Purification and properties of DNase γ from apoptotic rat thymocytes

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We previously identified three distinct DNA endonucleases, DNases α, β and γ, present in rat thymocyte nuclei. On the basis of their enzymic and biochemical properties, γ-type DNase was regarded as a candidate for the apoptotic endonuclease. Here we purified DNase γ to apparent homogeneity from apoptotic rat thymocyte nuclei induced by X-irradiation and characterized its properties in detail. The purified DNase γ exhibited one predominant protein band on SDS/PAGE and an endonuclease activity in a zymography with an estimated molecular mass of 33 kDa. The molecular mass of the native form determined by G2000SW gel-filtration HPLC was 30 kDa. Amino acid analysis showed that the amino acid composition of DNase γ was similar to that of rat DNase I (molecular mass 32 kDa) but different with regard to alanine and lysine residues. The N-terminal amino acid sequence of DNase γ was revealed to be not identical with that of rat DNase I. In accordance with previous studies, homogeneously purified DNase γ requires both Ca^{2+} and Mg^{2+} for activity. This requirement could be partially supplied by Mn^{2+}. Of the bivalent metal ions tested, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} inhibited DNase γ activity. These bivalent cations also suppressed apoptotic DNA fragmentation in rat thymocytes irradiated by X-rays. The same order of inhibitory ability was observed for these bivalent metal ions *in vivo* (in intact cells) and *in vitro*, suggesting that the suppression of apoptotic DNA fragmentation at the cellular level is due to the inhibition of DNase γ. DNase γ activity was found to exist at high levels in spleen, lymph node, thymus, liver and kidney, but little was present in brain, heart or pancreas. On the basis of these findings, together with previous data, we conclude that DNase γ is a novel DNase I-like endonuclease responsible for internucleosomal cleavage of chromatin during thymic apoptosis.

INTRODUCTION

Two distinct patterns of cell death have been recognized, necrosis and apoptosis [1–3]. Necrosis is passive cell death that is thought to occur only in pathological situations. On the other hand, apoptosis is an active suicidal process in which cells die in a genetically controlled manner, and is important in a variety of physiological circumstances in multicellular organisms [1–8]. Cell death by apoptosis is characterized by two outstanding features, specific morphological changes and nuclear DNA fragmentation [1–3,7,8]. The internucleosomal cleavage of chromosomal DNA, which is suggested to be catalysed by a constitutive DNA endonuclease, is the biochemical feature most commonly associated with apoptotic processes leading to cell death [9–13]. Identification of the DNA endonuclease responsible is therefore important to the understanding of the mechanisms by which apoptosis occurs.

At present, Ca^{2+}/Mg^{2+}- or Ca^{2+}/Mn^{2+}-dependent endonucleases have been suggested as candidates for the apoptotic endonuclease [14–19]. An acidic endonuclease, DNase II, has also been proposed to be involved [20,21]. We previously reported that the nuclei of rat thymocytes contain at least three types of endonuclease, tentatively named DNases α, β and γ [14]. The partially purified DNases α and β are bivalent-cation-independent acidic endonucleases that cleave chromatin DNA with 3′-phosphoryl (P)/5′-hydroxyl (OH) ends [15]. On the other hand, DNase γ is a Ca^{2+}/Mg^{2+}-dependent endonuclease that produces DNA fragments with 3′-OH/5′-P ends [15]. Several lines of evidence indicate the involvement of DNase γ in nucleosomal DNA fragmentation during apoptosis [14,15]. First, a high activity of DNase γ is still present in apoptotic thymocytes. Secondly, DNase γ is strongly inhibited by Zn^{2+}, which is known to suppress apoptotic DNA fragmentation in intact cells. Thirdly, after incubation of X-irradiated thymocytes in the presence of cycloheximide, nucleosomal DNA fragmentation scarcely occurs, in spite of the existence of DNase γ (DNases α and β disappear from the cycloheximide-treated cell nuclei). When cycloheximide is removed and the thymocytes are incubated further, apoptotic DNA fragmentation occurs, even though the thymocytes contain only DNase γ. Fourthly, endolabelling analysis shows that DNA fragments digested by DNases α, β and γ have 3′-P/5′-OH, 3′-P/5′-OH and 3′-OH/5′-P end groups respectively. Of these, only the DNA ends produced by DNase γ are the same as those of nucleosomal fragments formed at the cellular level during thymic apoptosis.

Nothing definite is, however, known about the properties of purified γ-type DNase present in apoptotic cells or the genomic structure of its gene. In order to solve these problems, we attempted to purify DNase γ extensively from apoptotic rat thymocytes. Here we report the purification to apparent homogeneity of DNase γ and the characterization of its physical and catalytic properties. This paper also provides a reproducible and efficient method for the purification of DNase γ present in apoptotic cell nuclei. The properties of the purified DNase γ are compared with other putative apoptotic endonucleases so far purified.

EXPERIMENTAL

Animals and materials

Adult male albino Wistar-strain rats were obtained from the National Institute of Radiological Sciences, Chiba, Japan.

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RPMI 1640 and fetal calf serum were from Nissui and Flow Laboratories respectively. S-Sepharose was from Pharmacia. DEAE5PW, CM5PW, heparin5PW and G2000SW were from Tosoh. RNase A, proteinase K, dexamethasone, bovine DNase I, bovine DNase II, G-actin and calf thymus DNA were from Sigma. All other reagents were of the highest purity available.

