded in the presence of 1 mM Ca^{2+} and Mg^{2+}. These results are shown in Table I. CypA and CypB had optimal nuclease activity with a combination of Ca^{2+} and Mg^{2+}, whereas CypC was most active in the presence of Mg^{2+} alone. Under all circumstances, CypC displayed the greatest efficiency at DNA degradation, with a 100 times greater DNA degrading activity than CypA or CypB under the same experimental conditions (data not shown). Interestingly, this difference in activity was not appreciated in the previous experiments performed under denaturing conditions (29) and probably reflects an inefficient renaturation of CypC, the highest molecular weight protein of

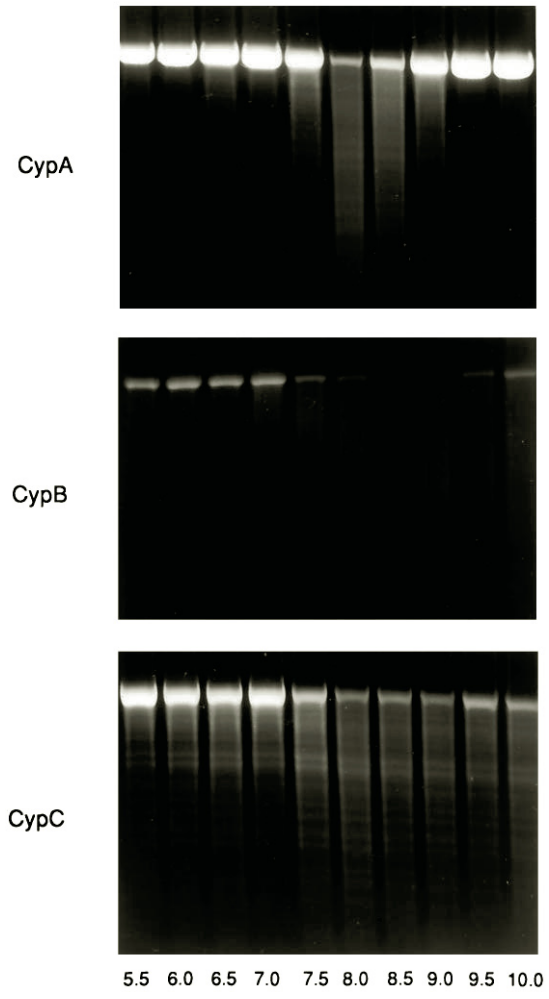


FIG. 2. **pH dependence of cyclophilin nuclease activity.** The solution nuclease assays were performed with a double-stranded linearized pUC18 plasmid substrate as described in the legend to Fig. 1, with the exception that buffers of differing pH values were substituted in place of the 20 mM Tris, pH 8.0, buffer. The buffers were all 20 mM and included MES (pH 5.5 and 6.0), HEPES (pH 6.5, 7.0, and 7.5), Tris (pH 8.0 and 8.5), and CAPS (pH 9.5 and 10.0). The amount of DNA degraded in each case was measured densitometrically, and the results from three separate experiments for each Cyp revealed optimal pH values to be pH 8.0 for CypA, pH 8.5 for CypB, and pH 9.5 for CypC.

TABLE I
Calcium and magnesium ion activation of cyclophilin nuclease activity

The amount of DNA degraded by cyclophilins A, B, and C in the presence of 1 mM Ca^{2+} alone or 1 mM Mg^{2+} alone was compared as a percentage of the amount of DNA degraded in the presence of 1 mM both Ca^{2+} and Mg^{2+}. The assays and quantitation procedures were performed as described under "Experimental Procedures."

Reaction conditions	CypA	CypB	CypC
1 mM Ca^{2+}/Mg^{2+}	100%	100%	100%
1 mM Ca^{2+}	61.8	80.2	70.8
1 mM Mg^{2+}	45.5	42.4	184.8

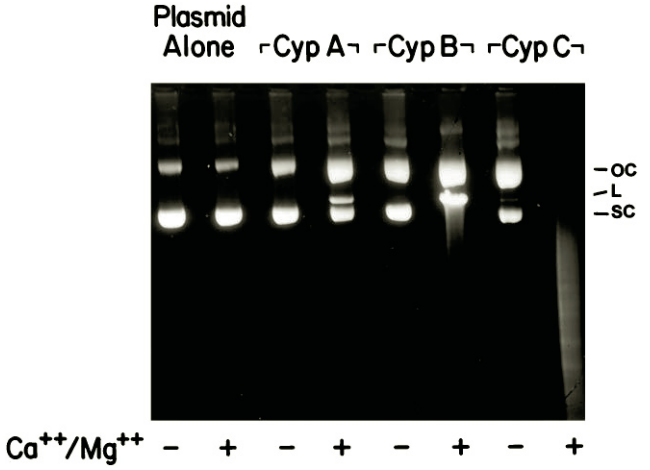


FIG. 3. **Cyclophilins demonstrate an endonucleolytic activity by degrading supercoiled plasmid.** 1 μg of supercoiled pCTF1 plasmid (3 nmol of phosphodiester bonds) was incubated with 40 pmol of CypA, 40 pmol of CypB, or 20 pmol of CypC for 3 h at 37 °C in the absence (−) and presence (+) of 1 mM Ca^{2+}/Mg^{2+}. Cleavage occurred in the samples containing Cyps and ions, and some nicking activity is apparent in the *CypC*, $-Ca^{++}/Mg^{++}$ sample. CypA and CypB demonstrate a nicking activity in the presence of ions by converting the supercoiled (*SC*) form to open circular (*OC*) and linear (*L*) forms. CypC produces low molecular weight fragments in the same time period. The cleavage of supercoiled DNA by the Cyps indicates an endonucleolytic capacity.

the three studied. These optimal conditions were applied to further characterizations of the nuclease activity and the interaction of Cyps with DNA.

