Ionic regulation of endonuclease activity in PC12 cells

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We have investigated the Ca^{2+} dependency of DNA degradation into nucleosome-sized fragments in intact chromaffin-like PC12 cells and PC12 nuclear fractions. In intact cells we were unable to trigger DNA fragmentation by inducing either transient or sustained elevations of cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_i$) with the Ca^{2+} ionophore ionomycin. On the contrary, DNA fragmentation was induced in intact cells by the intracellular Zn^{2+} chelator *NNN'*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN). To characterize further PC12 cell endonuclease activity, we then investigated digestion by purified PC12 cell fractions of exogenously added plasmids. In nuclear fractions two endonuclease activities were identified: an acidic (pH 5.0) endonuclease activity that was fully Ca^{2+} - and Mg^{2+} -independent; and a neutral (pH 7.6)

endonuclease activity that was Ca^{2+} -independent but Mg^{2+} -dependent. Both endonuclease activities were inhibited by Zn^{2+} . Nuclear membrane permeabilization greatly enhanced plasmid digestion at pH 7.6, but not at pH 5.0. This suggests that neutral endonuclease was located in a membrane-bound compartment, whereas acidic endonuclease was freely accessible to the substrate even in the presence of an intact nuclear membrane. In intact nuclei, digestion of genomic DNA could not be triggered by increasing the bivalent cation composition of the medium. On the contrary, in hypotonic medium we observed a large spontaneous nucleolytic DNA degradation that was increased by Zn^{2+} chelation. However, an acidic pH shift was a potent stimulus for DNA fragmentation in isotonic as well as hypotonic medium.

INTRODUCTION

Apoptosis is a highly regulated process of cell death that involves a chain of morphological and biochemical changes, among which is a peculiar degradation of genomic DNA into fragments that may vary in size from 30–50 kbp to small pieces of nucleosome size (180–200 bp) [1–5]. Enzymes responsible for apoptotic DNA cleavage have not yet been definitively identified, although some good candidates have been proposed. Topoisomerases are thought to trigger DNA cleavage into kbp fragments, while endonucleases are thought to be involved in the final degradation into nucleosomes [4–6]. Regulation of DNA-degrading enzymes by intracellular second messengers is poorly understood, although a pivotal role for known signal transduction pathways, such as changes in cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), cyclic AMP, pH or protein kinase C, have been suggested [7].

Not surprisingly, Ca^{2+} is one of the best candidates for the role of intracellular regulator of apoptosis. In many cells an increase in $[\text{Ca}^{2+}]_i$ induced by Ca^{2+} ionophores can stimulate apoptosis [8,9]. Recently, a few membrane receptors whose activation triggers Ca^{2+} fluxes as well as apoptosis have been characterized (e.g. the T-cell receptor in immature T cells [10] and the purinergic $\text{P}_{2\text{X}}/\text{P}_{2\text{Z}}$ receptors in a number of cell lines or primary cultures [11,12]). Furthermore, the Ca^{2+} -releasing agent thapsigargin has been shown to promote apoptosis [13], while cell-permeant Ca^{2+} -chelating agents delayed this process in some cell systems [14]. The oncoprotein Bcl-2 has also been proposed to prevent apoptosis by altering cytoplasmic Ca^{2+} compartmentalization [15]. However, the role of Ca^{2+} seems to be different in other cellular systems, where an increase in $[\text{Ca}^{2+}]_i$ appears to prevent rather than induce apoptosis and DNA degradation [16].

The final intracellular target for Ca^{2+} is the endonuclease that degrades DNA into nucleosomes. Three candidate nucleases with different ionic requirements have been proposed to participate in apoptosis: DNase I, DNase II and a novel 18 kDa

nuclease called nuc18 [17–19]. In isolated thymocyte nuclei, generation of the typical DNA ladder requires an increase in the intranuclear Ca^{2+} concentration and the presence of Mg^{2+} [20], while in a tumour cell line recently investigated a pH-dependent endonuclease appears to be active [18].

PC12 cells are a model widely used to study the mechanism of apoptotic cell death. This cell type can be induced to undergo apoptosis by growth factor deprivation through a process that appears to require endonuclease activation [21,22].

In this report we have investigated the process of DNA degradation in intact PC12 cells and purified PC12 cell fractions with the aim of characterizing ion regulation of endonuclease activity. Our results suggest that DNA degradation into nucleosome-sized fragments is Ca^{2+} -independent in PC12 cells. Furthermore, we have identified two different Zn^{2+} -inhibited nuclease activities in nuclear and microsomal fractions with different pH optima and Ca^{2+} , Mg^{2+} -dependency.