Preparation of thymocytes

Rat thymus glands were removed from anaesthetized animals, minced and washed in Krebs–Ringer phosphate buffer to obtain thymocytes. The cell suspension was filtered through 200 μm nylon mesh and centrifuged at 200 ***g*** for 10 min. The precipitated cells were resuspended in fresh Krebs–Ringer phosphate buffer.

Induction of apoptosis

Thymocytes were seeded at 2×10^8 cells/ml in Krebs–Ringer phosphate buffer containing 10 mM glucose. Apoptosis was induced by incubating the cells at 37 °C for 4 h after X-irradiation. Irradiation was carried out at 0–4 °C with 200 kVp X-rays of ^{60}Co operating at 20 mA and filtered through 0.5 mm Cu plus 0.5 mm Al at a dose of 10 Gy as described previously [22]. Cell morphology was analysed by transmission electron microscopy as described previously [8].

Analysis of DNA fragmentation

DNA fragmentation was analysed as described previously [14,15]. Cells (1×10^6) were lysed in buffer L [50 mM Tris/HCl, pH 7.8, 10 mM EDTA and 0.5 % (w/v) sodium *N*-lauroylsarcosinate]. The lysates were incubated sequentially with 0.5 mg/ml RNase A at 50 °C for 60 min and 0.5 mg/ml proteinase K at 50 °C for 60 min. The resulting DNA preparations were analysed by electrophoresis in 2 % agarose gels containing 0.5 μg/ml ethidium bromide. The DNA-fragmentation pattern was examined in photographs taken under UV illumination. Percentage fragmentation was determined by densitometry as described previously [14,15].

Extraction of DNases

All operations were performed at 0–4 °C unless otherwise indicated. Apoptotic thymocyte nuclei were isolated by homogenizing the X-irradiated thymocytes for 10 strokes in a Teflon/glass homogenizer in buffer A (10 mM Tris/HCl, pH 7.8, 3 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.3 mM PMSF) containing 0.1 % Nonidet P40. When other organs were used, they were rinsed with 10 vol. of buffer A containing 0.1 % Nonidet P40 and cut into small pieces, then homogenized first in a Waring blender followed by 10 strokes in a Teflon/glass homogenizer. The homogenate was filtered through 200 μm stainless-steel mesh and the nuclei were collected by centrifugation at 400 ***g*** for 5 min. Isolated nuclei were then resuspended in 8 vol. of buffer A containing 0.5 M $(NH_4)_2SO_4$, and sonicated ($\times 3$) with a Branson sonifier at an output control setting of 1 for 10 s. The mixtures were next stirred on ice for 30 min, and the nuclear debris was removed by centrifugation at 150000 ***g*** for 60 min. The supernatant was taken as the nuclear extract.

Assay of endonuclease

DNase γ activity was measured as described previously [14,15]. Briefly, a 1 μl aliquot of enzyme fraction was added to 30 μl of reaction mixture containing 50 mM Mops/NaOH, pH 7.2, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM PMSF and 5×10^5 HeLa S3 cell nuclei (substrate), and the incubations were carried out at 37 °C for 60 min unless otherwise noted. In control assays, 1 μl of buffer used for enzyme preparation was added in place of the enzyme fraction. Reactions were terminated by chilling on ice. The resulting reaction mixture was centrifuged at 20000 ***g*** for 10 s and the precipitated nuclei were lysed in buffer L. The DNA was prepared by successive treatments with 0.5 mg/ml RNase A at 50 °C for 20 min and 0.5 mg/ml proteinase K at 50 °C for 30 min and then loaded on to a 2 % agarose gel containing 0.5 μg/ml ethidium bromide. DNase activity was detected as DNA ladders in photographs taken under UV illumination, and was quantified as percentage fragmentation by densitometry as described previously [14,15].

No spontaneous ladder formation was observed by incubation of HeLa S3 nuclei alone under these conditions (see Figures 2, 4 and 5).

DNase activity gel system

For the identification of DNase activity and the estimation of its molecular mass, the activity gel system (zymography) for DNase [23] was used with several modifications as described previously [14,15]. The purified DNase was electrophoresed in standard SDS/polyacrylamide gels containing 200 μg/ml native calf thymus DNA as described by Laemmli [24]. After electrophoresis, the gels were washed with 10 mM Tris/HCl, pH 7.8, containing 5 mM 2-mercaptoethanol at 50 °C for 1 h to remove SDS, and then with 10 mM Tris/HCl, pH 7.8, at 4 °C overnight to allow renaturation of proteins. The gels were then incubated in 10 mM Tris/HCl, pH 7.8, or 10 mM Mes/NaOH, pH 5.6, containing the indicated concentrations of $CaCl_2$, $MgCl_2$ and/or $ZnCl_2$ at 37 °C overnight and in 10 mM Tris/HCl, pH 7.8, containing 0.5 μg/ml ethidium bromide for an additional 30 min. Apparent nuclease activities were detected as dark areas on a fluorescent background after trans-illumination of the gels with UV light. The calibration curve constructed using molecular-mass standards was not affected by the presence of 200 μg/ml double-stranded DNA in the SDS/polyacrylamide gels.

Analysis of amino acid composition

The amino acid composition of the purified enzyme was determined as described previously [25]. Samples were hydrolysed with 6 M HCl at 110 °C for 24 and 48 h.

Determination of amino acid sequence

The enzyme fraction from the second heparin5PW HPLC (see below) was subjected to SDS/PAGE (10 % gel). After electrophoresis, proteins in the gel were electrotransferred to PVDF membranes (Immobilon P®) in transfer buffer (10 mM *N*-cyclohexyl-3-aminopropanesulphonic acid/NaOH, pH 11.0, and 10 % methanol) at 200 mA for 60 min. The DNase γ (33 kDa) band was identified by Ponceau S staining (0.1 % Ponceau S and 1 % acetic acid) and cut out. The membrane strip containing approx. 10 pmol of DNase γ was subjected to amino acid sequencing (ABI model 470A).