Cyps Have Endonucleolytic Activity—Substitution of supercoiled DNA for linear DNA as substrate in the solution nuclease assay allows for discrimination of Cyp endo- or exonuclease activity. The addition of supercoiled plasmid to the digestion assays revealed that it could be cleaved by CypA, -B, and -C (Fig. 3), indicating that each pure protein had endonucleolytic activity. Interestingly, both nicking and linearizing activities were observed in both the presence and absence of added ions. CypA and CypB, which degrade at a slower rate than CypC, clearly demonstrate a nicking activity by the conversion of the supercoiled substrate (*SC*) to both open circular (*OC*) and linear (*L*) forms prior to degradation to lower molecular weight forms (Fig. 3, *CypA*, $+Ca^{++}/Mg^{++}$ *lane*, and *CypB*, $+Ca^{++}/Mg^{++}$ *lane*). CypC shows only a conversion to low molecular weight DNA in the same time period (Fig. 3, *CypC*, $+Ca^{++}/Mg^{++}$ *lane*).

CypB Binds to DNA in the Absence of Ca^{2+} and Mg^{2+}—To explore the interaction between Cyps and DNA more directly, we took advantage of the different efficiencies of DNA degradation by the different Cyps. While CypC demonstrates a clear nucleolytic activity in the absence of added ions, CypA and CypB show much lower levels of DNA degradation in the absence of ions. Thus, by incubating CypA and CypB with DNA in the absence of added ions, we were able to observe a direct interaction between protein and the DNA substrate. Several different approaches were employed, including gel shift assays and electron microscopy, which allowed for a clear picture of Cyp/DNA interaction. Fig. 4*A* shows a gel shift assay with CypB and radiolabeled linear pUC18 as a substrate. The *CypB lane* shows a band of slower migrating DNA, indicating that it is bound to the protein. Electron micrographs of linear pUC18 and CypB incubated in a similar manner, *i.e.* without added ions, reveal CypB bound at discrete sites along the DNA strand (Fig. 4*B*). These are the first such images to show direct interaction between CypB and linear DNA. The stochastic arrangement of the CypB multimers along the DNA substrate suggests that some specificity may be involved in Cyp binding to DNA.

Inhibitors of Cyp Nuclease Activity—An important property of a nuclease is its ability to be regulated *in vivo*. There are several agents, such as zinc (45) and aurintricarboxylic acid (46), that are well known inhibitors of both apoptosis and nucleases. We have previously shown that these two agents are capable of inhibiting Cyp nuclease activity in the radioactive gel assay at 2 and 1 mM, respectively (29). In the solution