MATERIALS AND METHODS

Cell culture

PC12 cells were cultured in Bibby plastic flasks (Bibby Sterilin Ltd, Staffs., U.K.) in RPMI 1640 (PAA M.B.H., Linz, Austria) supplemented with 2 mM glutamine, 10% (v/v) heat-inactivated horse serum (Gibco BRL, Paisley, U.K.), 5% (v/v) heat-inactivated fetal-calf serum (Gibco BRL), penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) as described previously [23].

Isolation of nuclei

PC12 cells were gently detached, centrifuged (600 g) and suspended in PBS, then centrifuged once more and the pellet resuspended in a double volume of solution containing 250 mM sucrose, 5 mM Tris/HCl, 2.5 mM KCl, 5 mM MgCl_2 , 1.4 mM

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; TPEN, *NNN'*-tetrakis(2-pyridylmethyl)ethylenediamine; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid acetoxymethyl ester.

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PMSF, pH 7.5, (buffer A). Cells were lysed by repeated (at least 30) passages through a G22 $1\frac{1}{4}$ gauge needle. A double volume of a solution containing 2.3 M sucrose, 5 mM Tris/HCl, 2.5 mM KCl, 5 mM $MgCl_2$, 1.4 mM PMSF, pH 7.5, (buffer B) was added and mixed strongly. This broken cell suspension was layered on to a one-third vol. of buffer B and centrifuged at 60000 g for 1 h at 4 °C in a swing-out rotor. The pellet was resuspended in a solution containing 1 M sucrose, 50 mM Tris/HCl, 2.5 mM KCl, 5 mM $MgCl_2$, pH 7.5, (buffer C), and further centrifuged at 2500 g for 10 min at 4 °C. The pellet was resuspended in a solution containing 250 mM sucrose, 50 mM Tris/HCl, 2.5 mM KCl, 5 mM $MgCl_2$, pH 7.5, (buffer D), and centrifuged at 250 g for 10 min at 4 °C. This final pellet was resuspended at approx. 2 mg/ml in a solution containing 300 mM sucrose, 5 mM imidazole, pH 7.5, and stored at -70 °C until used.

Cellular fractionation

PC12 cells were gently detached, washed once with PBS, suspended in a buffer containing 300 mM sucrose, 5 mM imidazole, 0.5 mM EDTA, 1 mM PMSF (buffer E) and lysed by repeated passages (at least 20) through a G22 $1\frac{1}{4}$ gauge needle. Broken cell suspension was centrifuged at 1200 g for 10 min at 4 °C. The supernatant was collected and centrifuged at 10000 rev./min in an Eppendorf 5415 C bench microcentrifuge for 10 min at 4 °C (mitochondrial pellet). The supernatant was centrifuged at 360000 g at 4 °C for 20 min, the pellet (microsomes) resuspended (2 mg/ml) in a solution containing 300 mM sucrose, 5 mM imidazole, 0.5 mM EDTA, pH 7.5, and stored at -70 °C until used.

Nuclear extracts

Nuclei extraction was performed in a solution containing 400 mM NaCl, 20 mM Tris/HCl, 1 mM EDTA, pH 7.5, for 1 h on ice. In some experiments, nuclear extract was obtained in this medium in the presence of 1 or 0.5% Triton X-100. After extraction, samples were centrifuged at 110000 g for 1 h at 4 °C. Supernatants were stored at -70 °C until used.

Nuclei autolysis

Nuclei, at a concentration of 2.7 μg of protein/ μl , were suspended in a solution containing 125 mM KCl, 20 mM Hepes, 5 mM NaCl, 5 mM $NaHCO_3$, 1 mM $MgSO_4$, pH 7.6 or 5.0 (isotonic medium). Alternatively, they were incubated in a Tris-azide (TA) buffer containing 40 mM Tris/HCl and 0.02% azide (hypotonic medium) at pH 7.6 or 5.0. Incubations at pH 5.0 were also carried out in a Hepes (40 mM)-buffered solution. After an incubation of 2.5 h, the reaction was stopped by addition of 5 $\times$ ice-cold loading buffer [aqueous solution of 0.25% Bromophenol Blue/0.25% xylene cyanol/30% (w/v) glycerol].