Organ specificity of DNase γ

Nuclear extracts prepared as described above and aliquots from the extracts containing 0.5 mg of protein were diluted with 4 vol. of buffer N (20 mM Tris/HCl, pH 7.8, 1 mM 2-mercaptoethanol, 0.1 mM PMSF and 5 % ethylene glycol) and filtered through a 0.45 μm pore size filter. This fraction was applied to a CM5PW column equilibrated with buffer N. Proteins were eluted with a linear gradient of 0–1 M KCl in buffer N at a flow rate of 0.3

ml/min and fractions were collected every 30 s. DNase γ was eluted at 0.55 M KCl [14,15], and 1 μl aliquots of the fraction (150 μl; approx. 10 μg/ml protein) were applied to the DNA-fragmentation assay. Enzyme reaction was performed at 37 °C for 20 min. Protein quantification was carried out using a protein assay kit (Bio-Rad) according to the manufacturer's instructions.

RESULTS

Purification of DNase γ from apoptotic rat thymocytes

Electron-microscopic analysis showed that incubation of rat thymocytes after X-irradiation resulted in typical apoptotic morphological changes such as disappearance of cell-surface microvilli, and nuclear condensation and fragmentation. After 4 h incubation, apoptotic cells constituted 65 % of the total population. These morphological changes were accompanied by DNA fragmentation as judged by nucleosomal ladders on agarose-gel electrophoresis of genomic DNA. At this period, about 70 % of DNA was fragmented to nucleosomal oligomers as determined by densitometric analysis. The apoptotic thymocytes thus prepared were used as the source of DNase γ purification.

All operations were performed at 0–4 °C unless otherwise indicated. For the purification of DNase γ, nuclei were first isolated from 2×10^{10} apoptotic rat thymocytes. Nuclear extract was prepared as described in the Experimental section. The solubilized nuclear enzyme fraction was used in the subsequent chromatographies.

Step 1: S-Sepharose column chromatography

The nuclear extract (38 ml; 11.4 mg of protein) was mixed with 20 ml of S-Sepharose pre-equilibrated with buffer N′ [20 mM Tris/HCl, pH 7.8, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, and 10 % ethylene glycol) and the mixture was diluted with 4 vol. of buffer N′. The S-Sepharose suspension was stirred continuously for 30 min and then allowed to settle. The supernatant was discarded by decantation and the S-Sepharose was washed once by resuspension and settling in buffer N′. The washed S-Sepharose was then packed into a column. The column was washed with 2 bed vol. of the same buffer and developed with a linear gradient of 0–1 M KCl in buffer N′ at a flow rate of 2 ml/h. DNase activity was eluted in a broad peak at around 0.6 M KCl. The active fractions were pooled (14 ml; 1.86 mg of protein) and diluted with 2 vol. of buffer N and subjected to the next column chromatography after filtration through a 0.45 μm pore size filter.

Step 2: DEAE5PW/CM5PW HPLC

The above enzyme fractions were subjected to HPLC on CM5PW linked in series after a DEAE5PW column equilibrated with buffer N. DNase activities passed through DEAE5PW and were adsorbed on to CM5PW. The proteins retained in the CM5PW column were eluted with a linear gradient of 0–1 M KCl in buffer N at a flow rate of 0.3 ml/min. DNase γ activity was eluted at 0.55 M KCl.

Step 3: First heparin5PW HPLC

The enzyme fractions obtained above were pooled (1 ml; 560 μg of protein) and diluted with an equal volume of buffer N, and then subjected to heparin5PW HPLC in a column equilibrated with buffer N. Proteins were eluted with a linear gradient of 0–1 M KCl in buffer N at a flow rate of 0.3 ml/min.

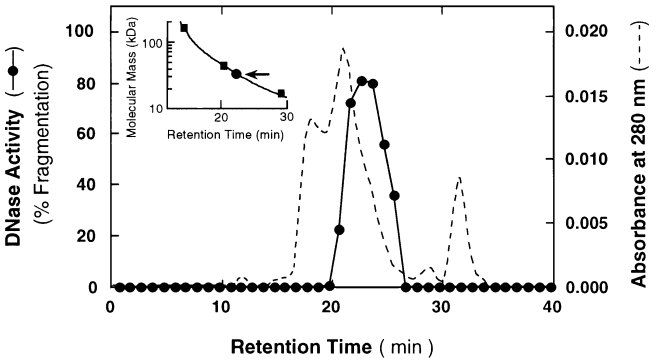

Figure 1 G2000SW gel-filtration HPLC of DNase γ

DNase γ separated by the first heparin5PW HPLC was subjected to G2000SW HPLC as described in the Experimental section. The inset indicates the calibration curve of molecular-mass standards, IgG (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa). The peak position of DNase γ activity is indicated by an arrow.

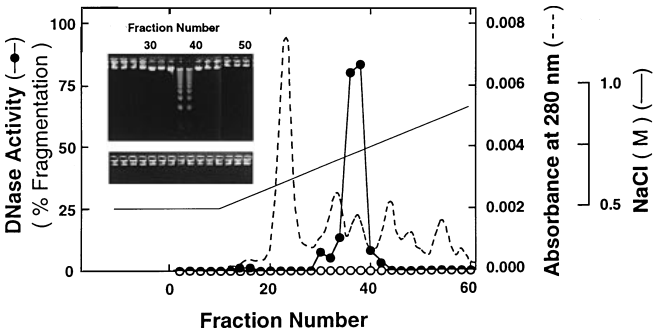

Figure 2 Elution profile of the second heparin5PW HPLC of DNase γ

The measurement of DNase γ activity with (●) or without (○) fractions separated by the second heparin5PW HPLC were performed as described in the Experimental section. Results of DNA fragmentation assayed with or without fractions are illustrated in the upper and lower panels respectively of the inset.