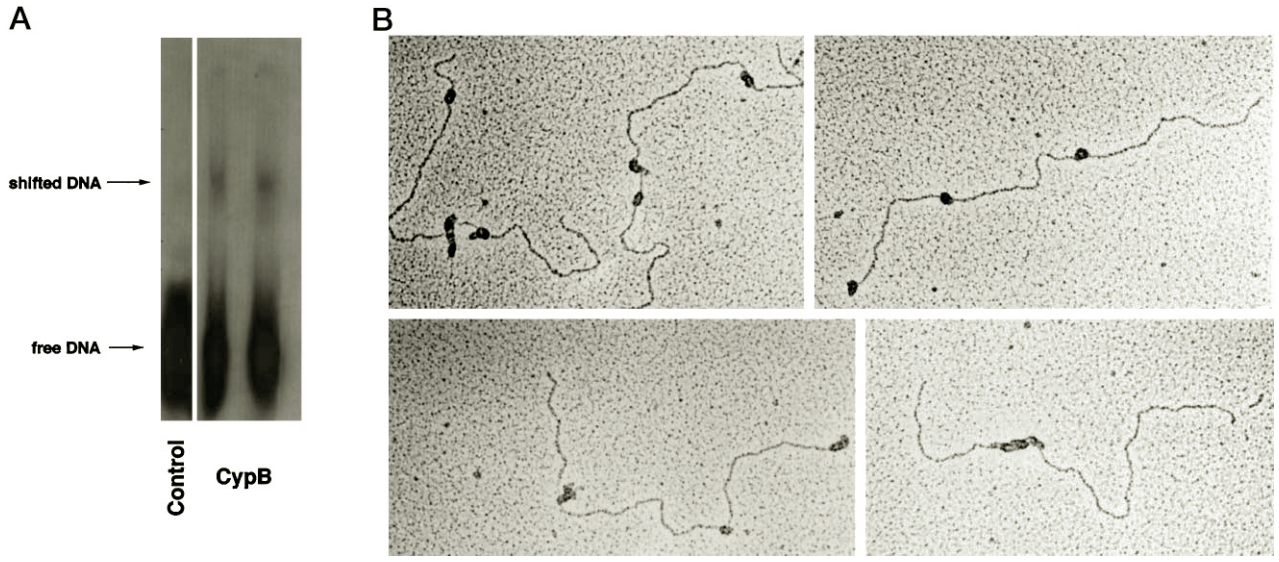


FIG. 4. **CypB interaction with linearized DNA.** *A*, cyclophilin B shifts DNA migration in a nondenaturing gel. The gel shift assay was performed as described under "Experimental Procedures." The *left lane* contains labeled oligonucleotides, and the *right two lanes* are duplicate samples of the oligonucleotide incubated with CypB. The slower migrating band in these two samples demonstrates binding of CypB to DNA. *B*, electron microscopy reveals that CypB binds at discrete sites along linear pUC18. 2 μg of (4.8 μM) CypB were incubated with 2.7 μg of (56 nM) linear pUC18 in 20 mM Tris-HCl (pH 7.5) without ions for 2 h prior to fixing and preparing for electron microscopy. These electron micrographs show multimers of CypB binding at certain sites along the DNA strand. The discrete binding of CypB suggests that Cyps have preferred binding sites along the strand of DNA.

nuclease assay, 100 μM zinc completely blocked DNA degradation by each Cyp (data not shown). However, we were interested in determining whether more physiologically relevant factors, such as certain common ions, could also attenuate Cyp nuclease activity. We chose to examine the effects of K^+ and Na^+ on Cyp nuclease activity because ion fluxes have recently been shown to be involved in apoptosis (47, 48). When either K^+ or Na^+ was added to a solution nuclease assay containing 1 mM Ca^{2+} and Mg^{2+}, there was a dose-dependent decrease in the amount of DNA degraded, with IC_{50} values at approximately 79 mM K^+ and 48 mM Na^+ (Table II). Lower concentrations of each ion resulted in partial inhibition of nuclease activity from Cyps in a dose-dependent manner. The effect of a specific Cyp-binding agent was then assessed for its ability to inhibit or enhance Cyp nuclease activity.

CsA Does Not Inhibit Cyp Nuclease Activity—The binding of the immunosuppressant drug CsA to Cyp is known to inhibit the peptidylprolyl isomerase activity of Cyps (16, 49). We also observe CsA inhibition of peptidylprolyl isomerase activity. As shown in Fig. 5*A*, when the Cyp is incubated at a 1:1 molar ratio with CsA for 15 min prior to being added to the assay reaction, there is a complete inhibition of the Cyp isomerase activity (see "Experimental Procedures"). However, when CsA is added to the solution nuclease assay, we do not observe any change in the DNA degradation profile (Fig. 5*B*). These results demonstrate that the Cyps we were evaluating for nuclease activity have isomerase activity and, further, that these Cyps are capable of binding to CsA with a well characterized response. In addition, these data show for the first time that the nuclease activity of Cyps is separate from the isomerase activity.

Cyp Is Located in the Nucleus of Control and Apoptotic Thymocytes—While our previous work demonstrated a surprising homology between NUC18, a nuclease isolated from apoptotic rat thymocyte nuclei, and Cyp proteins (29), it was not clear whether Cyps were nuclear proteins in rat thymocytes. To address this question, we developed antibodies directed against Cyp proteins. Western blot analyses of nuclear and cytoplasmic extracts from rat thymocytes could then be performed. The results (Fig. 6) showed an 18-kDa band that reacts to these

TABLE II

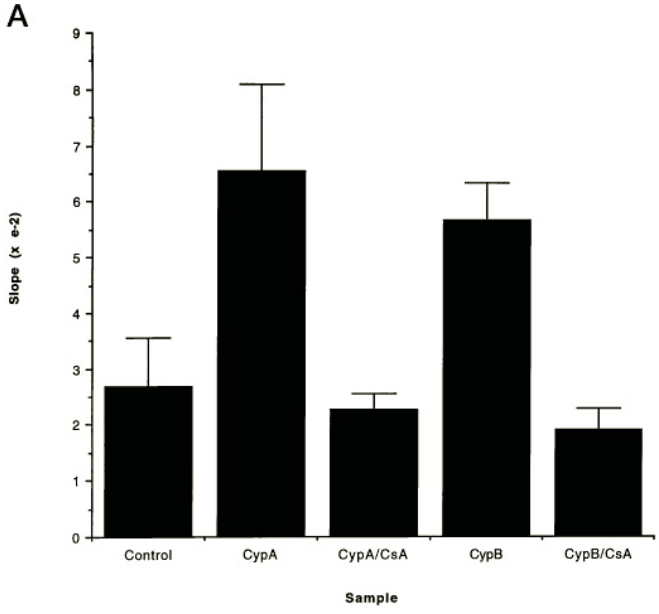
IC_{50} of potassium and sodium ions on cyclophilin nuclease activity

NaCl and KCl were added at increasing concentrations to the solution nuclease assay, which also contained 1 mM Ca^{2+} and Mg^{2+}. The IC_{50} for Na^+ and K^+ were determined by quantitating the amount of DNA degraded in the presence of each ion at 0, 1, 10, and 100 or 150 mM, depending on the ion. Na^+ showed complete inhibition at 100 mM and K^+ at 150 mM. The percentage of DNA degraded was plotted as a function of the concentration of the inhibitory ion. The concentration of ion at 50% degradation was then determined from the slope of that line. Three separate experiments for each ion were performed.