DNA extraction

DNA from whole cells was extracted with the salting out technique as previously described [24], while DNA from isolated nuclei was extracted with phenol-chloroform [25]. DNA was run on a 1% agarose gel (ICN Biomedicals GmbH, Meckenheim, Germany).

Plasmid preparation

The plasmid YNH24, a 4.69 kb pUC18 derivative, contains a 2 kb insert of a marker sequence of the chromosome 2p (D2S44) [26]. A sample (200 ng) of plasmid DNA, which was prepared

according to standard procedures [27], was ^{32}P -labelled by using a random primer labelling kit (Boehringer, Mannheim, Germany) in the presence of [α - ^{32}P]dCTP (3000 Ci/mmol).

Determination of ^{32}P -labelled plasmid digestion

Cellular fractions were incubated with labelled plasmid at 37 °C for different times in a reaction medium containing 40 mM Tris, 0.5 mM Ca^{2+} , 1 mM Mg^{2+} and 0.02% azide. Samples were run in a 1% agarose gel, the area corresponding to the intact plasmid cut out and counted in a liquid scintillation counter. Basal serum endonuclease activity was quantitatively assayed by checking digestion of labelled plasmid in the presence of complete (10% heat-inactivated horse and 5% heat-inactivated fetal-calf serum) RPMI medium. The assay was run at 37 °C for 10 min at pH 5.0 (in either 40 mM Tris/HCl or 40 mM Hepes buffer) and 30 min at pH 7.6 (in 40 mM Tris/HCl buffer). Under these conditions, approx. 15% of the plasmid was digested at pH 7.6, while no digestion was detected at pH 5.0.

Intracellular Ca^{2+} measurement

Changes in $[Ca^{2+}]_i$ were measured with the intracellular fluorescent indicator fura-2/AM as described previously [11].

RESULTS

Several lines of evidence suggest that an increase in $[Ca^{2+}]_i$ is a crucial trigger of apoptosis. PC12 cell monolayers were stimulated with the Ca^{2+} ionophore ionomycin for different lengths of time in Ca^{2+} -containing RPMI medium and morphological and bio-

Figure 1 Effect of ionomycin and TPEN on PC12 cell morphology

Cells (10^6 /ml) were incubated in RPMI medium alone (panels a and b), in the presence of 5 μM ionomycin (panels c and d) or 20 μM TPEN for 24 h (panels e and f). At the end of the incubation, monolayers were either stained with propidium iodide (10 μM) and photographed under fluorescent light, or directly photographed under phase contrast with a fluorescence Olympus microscope equipped with a 40 $\times$ objective. Bar = 25 μm .

Figure 2 Effect of Ionomycin and TPEN on DNA fragmentation

PC12 cells ($5 \times 10^6/\text{ml}$) were incubated in complete (10% horse and 5% calf serum) RPMI medium for 24 h in the presence of increasing concentrations of ionomycin (a) or TPEN (b). At the end of this incubation time, an aliquot was withdrawn for measurement of Eosin Red permeability (percentage values are reported on the lower abscissa) and DNA was isolated from the remaining sample as described in the Materials and methods section.

chemical signs of apoptosis were investigated. As shown in Figure 1 (panel c), the Ca^{2+} ionophore caused clearcut morphological alterations after 24 h of incubation that were paralleled by a decrease in cell viability. However, after propidium iodide staining, no morphological nuclear changes suggestive of the occurrence of apoptosis were detected (Figure 1, panel d). Control cells are shown in panels (a) and (b). We recently reported that chelation of intracellular Zn^{2+} by the membrane-permeant heavy-metal chelator $\text{NNN}'\text{N}'$ -tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) was a powerful apoptotic stimulus in human peripheral lymphocytes [28]. Figure 1 (panels e and f), reports that TPEN is also an apoptotic stimulus for PC12 cells, in which it causes cytoplasmic fragmentation and nuclear condensation (arrows). Analysis of nuclear DNA confirmed the morphological observations (Figure 2). Ionomycin, tested over a range of concentrations (Figure 2a, lanes 3–7), did not trigger DNA

Figure 3 Ca^{2+} chelation triggers DNA fragmentation

PC12 cells were incubated as described in Figure 2 either in Ca^{2+} (0.42 mM) supplemented (lanes 2, 3 and 4) or in Ca^{2+} -free RPMI medium (lanes 5, 6 and 7). Bivalent cations were removed by treating this medium with a Chelex 100 (200–400 mesh) resin (Bio-Rad, Richmond, CA, U.S.A.). In some experiments, cells were also loaded with BAPTA/AM (lanes 3, 4, 5 and 7).

cleavage into nucleosome-sized fragments, although it clearly had a cytotoxic effect, as indicated by increased cell membrane permeability to the vital stain Eosin Red; on the contrary, TPEN was a very active fragmenting agent (Figure 2b) at optimal concentrations of 10 and 20 μM (lanes 6 and 7).