Step 4: G2000SW gel-filtration HPLC

The enzyme fractions (1.2 ml; 72 μg of protein) were concentrated to 800 μl with Ultrafree C3-LCC (Millipore) by centrifugation at 5000 ***g*** and then subjected to gel-filtration HPLC on G2000SW equilibrated with 0.5 M NaCl in buffer M (20 mM Mes/NaOH, pH 5.6, 1 mM 2-mercaptoethanol, 0.1 mM PMSF and 5 % ethylene glycol). Elution was carried out with the same buffer at a flow rate of 0.5 ml/min (Figure 1). The molecular-mass standards used for calibration were IgG (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa) (Figure 1, inset).

Step 5: Second heparin5PW HPLC

The enzyme fractions were pooled (3.0 ml; 21 μg of protein) and subjected directly to heparin5PW HPLC in a column equilibrated with buffer M containing 0.5 M NaCl, and the proteins were eluted with a linear gradient of 0.5–1.0 M NaCl in buffer M at a flow rate of 0.3 ml/min. DNase γ activity was eluted in a single peak at 0.7 M NaCl (Figure 2). Results of the DNA fragmentation assay are shown in the insets.

The active fractions were pooled (1.2 ml; approx. 300 ng of protein) and desalted by dialysis against buffer M and stored at

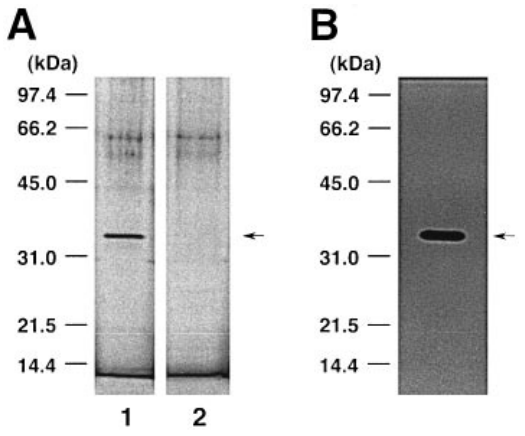

Figure 3 SDS/PAGE and activity gel analysis of DNase γ

The purified DNase γ was subjected to SDS/PAGE (**A**) and activity gel system (**B**) as described in the Experimental section. The bands representing the DNase γ protein (**A**) and activity (**B**) are marked by arrows. SDS/PAGE was performed with (lane 1) or without (lane 2) enzyme preparation. The faint bands of about 60 kDa are ghost backgrounds.

Table 1 Amino acid compositions of rat DNase I and DNase γ

Values are averages after 24 h and 48 h hydrolysis. Basic means the sum of Lys, His and Arg, and acidic means the sum of Asx and Glx. The values for DNase I are calculated from the amino acid sequence listed in the SWISS PROT database (P21704).

	Amount (mol%)	
Amino acid	DNase γ	DNase I
Asx (Asp + Asn)	12.15	10.30
Thr	5.01	6.11
Ser	9.21	9.16
Glx (Glu + Gln)	8.73	8.78
Gly	3.77	3.43
Ala	4.82	8.40
Val	8.31	8.78
Met	1.82	1.52
Ile	5.44	6.49
Leu	7.05	7.63
Tyr	4.31	6.11
Phe	6.10	4.58
Lys	9.75	3.05
His	1.31	1.91
Arg	6.81	6.11
Pro	3.11	4.96
Basic	17.87	11.07
Acidic	20.88	19.08
Acidic/basic	1.17	1.72

0–4 °C. On SDS/PAGE and silver staining, the purified enzyme appeared homogeneous (Figure 3A, lane 1). This enzyme preparation was used in the following studies.

Determination of molecular mass

The molecular mass of DNase γ was determined by both SDS/PAGE and the activity gel system. The extensively purified DNase γ from the second heparin5PW HPLC showed one protein band on an SDS/polyacrylamide gel stained with silver (Figure 3A, lane 1). On the basis of the mobility of DNase γ relative to the protein standards, the molecular mass was estimated to be 33 kDa. This result is consistent with the result of the activity gel system (Figure 3B) and with that of partially purified DNase γ [15]. On G2000SW gel-filtration HPLC (Figure 1). DNase γ appeared as a single 30 kDa peak, suggesting that the enzyme is a monomeric polypeptide.

Table 2 N-Terminal amino acid sequences of DNase γ and DNase I

The N-terminal amino acid sequence of DNase γ was analysed as described in the Experimental section. The result was aligned with DNase I protein sequences listed in the SWISS PROT protein database. The accession numbers of DNase I are P24855 (human), P00639 (bovine), P11936 (pig), P11937 (sheep), P49183 (mouse) and P21704 (rat). Conserved amino acid residues are shaded. Amino acids are expressed by the one-letter code and X indicates a residue that could not be identified.