	IC_{50} K^+	IC_{50} Na^+
CypA	82.6 ± 6.8	44.1 ± 3.6
CypB	76.3 ± 8.8	51.6 ± 0.6

antibodies, revealing that Cyp is located in both the nucleus and the cytoplasm of rat thymocytes. Additionally, we were also interested in determining whether Cyp levels in either compartment were altered following stimulation of apoptosis. Nuclear and cytoplasmic extracts were prepared from thymocytes isolated at increasing time points following dexamethasone treatment of rats (0–4 h) and analyzed by Western blotting. These results indicated that there were no changes in Cyp levels in either the nucleus or cytoplasm during the early stages of apoptosis. The Western blot for the 4-h time point is also shown in Fig. 6. Thus, we observed that Cyps are located in the nucleus as well as the cytoplasm of rat thymocytes and that the levels of the protein are unchanged during dexamethasone-induced apoptosis.

Cyps Produce 3′-OH Termini—After characterizing a variety of aspects of nuclease activity from Cyps to compare with apoptotic nucleases, we next wanted to determine what type of termini was produced in the Cyp-fragmented DNA, because apoptotic DNA fragmentation is associated with the formation of 3′-OH termini (50). Terminal deoxynucleotidyltransferase specifically attaches free nucleotides to 3′-OH termini of DNA, so we could determine if Cyps caused the formation of free 3′-OH groups by attempting to label Cyp-cleaved DNA products with terminal deoxynucleotidyltransferase and [^{32}P]dCTP. These assays revealed an increase in the number of 3′-OH termini in the digested *versus* the control samples (Fig.



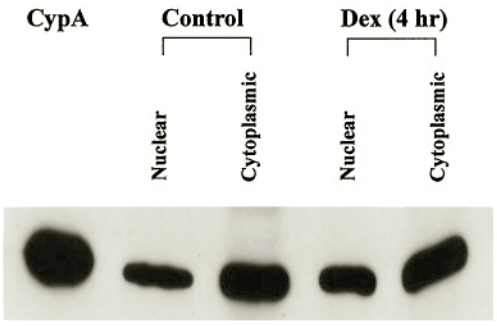


FIG. 6. **Cyclophilin is located in the nucleus and the cytoplasm of nonapoptotic and apoptotic thymocytes.** Western blot analyses of rat thymocyte extracts from nonapoptotic (*Control*) and apoptotic (*Dex*) nuclear and cytoplasmic extracts are shown. The thymocyte extracts from adrenalectomized rats that were either untreated or injected with dexamethasone 4 h prior to preparation were harvested, fractionated, and assayed as described under "Experimental Procedures." Affinity-purified anti-CypA antibodies recognized an 18-kDa protein in the nucleus and cytoplasm of apoptotic and nonapoptotic thymocytes, demonstrating that Cyps are located in both the nucleus and the cytoplasm. The "CypA" lane contains 2 μg of purified recombinant CypA.

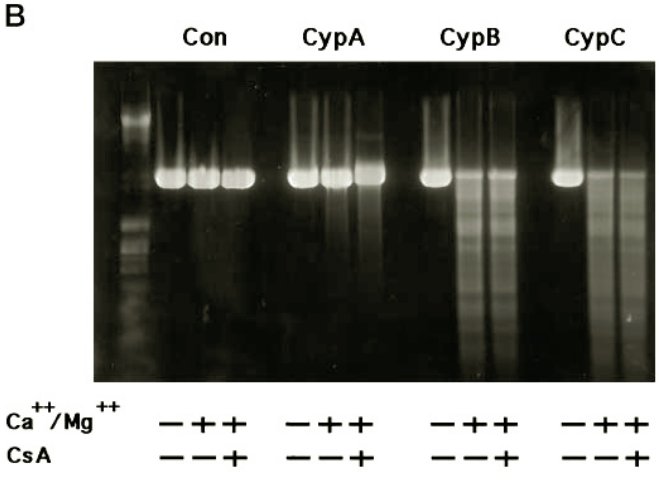


FIG. 5. **Cyclosporin A does not inhibit Cyp nuclease activity.** *A*, the results of the peptidylprolyl isomerase assays (described under "Experimental Procedures") with CypA and CypB. When CsA is added to the isomerase assays at a 1:1 molar ratio with the Cyp, there is a decrease in the rate of conversion from *cis* to *trans* in the substrate. Taken together, these data indicate that the nuclease activity of Cyps is separate from its isomerase activity. *B*, a series of solution nuclease assays were performed in the presence of no protein, CypA (202 pmol), CypB (48 pmol), and CypC (10 pmol), with DNA alone (14 nmol of phosphodiester bonds); DNA plus 1 mM Ca^{2+}/Mg^{2+}; or DNA, ions, and CsA (CsA was at a 1:1 molar ratio with the Cyp in each sample). These data show that the addition of CsA does not alter the amount of Cyp DNA degradation.