A few laboratories have recently reported the occurrence of DNA fragmentation in the absence of $[\text{Ca}^{2+}]_i$ increases or at $[\text{Ca}^{2+}]_i$ significantly below physiological resting levels. Thus, we tested the effect of chelation of extracellular and intracellular Ca^{2+} .

Figure 3 shows that mere incubation in the absence of external Ca^{2+} sufficed to trigger DNA fragmentation (lane 5). A 24 h incubation in the absence of extracellular Ca^{2+} brought about a decrease of $[\text{Ca}^{2+}]_i$, as measured by fura-2, to about 20 nM (results not shown). We checked whether a more dramatic $[\text{Ca}^{2+}]_i$ decrease obtained with the membrane-permeant chelator bis-(*o*-aminophenoxy)ethane- $\text{NNN}'\text{N}'$ -tetra-acetic acid acetoxymethyl ester (BAPTA/AM) would enhance DNA degradation. As shown by Figure 3 (lanes 6 and 7), incubation in the presence of BAPTA did not significantly potentiate DNA degradation. Rather interestingly, BAPTA did not cause DNA degradation in the presence of external Ca^{2+} (Figure 3, lanes 3 and 4).

These rather unexpected results prompted a more detailed investigation of endonuclease activity in PC12 cells. In order to be able to rigorously investigate cation dependency of putative nuclear endonucleases, we measured digestion of exogenous plasmids by nuclear extracts. Figure 4(a) shows agarose gel electrophoresis of plasmid incubated together with PC12 nuclear extracts in the presence of different cation concentrations. In the complete absence of bivalent cations (no added Ca^{2+} and Mg^{2+} , 2 mM EDTA) there was no plasmid digestion (Figure 4a, lane 2), although shift of the band to a higher molecular mass suggests that some nicking, and subsequent relaxation of supercoiling, occurred. Omission of EDTA or an increase of Ca^{2+} up to 2 mM did not cause plasmid degradation (Figure 4a, lanes 3–5). On the contrary, increasing the Mg^{2+} concentration from 0.05 to 1 mM caused complete plasmid degradation (Figure 4a, lanes 7–10).

Figure 4 Digestion of exogenous plasmid by isolated nuclear extract

PC12 cell nuclear extracts were incubated for 2.5 h at 37 °C in the presence of 1 µg of exogenous plasmid (pl) at pH 7.6 (a) or 5.0 (b) and various bivalent cation concentrations as described in the Materials and methods section. At the end of this incubation, samples were subjected to agarose (1%) gel electrophoresis.

Mg²⁺ alone was sufficient to cause endonuclease activation as the plasmid was also completely degraded in the presence of 2 mM EGTA (lane 15). Changes in the Ca²⁺ concentration in the presence of 1 mM Mg²⁺ did not affect plasmid digestion (Figure 4a, lanes 11–14). Plasmid digestion was fully inhibited by 1 mM Zn²⁺ (result not shown). It has been recently suggested that a drop in intracellular pH might be a key activator of endonuclease activity. Thus we investigated plasmid digestion at pH 5.0 (Figure 4b). At this pH we observed near complete plasmid degradation irrespective of the presence of bivalent cations, in fact increasing the Ca²⁺ concentration inhibited, if anything, plasmid digestion (Figure 4b, lanes 3–7). As expected, endonuclease activity was fully blocked by Zn²⁺ at an optimal concentration of 1 mM (Figure 4b, lanes 8–11).