	Species	N-Terminal sequence
DNase γ	Rat	LRLXSFNVRSFG
DNase I	Rat	LRIAAFNIRTFG
	Human	LKIAAFNIQTFG
	Bovine	LKIAAFNIRTFG
	Pig	LRIAAFNIRTFG
	Sheep	LKIAAFNIRTFG
	Mouse	LRIAAFNIRTFG

Amino acid composition

The amino acid composition of the purified DNase γ is shown in Table 1, together with that of rat DNase I. The values for Thr and Ser were extrapolated and those for Trp and Cys were not determined. The amino acid composition of DNase γ is similar to that of rat DNase I, but different with respect to the Ala and Lys residues. DNase γ had relatively high contents of acidic (Asx and Glx) and basic (Lys, His and Arg) amino acid residues. The ratio of acidic to basic amino acids was 1.17, which is quite different from that of DNase I.

Amino acid sequence

The N-terminal amino acid of the purified DNase γ was detected, suggesting that the N-terminus is not blocked. The N-terminal amino acid sequence was determined to be LRLXSFNVRSFG, the fourth amino acid remaining undetermined. Although no identical sequence is listed in the SWISS PROT protein database, the N-terminal sequence has high homology to that of rat DNase I (Table 2). An alignment of the amino acid sequences of DNase I from various species is also shown in Table 2.

Optimum pH

Figure 4 shows the pH-dependence of DNase γ activity in the DNA-fragmentation assay system using HeLa S3 cell nuclei as the substrate. DNase γ activity was observed in the neutral pH range with a maximum at pH 7.2 in Mops/NaOH buffer. In the absence of DNase γ, no appreciable DNA fragmentation was observed over the pH ranges tested, suggesting that little DNase activity is present in the substance of isolated HeLa S3 cell nuclei.

Effects of bivalent cations

The homogeneously purified DNase γ required both Ca^{2+} and Mg^{2+} for activity, the optimal concentrations of both being

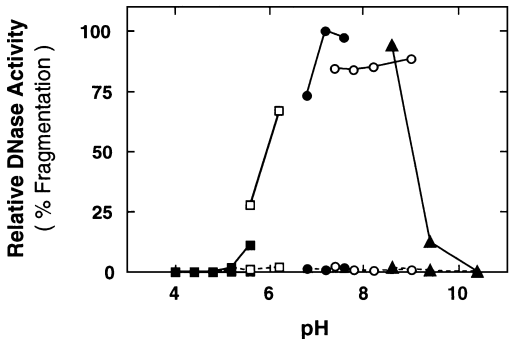

Figure 4 Effects of pH on DNase γ activity

The activity of purified DNase γ was measured by the DNA-fragmentation assay as described in the Experimental section, except that the following buffers were used; acetate/KOH (■) (pH 4.0, 4.4, 4.8, 5.2 and 5.6), Mes/NaOH (□) (pH 5.6 and 6.2), Mops/NaOH (●) (pH 6.8, 7.2 and 7.6), Tris/HCl (○) (pH 7.4, 7.8, 8.2 and 9.0) and *N*-cyclohexyl-3-aminopropanesulphonic acid/NaOH (▲) (pH 8.6, 9.4 and 10.4). Incubation was carried out with (——) and without (– – – –) the enzyme source.

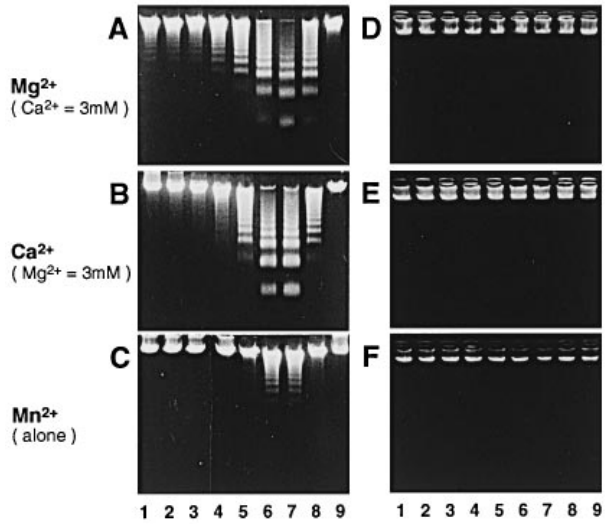

Figure 5 Bivalent cation requirements of DNase γ

The activity of purified DNase γ was measured by the DNA-fragmentation assay as described in the Experimental section. Reactions were performed with (**A**, **B** and **C**) or without substrate (HeLa S3 cell nuclei alone) (**D**, **E** and **F**) DNase γ in the presence of 3 mM $CaCl_2$ and increasing concentrations of $MgCl_2$ (**A**, **D**), in the presence of 3 mM $MgCl_2$ and increasing concentrations of $CaCl_2$ (**B**, **E**), or increasing concentrations of $MnCl_2$ alone (**C**, **F**). The concentrations of $MgCl_2$, $CaCl_2$ and $MnCl_2$ were 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 mM (lanes 1–9 respectively).

1–3 mM (Figures 5A and 5B). Only Mn^{2+} could substitute for Ca^{2+} and Mg^{2+} (Figure 5C); about 50% of full activity was achieved at concentrations of 1–3 mM Mn^{2+}. Fe^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} were all not able to substitute (results not shown). Since no DNA fragmentation was seen in the control (without enzyme) assays (Figures 5**D**–5**F**), DNase activities detected by the DNA-fragmentation assay were not due to activation of endogenous endonuclease(s) present in HeLa S3 cell nuclei as substrates.