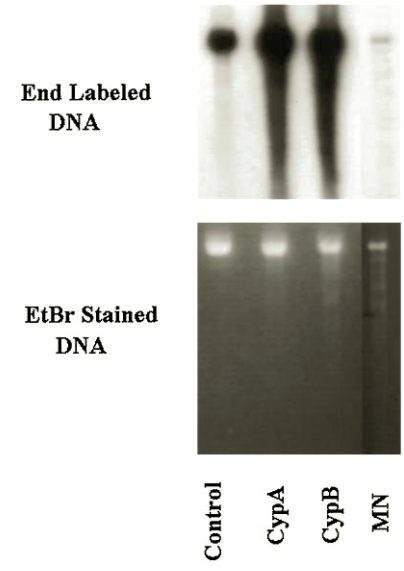


FIG. 7. **Cyclophilins produce 3′-OH termini in linear double-stranded DNA.** Following incubation with either no protein, cyclophilin A (*CypA*), cyclophilin B (*CypB*), or micrococcal nuclease (*MN*), DNA was labeled with terminal deoxynucleotidyltransferase and [^{32}P]dCTP. The *lower panel* (*EtBr Stained DNA*) shows the DNA after electrophoresis through a 1% agarose gel. Because of the purification step prior to running the gel, much of the degraded, low molecular weight fragments were lost. After the gel was dried and exposed to film, the remaining low molecular weight DNA from the cyclophilin-degraded samples were readily apparent. The increase in the amount of end labeling in these samples as compared with the untreated sample (*Control lane*) or MN-treated sample (*MN lane*), which produces 3′-PO_4 termini, indicates an increase in the production of 3′-OH ends by the Cyps.

7; *cf. Control lane* with *CypA* and *CypB lanes*). The DNA digested with micrococcal nuclease, which produces termini with 3′-phosphate groups does not label with terminal deoxynucleotidyltransferase (Fig. 7, *MN lane*). With the generation of 3′-OH termini, Cyps exhibit a similarity with the nuclease (or nucleases) activated during apoptosis.

CypC Generates 50-kb DNA Fragments in Chromatin—With the solution nuclease assay, we have been able to show that many aspects of Cyp nuclease activity are similar to that of apoptotic nucleases. To further explore the notion that Cyps may be nucleolytically active during apoptosis, we investigated the most active Cyp, CypC, for its ability to digest chromatin DNA in the HeLa nuclei assay. Because there are two different types of DNA degradation observed during apoptosis (50-kb fragments and smaller, internucleosomally cleaved fragments (51)), we looked for both DNA degradation patterns in HeLa DNA following incubation with CypC. As shown in Fig. 8, the addition of CypC to HeLa nuclei in the presence of Ca^{2+} and Mg^{2+} completely eliminated the high molecular weight chromatin (*panel B*), as shown by loss of DNA in the well and the compression band. The addition of CypC also generated a 50-kb band of DNA from the HeLa chromatin. Additionally, an increase in low molecular weight DNA is observed (*panel A*), although internucleosomal degradation patterns were not observed. This assay demonstrates that CypC is capable of acting

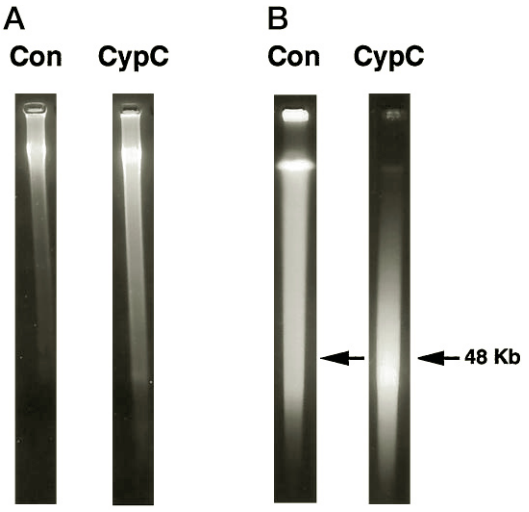


FIG. 8. **Cyclophilins generate 50-kb fragments in HeLa chromatin.** 500 nM CypC were added to 2×10^6 HeLa nuclei in the presence of Ca^{2+} and Mg^{2+} and incubated for 5 h at room temperature. After incubation, the samples were analyzed for the production of both large chromatin breaks as well as internucleosomal breaks, as described under "Experimental Procedures." The internucleosomal cleavage assay (*A*) reveals that lower molecular weight DNA is generated by the addition of CypC to the HeLa nuclei; however, the "ladder pattern" of internucleosomally cleaved DNA characteristic of apoptosis is not apparent. The 50-kb fragments produced in the CypC-treated samples are shown in *B*. The non-CypC-treated nuclei samples (*Con*) are shown to the *left* in each *panel* and display some background chromatin cleavage in both cases.

on chromatin DNA and generating a 50-kb product, similar to what is observed during apoptosis.