In Figure 5 we quantitatively investigated the localization of endonuclease activity in different nuclear fractions by using ³²P-labelled plasmids as substrate. A comparison of Figures 5(a) and 5(b) shows that plasmid digestion occurred to a larger extent at pH 5.0 than at pH 7.6, especially when intact nuclei (open squares) were used. Furthermore, the two enzyme activities appeared to be differently localized in the nucleus. Plasmid digestion at pH 7.6, but not at pH 5.0, was greatly enhanced by solubilization of the nuclear envelope, suggesting localization

Figure 5 Effect of detergent treatment on plasmid digestion by isolated nuclei

PC12 cell nuclei were isolated as described in the Materials and methods section and incubated in the presence of ³²P-labelled plasmid as described in Figure 4 at pH 5.0 (a) or 7.6 (b). Symbols represent nuclear extract (◇), intact nuclei (□), nuclei solubilized with 0.5% (▲) or 1% (◆) Triton X-100, samples were treated with 1% Triton X-100 only during plasmid digestion.

Figure 6 Plasmid digestion by nuclear and microsomal nucleases at pH 5.0 and 7.6

PC12 nuclei and microsomes, isolated as described in the Materials and methods section, were incubated together with ³²P-labelled plasmid as described in Figure 4. Nuclear fraction incubated at pH 5.0 (□) or 7.6 (■); microsomal fraction incubated at pH 5.0 (△) or 7.6 (▲).

within a membrane-bound compartment for the neutral but not for the acidic enzyme.

Endonuclease activity was also found in the microsomal

Figure 7 Effect of changes in ionic composition of the medium on endogenous DNA digestion in isolated nuclei

PC12 nuclei were isolated as described in the Materials and methods section. Incubation was performed either in isotonic (a), or hypotonic (b) medium (for ion composition of isotonic and hypotonic media see the Materials and methods section). (a) Lane 2, isotonic medium; lane 3, 2 mM Ca^{2+} and Mg^{2+} ; lane 4, 20 μM TPEN; lane 5, pH 5.0. (b) Lane 2, hypotonic medium; lane 3, 2 mM EDTA; lane 4, 2 mM Ca^{2+} and Mg^{2+} ; lane 5, 20 μM TPEN; lane 6, 2 mM Ca^{2+} and Mg^{2+} , pH 5.0; lane 7, 2 mM Ca^{2+} and Mg^{2+} , 1 mM Zn^{2+} , pH 5.0. Autodigestion of genomic DNA was carried out for 2.5 h at 37 °C.

fraction, although to a slightly lower level than in the nuclei (Figure 6).

Digestion of exogenously added plasmids allows rigorous quantitative measurement of endonuclease activity, but can not be assumed to be representative of digestion of endogenous genomic DNA. Therefore we investigated ionic regulation of endogenous DNA degradation in isolated intact nuclei. In nominally Ca^{2+} -free medium, there was no DNA degradation (Figure 7a, lane 2). Likewise, and in contrast to previous reports obtained in thymocyte nuclei [20], we were unable to trigger

DNA digestion by incubating the nuclei in the presence of Ca^{2+} (Figure 7a, lane 3). Also, DNA was not fragmented by other treatments that were efficacious in intact cells, such as chelation of extracellular Ca^{2+} with EGTA (results not shown) or addition of TPEN (Figure 7a, lane 4). The sole experimental condition permissive for DNA digestion under isotonic conditions was incubation at pH 5.0 (lane 5). On the contrary, in hypotonic conditions (Figure 7b) we observed a spontaneous DNA fragmentation (lane 2) that was inhibited by $\text{Ca}^{2+}/\text{Mg}^{2+}$ (compare lanes 3 and 4) and enhanced by TPEN (lane 5). At pH 5.0, $\text{Ca}^{2+}/\text{Mg}^{2+}$ inhibition was overcome, but Zn^{2+} was still partially inhibitory (Figure 7b, lanes 6 and 7).

DISCUSSION

The most striking biochemical change observed during apoptosis is digestion of genomic DNA [1–3]. It is believed that this process initially generates kbp fragments that are subsequently further degraded into nucleosome-sized (about 200 bp) fragments easily detected as a DNA ladder in a standard agarose gel electrophoresis [1–6]. In spite of intense investigation very little is known about the regulation of enzymes involved in this DNA degradative process. Several recent reports suggest that DNA cleavage into large fragments is probably due to topoisomerase II, an enzyme located at a 300 kbp interval along chromatin, a location that would favour DNA attack during the early stages of apoptosis [4–6,29]. Once these large fragments are generated and detached from the nuclear scaffold, endonucleolytic digestion also occurs and small fragments appear.