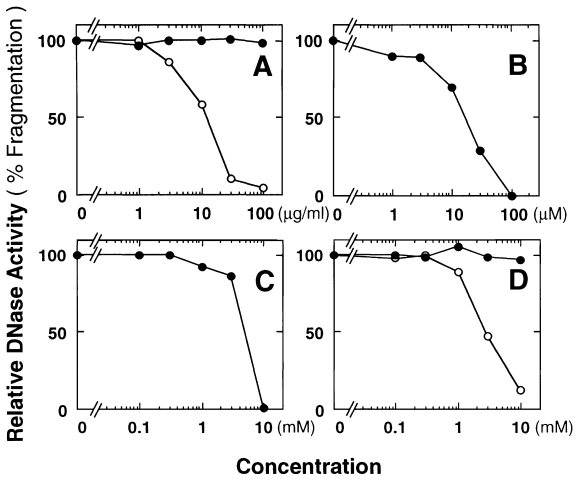

Figure 6 Effects of DNase inhibitors on DNase γ

The activity of purified DNase γ (●) was measured by the DNA-fragmentation assay in the presence of various concentrations of G-actin (**A**), aurintricarboxylic acid (**B**), sodium citrate (**C**) or $MgSO_4$ (**D**). The inhibition profiles of bovine DNase I by G-actin (**A**) and bovine DNase II by $MgSO_4$ (**D**) are indicated by open symbols as positive controls. The activities of DNase I and DNase II were assayed by the same method as for DNase γ, except that, in the case of DNase II, Mes/NaOH, pH 5.6, was used in the reaction buffer instead of Mops/NaOH, pH 7.2.

Effects of DNase inhibitors

The effects of known endonuclease inhibitors on the purified DNase γ were examined. DNase γ was not inhibited by up to 100 μg/ml G-actin, an inhibitor of DNase I [26,27] (Figure 6A). Aurintricarboxylic acid, a general inhibitor of nucleases [28], inhibited DNase γ activity completely at a concentration of 100 μM (Figure 6B). DNase γ was inhibited by sodium citrate, an inhibitor of DNase I [29], with an IC_{50} of 4 mM (Figure 6C). In contrast, SO_4^{2-} ion, an inhibitor of DNase II [30], did not inhibit DNase γ (Figure 6D).

Inhibition by bivalent cations

Enzyme level

We previously reported that Zn^{2+} strongly inhibits partially purified DNase γ activity [14,15], but it was not known whether Zn^{2+} and other metal ions suppress purified DNase γ activity. Therefore we tested the effects of several bivalent cations on purified DNase γ. As shown in Figure 7(A), the enzyme was found to be sensitive to Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+}, with IC_{50} values of 40, 130, 150 and 270 μM respectively. On the other hand, up to 3 mM Fe^{2+} had essentially no effect (results not shown).

Cellular level

Figure 7(A) shows that the purified DNase γ was inhibited by Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} ions in that order. This prompted us to test the effects of these metal ions on apoptotic DNA fragmentation at the cellular level (*in vivo*). Figure 7(B) shows the dose effects of these bivalent cations on irradiation-induced apoptotic DNA fragmentation in rat thymocytes. The formation of a nucleosomal DNA ladder was suppressed in a dose-

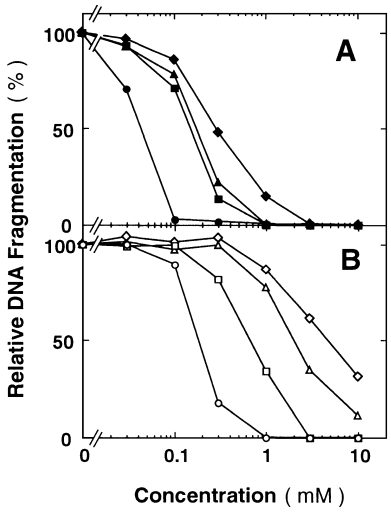

Figure 7 Inhibition of DNA fragmentation by bivalent metal ions *in vitro* and *in vivo* (at the cellular level)

HeLa S3 cell chromosomal DNA was digested by the DNA-fragmentation assay with purified DNase γ in the presence of the indicated concentrations of $CoCl_2$ (◆), $NiCl_2$ (▲), $CuCl_2$ (■) or $ZnCl_2$ (●) (**A**). X-irradiated thymocytes were incubated in a suspensions culture under 5% CO_2 atmosphere at 37 °C for 4 h in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum in the presence of the indicated concentrations of $CoCl_2$ (◇), $NiCl_2$ (△), $CuCl_2$ (□) or $ZnCl_2$ (○) (**B**). Digested DNA produced *in vitro* and *in vivo* was then analysed by agarose-gel electrophoresis and percentage fragmentation was determined as described in the Experimental section.

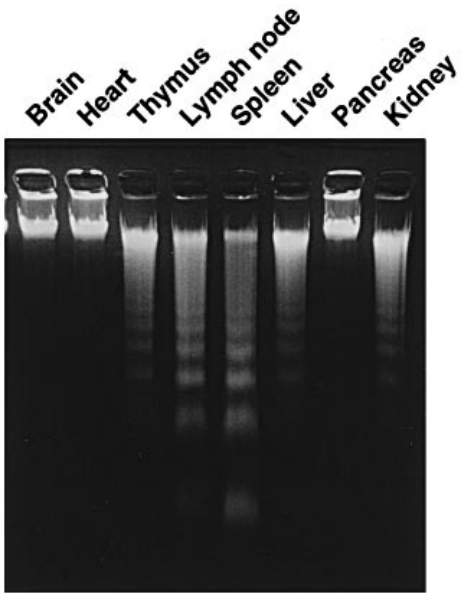

Figure 8 Organ specificity of DNase γ

DNase γ present in nuclear extracts prepared from each organ was separated from DNases α and β by CM5PW HPLC. Aliquots of the extracts containing 0.5 mg of protein were applied to a CM5PW column. Endonuclease activity in DNase γ fractions eluted at 0.55 M KCl was assayed at 37 °C for 20 min as described in the Experimental section.

dependent manner by these ions. The order of inhibition efficiency *in vivo* was the same as that observed *in vitro* (Figure 7A); IC_{50} was achieved at 180 μM Zn^{2+}, 650 μM Cu^{2+}, 1850 μM Ni^{2+} or 4500 μM Co^{2+}. The same results were obtained with dexamethasone-induced thymic apoptosis (results not shown).