DISCUSSION

Cyps comprise a highly abundant family of proteins (1) that are expressed in every organism studied to date. They possess peptidylprolyl *cis-trans*-isomerase activity (16, 49), and have been shown to enhance the folding rate of several proteins *in vitro* (15–17). We recently reported a sequence and structural homology between Cyps and NUC18, an apoptotic nuclease, and demonstrated that several members of the Cyp family have an inherent nuclease activity (29). We have now further characterized the nuclease activity of Cyp and demonstrated that it is located in the nucleus and shares many characteristics with nucleases associated with apoptosis.

Although there is still some debate as to the pH in apoptotic cells, there are many reports of an apoptotic nuclease with a pH optimum in the 7.5–9.5 range (32, 52–54), similar to that observed for CypA, -B, and -C. These three Cyps display an ion-dependent nuclease activity that can be activated by either Ca^{2+} or Mg^{2+} or by a combination of both, although to different efficiencies in each. This Ca^{2+}/Mg^{2+} dependence correlates with what has been observed for apoptotic nucleases in other studies (32–35). Interestingly, we observe a much stronger activation of CypC by Mg^{2+} alone than by Mg^{2+} plus Ca^{2+}. Sun and Cohen (43) were recently able to distinguish two apoptotic nuclease activities (50-kb fragmentation from internucleosomal cleavage) by using Ca^{2+} and Mg^{2+} ions, such that the addition of Mg^{2+} alone to nuclei resulted in the digestion of DNA into 50-kb fragments, while internucleosomal cleavage was not observed until Ca^{2+} was added in conjunction with the Mg^{2+}. Thus, the strong activation of CypC by Mg^{2+} alone, plus the observation that CypC addition to isolated nuclei results in the formation of 50-kb fragments of chromatin DNA, suggests that CypC could be responsible for the cleavage of DNA into 50-kb fragments. We also observe inhibition of Ca^{2+}/Mg^{2+}-stimulated nuclease activity at physiologically relevant levels with the ions Na^+ and K^+. The normal intracellular concentration of K^+ is approximately 140 mM, a concentration high enough to inhibit Cyp nuclease activity; however, the decrease in the intracellular K^+ concentration during the shrinkage of cells undergoing apoptosis (47)[4] results in conditions in which Cyps become active nucleases. The inhibitory effect of Na^+ at concentrations that correspond to the higher levels observed in necrotic cells (55) suggests that Cyps would not act during necrotic cell death. Thus, these two facts together imply that Cyps can act as apoptotic-specific nucleases. An important finding first discovered in our study is the production of 3′-OH termini by Cyp, which corresponds to what has been observed in many cases of apoptosis (50). In fact, the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method, a technique widely used to detect apoptotic cells *in situ* (50), is based on the overproduction of 3′-OH ends in apoptotic cells. Thus, these data demonstrate that the characteristics of Cyp nuclease activity are suggestive of an apoptotic nuclease.

Because the catalytic activity most associated with Cyps is peptidyl-prolyl isomerization, we were interested in determining whether the novel nuclease activity of Cyps was related to the isomerase activity. The addition of the high affinity ligand CsA to the solution nuclease assay did not affect the ability of Cyps to degrade DNA, whereas it completely blocked the isomerase activity. These results indicate that the nuclease and isomerase properties of Cyps are distinct.

Although the rate of isomerization by the Cyps in *in vitro* assays shows a high efficiency (56), Cyps degrade DNA at a lower rate compared with the well characterized micrococcal nuclease. This highly efficient enzyme (which has a turnover rate of 95 s^{-1}; Ref. 57) degrades DNA approximately 1000 times faster than CypC. CypC, in turn, displays a nuclease activity approximately 100 times greater than CypA and CypB. There are several potential reasons for this apparently slower rate of Cyp nuclease activity. The first is that the proteins used in these assays are recombinant forms and therefore may be missing an essential post-translational modification that enhances their nucleolytic activity. Another possibility is that complete activation of the Cyps requires a co-factor or additional protein that was absent from our solution nuclease assays. We have shown that multimers of CypB bind to a strand of DNA with some specificity. It may be that an additional protein will enhance the DNA binding and nucleolytic activities of Cyp. Indeed, Cyp binds only weakly to calcineurin by itself, but it shows great affinity for calcineurin with the addition of CsA (58). Thus, there are several possibilities to account for the rate of DNA degradation by Cyps. Nevertheless, it was important for us to determine if the nuclease activity of Cyps was sufficient to account for the levels of DNA degradation observed during apoptosis *in vivo*. To completely convert chromatin, which contains 6×10^9 nucleotides (59), to 50-kb fragments, 60,000 phosphodiester bonds must be cleaved. Human thymocytes contain approximately 540,000 molecules of CypA (based on an average of 1.6 μg of CypA/mg of cell protein) (8). Therefore, only 60,000, or 11%, of all CypA molecules in the thymocyte need to be in contact with the chromatin and to make one disruption of the phosphodiester bond to completely reduce the chromatin to 50-kb fragments. Thus, the number of CypA molecules is more than adequate to at least account for the complete degradation of the genome to 50-kb fragments during apoptosis.

