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 nuclease 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 optima 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 \textit{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 nuclear 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 extensivly 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-
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 thymus isolated from adrenalectomized male Sprague Dawley rats. Control thymocytes were prepared through a Nitex mesh filter, and the cells were washed by centrifugation at 4 °C (5 min, 4,000 rpm). The cells were resuspended in fresh phosphate-buffered saline and passed through another Nitex mesh filter. After a second centrifugation, the cells were resuspended in 5 ml of phosphate-buffered saline and counted with a Coulter counter. 1 × 10^6 cells were removed and spun down in a microcentrifuge tube. Resuspension of the cells in 200 μl of 10 mM MgCl₂, 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 MgCl₂/Nonidet P-40 solution. The extracts were prepared by the addition of an equal volume of a lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and boiling 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, 2.35 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 (2890 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 Ncol or XhoI (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 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 μg CypA, 4.5 μg CypB, 4.4 μg CypC, and 50 μg pUC18 (30 μg plasmid DNA plus 10 μg supercoiled 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.

**Papinidylprolyl Isomerase Assay**—The cis-trans-papinidylprolyl isomerase assay was performed as described previously (39). 50 μM n-succinyl-Ala-Ala-Pro-Phe-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 n-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 probe 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). Briefly, HeLa cells were grown in suspension culture (1–7 × 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 sulphate. HeLa nuclei were prepared by pelleting the cells and resuspending in 10 mM MgCl₂ (25 °C). The nuclei were then pelleted and resuspended in 50 mM Tris (pH 7.4). Aliquots (2 × 10^5 nuclei) were then added to tubes containing 50 mM Tris (pH 7.4), 2 mM MgCl₂, 1 mM CaCl₂ (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-cm² mold, and placed at 4 °C for 5 min to solidify. Plugs were then incubated in 100 mM EDTA, 1% (w/v) N-laurylsarcosine (10 ml) at 37 °C overnight before being transfer 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 × 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 × TBE, which was allowed to equilibrate overnight. After 19 h at 6.0 V/cm with a linear switch interval ramp from 0.5 to 45 s. The parameters were chosen to optimally separate DNA fragments ranging from 10 to 500 kb (42). The gel was then stained with ethidium bromide.

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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 Nonnaturating 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 nucleolytic activity from the nonnaturated recombinant protein. In this assay, the purified Cyp is incubated with linearized plasmid (pUC18) in the presence of 1 mM Ca²⁺ and 1 mM Mg²⁺. 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. 1A, Con panel, +EDTA, +Ca²⁺/Mg²⁺ lanes, respectively) do not contain detectable levels of degraded DNA. CypA and CypB show greater DNA degradation in the presence of Ca²⁺ and Mg²⁺ than in the absence of these ions (Fig. 1A, CypA and CypB panels), whereas CypC can also degrade substantial levels of DNA in the absence of any additional ions (Fig. 1A, CypC panel, +EDTA lane). The nucleolytic activity of CypC is, however, enhanced by the addition of the divalent cations (Fig. 1A, CypC panel, +Ca²⁺/Mg²⁺ lane). Similarly, single-stranded DNA is best degraded by CypA and CypB in the presence of Ca²⁺ and Mg²⁺ and by Cyp C in either the absence or the presence of these ions (Fig. 1B). 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 Cyp A, -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 Ca²⁺ and/or Mg²⁺ 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 CypC; however, further experimentation with a series of solution nuclease assays performed at increasing Ca²⁺ and Mg²⁺ 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 Ca²⁺ and Mg²⁺. These results are shown in Table I. CypA and CypB had optimal nuclease activity with a combination of Ca²⁺ and Mg²⁺, whereas CypC was most active in the presence of Mg²⁺ 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. 1. Cyclophilins A, B and C degrade linear double-stranded DNA and linear single-stranded DNA in a nonnaturating 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 CaCl₂ and MgCl₂ 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.
Cyclophilins and Apoptosis

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  |

**TABLE I**

Calcium and magnesium ion activation of cyclophilin nuclease activity

**Fig. 3.** Cyclophilins demonstrate an endonucleolytic activity by degrading supercoiled plasmid. 1 ¼g 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\(^{2+}\)/Mg\(^{2+}\) 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.

**Fig. 4.** 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. 4A 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. 4B). 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...
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 Ca²⁺ and Mg²⁺, there was a dose-dependent decrease in the amount of DNA degraded, with IC₅₀ 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. 5A, 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. 5B). 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 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 [32P]dCTP. These assays revealed an increase in the number of 3'-OH termini in the digested versus the control samples (Fig.
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.

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 nuclease) activated during apoptosis.

Cyclophilins and Apoptosis

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'-PO4 termini, indicates an increase in the production of 3'-OH ends by the Cyps.
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 peptidyl-prolyl cis-trans-isomerase activity (15, 49), and have been shown to enhance the folding rate of several proteins (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 (45) 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) 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 active 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 isomerase, 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 Cyp 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 in 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 phosphodiester break per 50-kb fragment. 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

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4 C. Bortner and J. Cidlowski, unpublished observations.
remained 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 nuclelease activity to occur. Thus, attenuation of K\(^+\) levels in the nucleus may be all that is necessary to activate Cyp nuclelease 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 nuclelease activity is that Cyps may have altered post-translational modification during apoptosis such as they are now capable of degrading the chromatin DNA. Recently, Chattellard-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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