Organ specificity of DNase γ

To evaluate the organ distribution of DNase γ, DNase γ activities present in the nuclear extracts of various rat organs were separated from DNases α and β by CM5PW HPLC, and measured using the DNA-fragmentation assay (Figure 8). DNase γ was found to exist at high levels in spleen, lymph node, thymus, liver and kidney: little was detected in brain, heart or pancreas.

DISCUSSION

In this study, we purified DNase γ to apparent homogeneity from irradiation-induced apoptotic rat thymocyte nuclei. Electron-microscopic analysis revealed that about 65% of irradiated thymocytes were turned apoptotic after 4 h incubation, which was accompanied by the same extent of nucleosomal DNA fragmentation. In nuclei of these apoptotic thymocytes, only γ-type DNase was retained [15]. So, we tried to purify the DNase γ present in apoptotic thymocyte nuclei.

To achieve complete purification, we improved the previous procedures in several points; the buffer system was changed from basic (Tris/HCl, pH 7.8) to acidic (Mes/NaOH, pH 5.6) conditions for G2000SW gel-filtration HPLC and the following second heparin5PW HPLC. These modifications greatly improved the purification results. Although almost all the proteins passed through the heparin5PW column under acidic conditions in the presence of 0.5 M NaCl, DNase γ was retained selectively on this column. Furthermore some proteins bound to the resin together with the DNase γ were separated by elution with the NaCl linear gradient. Thus the procedures described here enabled us to obtain DNase γ in sufficient purity and quantity to carry out extensive characterization.

By making the assumption that the molecular mass of the 33 kDa band on SDS/PAGE corresponds to DNase γ protein and by estimating the protein to be about 95% pure in the final preparation, the amount was calculated to be about 0.0008% of total cellular protein. We also made a rough estimate of the number of DNase γ molecules present per nucleus. We estimated that 2×10^{10} apoptotic thymocytes contain about 3×10^{13} molecules of DNase γ. The number of DNase γ molecules/nucleus is calculated to be about 1500.

Characterization of the homogeneously purified DNase γ revealed that this enzyme is a Ca^{2+}/Mg^{2+}- (or Mn^{2+}-) dependent neutral endonuclease (Figure 5). These results are consistent with previous studies on crude and partially purified samples [14,15]. The presence of Ca^{2+}/Mg^{2+}-dependent endonucleases in mammalian tissues has been reported and some of these enzymes have been extensively purified [16,19,31]. A Ca^{2+}/Mn^{2+}-dependent endonuclease (22 kDa) [18] and a Ca^{2+}/Mg^{2+}-dependent endonuclease named NUC18 (18 kDa) [16] have been purified from rat thymocyte nuclei, and suggested as candidates for the apoptotic endonuclease. These DNases bear a resemblance to DNase γ in their enzymic characteristics. However, both differ from DNase γ in their molecular masses.

DNase I (32 kDa), which is a well-known mammalian DNase of molecular mass similar to that of DNase γ (33 kDa), has been suggested to be involved in apoptosis [17]. Although some similarities have been found between rat DNase I and DNase γ in terms of enzymic character, they are different with respect to the following. (i) DNase I is active in the presence of a single bivalent cation, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+} [26,32], whereas DNase γ is not (Figure 5). Zn^{2+}, Ni^{2+} and Co^{2+} cannot

substitute for Ca^{2+}/Mg^{2+} required for DNase γ activity and are quite potent inhibitors. (ii) The organ and cellular distributions of DNase I are different from those of DNase γ (Figure 8). High levels of DNase I are known to be present in the endoplasmic reticulum of secretory organs, such as parotid gland, intestinal mucosa and submaxillary gland, but little is detected in thymus, spleen or liver [26,33], in which DNase γ is abundant. (iii) G-actin, a specific inhibitor of DNase I, failed to suppress the activity of DNase γ even at a concentration of 100 μg/ml. Although rat DNase I is known to be less sensitive to G-actin (as compared with DNase I from other species), the IC_{50} has been reported to be about 0.2 μM (8 μg/ml) [34]. (iv) The analyses of amino acid composition and N-terminal sequence of DNase γ clearly show the physical differences between DNase γ and DNase I. Although the amino acid composition of DNase γ is very similar to that of DNase I, two amino acid residues, Ala and Lys, are markedly different (the content of Ala is about 2-fold lower and Lys is more than 3-fold higher than those of DNase I). The N-terminal sequence of DNase γ provides unequivocal evidence that DNase γ is distinct from rat DNase I (Table 2). However, seven of the 11 N-terminal amino acid residues determined were found to be identical with those of DNase I. Importantly, Asp-7 and Arg-9 in DNase I, which have been suggested to be essential for enzyme activity [35,36], are conserved in DNase γ. These results suggest that DNase γ is a distinct but closely related endonuclease to DNase I.