How might Cyps become active as nucleases during apoptosis? We have already shown that the levels of the protein

[4] C. Bortner and J. Cidlowski, unpublished observations.

remain unchanged during apoptosis; therefore, we propose several potential activation mechanisms. The first potential activation mechanism involves the loss of K^+ ions at the onset of apoptosis. We have shown how the normal intracellular K^+ levels are sufficient to prevent Cyp activity and that a decrease in K^+ levels allows Cyp nuclease activity to occur. Thus, attenuation of K^+ levels in the nucleus may be all that is necessary to activate Cyp nuclease activity. An additional possibility is that the loss of K^+ ions stimulates release of Cyp from an inhibitory complex, freeing the active Cyp to act on the chromatin. In support of this idea, earlier work in our laboratory revealed that NUC18 was associated with a high molecular weight complex in nonapoptotic cells (60). Work performed in other laboratories (61), has shown that Cyp is part of a large multiprotein complex, which also suggests the possibility of an inhibitory complex for Cyp under nonapoptotic conditions. Another possible mechanism for activation of Cyp nuclease activity is that Cyps may have altered post-translational modification during apoptosis such that they are now capable of degrading the chromatin DNA. Recently, Chatellard-Gruaz *et al*. (62) demonstrated that isoforms of CypA were differentially expressed during keratinocyte differentiation, a process with many similarities to apoptosis. Additionally, Krummrei *et al*. (41), who describe CypA as a Zn^{2+}-dependent DNA-binding protein, suggest that CypA exists in two isoforms, one of which involves Zn^{2+}-dependent DNA binding and the other involving Zn^{2+}-independent isomerase and nucleolytic activity. Thus, there are several potential mechanisms to explain how Cyp might act as an apoptotic nuclease.

In summary, we have characterized several different aspects of Cyp nuclease activity, all of which suggest that Cyps could be apoptotic nucleases. For example, Cyps display a dependence on Ca^{2+} and Mg^{2+} for optimal activity, and they produce 3′-OH termini in the digested DNA. They are also capable of degrading a variety of DNA substrates and can generate 50-kb fragments in HeLa chromatin. We propose that Cyps are activated to degrade chromatin during apoptosis. Further analysis of the role of Cyp in apoptosis is ongoing.