It is not known how the activating signal for topoisomerase and/or endonuclease activation is transmitted into the nucleus, but intracellular ion changes have been long considered a likely mechanism of stimulation. Endonuclease(s) responsible for endonucleolytic cleavage were initially shown to be Ca^{2+} - and Mg^{2+} -dependent in isolated thymocyte nuclei [20]. Furthermore, in several cell models increases in $[\text{Ca}^{2+}]_i$ triggered by Ca^{2+} ionophores can trigger morphological changes typical of apoptosis and generation of nucleosome-sized fragments [8,9].

However, more and more evidence for Ca^{2+} -independent apoptosis is accumulating. For example, Ca^{2+} ionophore stimulation can block apoptosis dependent on interleukin-3 withdrawal and apoptosis in aged neutrophils [30,31]. A very interesting situation is that of neurons, that can be protected from apoptosis by a moderate $[\text{Ca}^{2+}]_i$ increase dependent on plasma membrane depolarization [32]. Furthermore, in other cell types it has been shown that DNA cleavage can be triggered by a reduction in $[\text{Ca}^{2+}]_i$ rather than an increase [33].

Our results raise a number of intriguing questions. First, the Ca^{2+} ionophore ionomycin was unable to cause DNA degradation under any condition tested, whether it triggered a transient or a long-lasting $[\text{Ca}^{2+}]_i$ increase. In fact, DNA degradation was reduced by removal of external Ca^{2+} , independently of buffering of $[\text{Ca}^{2+}]_i$ with the membrane-permeant chelator BAPTA/AM. Secondly, in nuclear and microsomal fractions we measured two endonuclease activities that were both Ca^{2+} -independent, although Zn^{2+} -inhibited. Thirdly, in contrast to previous results obtained in lymphocyte nuclei, we did not succeed in inducing degradation of endogenous genomic DNA in isolated intact PC12 nuclei by increasing the bivalent ion composition of the incubation medium.

We found very little endonuclease activity (whether acidic or neutral) in soluble nuclear extracts, while the great majority was located in the nuclear membrane fraction. This implies that endonucleases are not soluble, but rather membrane-bound, in agreement with the recent suggestion by Peitsch and colleagues

[17] that DNase I is located in the endoplasmic reticulum lumen. An intraluminal endonuclease location raises the issue of how this enzyme gains access to genomic DNA during apoptosis. In this respect it has been suggested that nuclear lamin breakdown is an early event in cell death [34], thus facilitating access of endonuclease to DNA, but this question clearly needs further, more detailed investigation.

One endonuclease described by us is active at acidic pH (5.0), is fully $\text{Ca}^{2+}/\text{Mg}^{2+}$ -independent and Zn^{2+} -inhibited. Thus, it resembles the acidic endonuclease recently described in Chinese hamster ovary nuclear extracts by Barry and Eastman [18]. However, these authors did not detect any further endonuclease activity above pH 6.50. In contrast, we identified a second endonuclease that was active at pH 7.6. This second enzyme was Ca^{2+} -independent, but Mg^{2+} -dependent and Zn^{2+} -inhibited. At the present stage we do not know whether the two activities refer to the same or two separate enzyme molecules; however, we think that the latter possibility is the most likely as the two endonuclease activities, besides being differently regulated by Mg^{2+} , also appeared to be differently affected by detergent treatment. Acidic nuclease activity was not increased in Triton X-100-treated nuclear fractions, while neutral nuclease activity was almost doubled. This might suggest that the acidic enzyme is located on the surface of the nuclear membrane, while the neutral one is located in a membrane-bound compartment.

Quite intriguingly, we were unable to trigger DNA fragmentation in isolated intact nuclei by changing the ion composition of the medium. Neither a Ca^{2+} increase or decrease (whether in the presence or absence of a Ca^{2+} ionophore), nor changes in Mg^{2+} concentration induced nucleolytic cleavage. On the contrary, an acidic pH shift caused a clearcut DNA degradation. In isolated nuclei TPEN was also a powerful stimulus for DNA degradation, but only when nuclei were incubated in hypotonic medium. This is an intriguing observation as this high-affinity Zn^{2+} chelator is thought to directly activate endonucleases by removing Zn^{2+} inhibition. The fact that in isolated nuclei, in contrast to intact cells, TPEN is active only when the DNA structure has been relaxed by hypotonicity, is evidence in support of the hypothesis that endonuclease activation by itself is not a sufficient stimulus for DNA cleavage. This observation suggests that under normal conditions the DNA filament is not accessible to endonuclease attack, unless its structure is relaxed.

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