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REFERENCES

1. Handschumacher, R., Harding, M., Rice, J., and Drugge, R. (1984) *Science* **226,** 544–546
2. Kieffer, L., Thalhammer, T., and Handschumacher, R. (1992) *J. Biol. Chem.* **267,** 5503–5507
3. Hornbogen, T., and Zocher, R. (1995) *Biochem. Mol. Biol. Int.* **36,** 169–176
4. Tropschug, M., Nicholson, D. W., Hartl, F.-U., Köhler, H., Pfanner, N., Wachter, E., and Neupert, W. (1988) *J. Biol. Chem.* **263,** 14433–14440
5. Liu, J., and Walsh, C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4028–4032
6. Hayano, T., Takahashi, N., Kato, S., Maki, N., and Suzuki, M. (1991) *Biochemistry* **30,** 3041–3048
7. Koletsky, A., Harding, M., and Handschumacher, R. (1986) *J. Immunol.* **137,** 1054–1059
8. Ryffel, B., Woerly, G., Greiner, B., Haendler, B., Mihatsch, M., and Foxwell, B. (1991) *Immunology* **72,** 399–404
9. Rassow, J., Mohrs, K., Koidl, S., Barthelmess, I., Pfanner, N., and Tropschug, M. (1995) *Mol. Cell. Biol.* **15,** 2654–2662
10. McDonald, M., Ardito, T., Marks, W., Kashgarian, M., and Lorber, M. (1992) *Transplantation* **53,** 460–466
11. Price, E., Zydowsky, L., Jin, M., Baker, C., McKeon, F., and Walsh, C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1903–1907
12. Kok, R., Christoffels, V., Vosman, B., and Hellingwerf, K. (1994) *Biochim. Biophys. Acta* **1219,** 601–606
13. Harding, M., Handschumacher, R., and Speicher, D. (1986) *J. Biol. Chem.* **261,** 8547–8555
14. Friedman, J., and Weissman, I. (1991) *Cell* **66,** 799–806
15. Kern, G., Kern, D., Schmid, F., and Fischer, G. (1995) *J. Biol. Chem.* **270,** 740–745
16. Fischer, G., Wittman-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. (1989) *Nature* **337,** 476–478
17. Steinmann, B., Bruckner, P., and Superti-Furga, A. (1991) *J. Biol Chem.* **266,** 1299–1303
18. Freskgard, P.-O., Bergenhem, N., Jonsson, B.-H., Svensson, M., and Carlsson, U. (1992) *Science* **258,** 466–468
19. Baker, E., Colley, N., and Zuker, C. (1994) *EMBO J.* **13,** 4886–4895
20. Xu, Q., Leiva, M., Fischkoff, S., Handschumacher, R., and Lyttle, C. (1992) *J. Biol. Chem.* **267,** 11968–11971
21. Sykes, K., Gething, M.-J., and Sambrook, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5853–5857
22. Marivet, J., Margis-Pinheiro, M., Frendo, P., and Burkard, G. (1994) *Plant Mol. Biol.* **26,** 1181–1189
23. Luban, J., Bossolt, K., Franke, E., Kalpana, G., and Goff, S. (1993) *Cell* **73,** 1067–1078
24. Thall, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C., Sodroski, J., and Gottlinger, H. (1994) *Nature* **372,** 363–365
25. Franke, E., Yuan, H., and Luban, J. (1994) *Nature* **372,** 359–362
26. Liu, J., Farmer, J., Lane, W., Friedman, J., Weissman, I, and Schreiber, S. (1991) *Cell* **66,** 1–9
27. Jain, J., McCaffrey, P., Miner, Z., Kerppola, T., Lambert, J., Verdine, G., Curran, T., and Rao, A. (1993) *Nature* **365,** 325–355
28. McCaffrey, P., Perrino, B., Soderling, T., and Rao, A. (1993) *J. Biol. Chem.* **268,** 3747–3752
29. Montague, J., Gaido, M., Frye, C., and Cidlowski, J. (1994) *J. Biol. Chem.* **269,** 18877–18880
30. Oberhammer, F., Wilson, J., Dive, C., Morris, I., Hickman, J., Wakeling, A., Walker, P., and Sikorska, M. (1993) *EMBO J.* **12,** 3679–3684
31. Walker, P., Weaver, V., Lach, B., LeBlanc, J., and Sikorska, M. (1994) *Exp. Cell Res.* **213,** 100–106
32. Ribeiro, J., and Carson, D. (1993) *Biochemistry* **32,** 9129–9136
33. Cohen, J., and Duke, R. (1984) *J. Immunol.* **132,** 38–42
34. Arends, M., Morris, R., and Wyllie, A. (1990) *Am. J. Pathol.* **136,** 593–608
35. Wyllie, A. (1980) *Nature* **284,** 555–556
36. Schneider, H., Charara, N., Schmitz, R., Wehrli, S., Mikol, V., Zurini, M., Quesniaux, V., and Movva, N. (1994) *Biochemistry* **33,** 8218–8224
37. Weigel, N., Schrader, W., and O'Malley, B. (1989) *Endocrinology* **125,** 2494–2501
38. Griffith, J., and Christiansen, G. (1978) *Annu. Rev. Biophys. Bioeng.* **7,** 19–35
39. Liu, J., Albers, M., Chen, C.-M., Schreiber, S., and Walsh, C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2304–2308
40. Schwartzman, R., and Cidlowski, J. (1993) *Endocrinology* **133,** 591–599
41. Krummrei, U., Bang, R., Schmidtchen, R., Brune, K., Band, H. (1995) *FEBS* **371,** 47–51
42. Birren, B., and Lai, E. (1993) *Pulsed Field Gel Electrophoresis*, Academic Press, Inc., San Diego, CA
43. Sun, X.-M., and Cohen, G. (1994) *J. Biol. Chem.* **269,** 14857–14860
44. Zhivotovsky, B., Cedervall, B., Jiang, S., Nicotera, P., and Orrenius, S. (1994) *Biochem. Biophys. Res. Commun.* **202,** 120–127
45. Duke, R., Chervenak, R., and Cohen, J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6361–6365
46. Hallick, R., Chelm, B., Gray, P., and Orozco, E. (1977) *Nucleic Acids Res.* **4,** 3055–3064
47. Beauvais, F., Michel, L., and Dubertret, L. (1995) *J. Leukocyte Biol.* **57,** 851–855
48. Bortner, C., and Cidlowski, J. (1996) *Am. J. Physiology*, in press
49. Takahashi, N., Hayano, T., and Suzuki, M. (1989) *Nature* **337,** 473–475
50. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. (1992) *J. Cell Biol.* **119,** 493–501
51. Bortner, C., Oldenburg, N., and Cidlowski, J. (1995) *Trends Cell Biol.* **5,** 21–26
52. Deng, G., and Podack, E. (1995) *FASEB J.* **9,** 665–669
53. Matsubara, K., Kubota, M., Kuwakado, K., Hirota, H., Wakazono, Y., Okuda, A., Bessho, R., Lin, Y.-W., Adachi, S., and Akiyama, Y. (1994) *Exp. Cell Res.* **213,** 412–417
54. Wyllie, A., Arends, M., Morris, R., Walker, S., and Evan, G. (1992) *Semin. Immunol.* **4,** 389–397
55. Johnson, E., and Ownby, C. (1994) *Comp. Biochem. Physiol. Toxicol. Endocrinol.* **107,** 359–366
56. Walsh, C., Zydowsky, L., and McKeon, F. (1992) *J. Biol. Chem.* **267,** 13115–13118
57. Serpesu, E., Shortle, D., and Mildvan, A. (1986) *Biochemistry* **26,** 1289–1300
58. Cardenas, M., Hemenway, C., Muir, R., Ye, R., Fiorentino, D., and Heitman, J. (1994) *EMBO J.* **13,** 5944–5957
59. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. (1989) *Molecular Biology of the Cell*, p. 388, Garland Publishing, New York
60. Gaido, M., and Cidlowski, J. (1991) *J. Biol. Chem.* **266,** 18580–18585
61. Ratajczak, T., Carrello, A., Mark, P. J., Warner, B. J., Simpson, R. J., Moritz, R. L., and House, A. K. (1993) *J. Biol. Chem.* **268,** 13187–13192
62. Chatellard-Gruaz, D., Saurat, J.-H., and Siegenthaler, G. (1994) *Biochem. J.* **303,** 863–867