Since apoptosis in rat thymocytes is known to be suppressed by Zn^{2+}, an endonuclease inhibitable by Zn^{2+} has been thought to be the candidate for an apoptotic DNase [10]. Here, the purified DNase γ was found to be inhibited by Zn^{2+} and also by Cu^{2+}, Ni^{2+} and Co^{2+} ions (Figure 7). Thus we examined the effects of these four metal ions on apoptotic DNA fragmentation at the cellular level. A comparison of these results reveals that the inhibition efficiencies of Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} on DNA fragmentation in intact cells (*in vivo*) and *in vitro* are in the same order (Figure 7). For each metal ion, the concentration required for half-maximum inhibition is higher *in vivo* than *in vitro*. This may be due to the abilities of the metal ions to penetrate into cells and their intracellular compartments. These facts therefore indicate that inhibition of apoptotic DNA fragmentation by Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} is due to the inhibition of DNase γ. These observations further support the involvement of DNase γ in thymic apoptosis.

Since active DNase γ is detected in the preparations from both apoptotic and non-apoptotic thymocytes, some activation steps are thought to exist. Possibly, DNase γ interacts with endogenous inhibitor(s) or activator(s) and/or is regulated by some post-translational modification(s), such as poly(ADP-ribosyl)ation or phosphorylation. In fact, Ca^{2+}/Mg^{2+}-dependent endonuclease activity has been shown to be regulated by poly(ADP-ribosyl)-ation [37,38]. Alternatively, DNase γ may be activated by proteolysis of its premature form. The elucidation of the regulatory mechanism of DNase γ at both the protein and gene levels will be important in understanding the common pathway of apoptosis. Further studies, including cDNA cloning and knock-out targeting of the DNase γ gene, are now under way to determine the structure and function of DNase γ.

Received 12 December 1996/29 April 1997; accepted 6 May 1997

We thank Professor Kenji Takahashi for helpful suggestions. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and funds from the Naito Foundation.

REFERENCES

1 Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) Br. J. Cancer **26**, 239–257
2 Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. (1980) Int. Rev. Cytol. **68**, 251–306
3 Tanuma, S. (1996) in Apoptosis Normal Development and in Cancer, (Sluyser, M., ed.), pp. 39–59, Taylor & Francis Ltd., London
4 Groux, H., Torpier, G., Monte, D., Mouton, Y., Capron, A. and Ameisen, J. C. (1992) J. Exp. Med. **175**, 331–340
5 Cohen, J. J., Duke, R. C., Fadok, V. A. and Sellins, K. S. (1992) Annu. Rev. Immunol. **10**, 267–293
6 White, E. (1996) Genes Dev. **10**, 1–15
7 Hale, A. J., Smith, C. A., Sutherand, L. C., Stoneman, V. E. A., Longthorne, V. L., Culhane, A. C. and Williams, G. T. (1996) Eur. J. Biochem. **236**, 1–26
8 Tanuma, S., Shiokawa, D., Tanimoto, Y., Ikekita, M., Sakagami, H., Takeda, M., Fukuda, S. and Kochi, M. (1993) Biochem. Biophys. Res. Commun. **194**, 29–35
9 Wyllie, A. H. (1980) Nature (London) **284**, 555–556
10 Cohen, J. J. and Duke, R. C. (1984) J. Immunol. **132**, 38–42
11 Arends, M. J., Morris, R. G. and Wyllie, A. H. (1990) Am. J. Pathol. **136**, 593–601
12 Peitsch, M. C., Mannherz, H. G. and Tschopp, J. (1994) Trends Cell Biol. **4**, 37–41
13 Wyllie, A. H., Arends, M. J., Morris, R. G., Walker, S. W. and Evan, G. (1992) Semin. Immunol. **4**, 389–397
14 Tanuma, S. and Shiokawa, D. (1994) Biochem. Biophys. Res. Commun. **203**, 789–797
15 Shiokawa, D., Ohyama, H., Yamada, T., Takahashi, K. and Tanuma, S. (1994) Eur. J. Biochem. **226**, 23–30
16 Gaido, M. L. and Cidlowski, J. A. (1991) J. Biol. Chem. **266**, 18580–18585
17 Peitsch, M. C., Polzar, B., Stepham, H., Crompton, T., MacDonald, H. R., Mannherz, H. G. and Tshopp, J. (1993) EMBO J. **12**, 371–377
18 Nikonova, L. V., Belelsky, I. P. and Umansky, S. R. (1993) Eur. J. Biochem. **215**, 893–901
19 Ribeiro, J. M. and Carson, D. A. (1993) Biochemistry **32**, 9129–9136
20 Barry, A. and Eastman, A. (1993) Arch. Biochem. Biophys. **300**, 440–450
21 Pérez-Sala, D., Collado-Escobar, D. and Mollinedo, F. (1995) J. Biol. Chem. **270**, 6235–6242
22 Yamada, T. and Ohyama, H. (1988) Int. J. Radiat. Biol. **53**, 65–75
23 Rosenthal, A. L. and Lacks, S. A. (1977) Anal. Biochem. **80**, 76–90
24 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
25 Tanuma, S. and Endo, H. (1990) Eur. J. Biochem. **191**, 57–63
26 Lacks, S. A. (1981) J. Biol. Chem. **256**, 2644–2648
27 Anai, M., Sasaki, M., Muta, A. and Miyagawa, T. (1981) Biochim. Biophys. Acta **656**, 183–188
28 Hallick, R. B., Chelm, B. K., Gray, P. W. and Orozco, Jr., E. M. (1977) Nucleic Acids Res. **4**, 3055–3064
29 Wiberg, J. S. (1985) Arch. Biochim. Biophys. **73**, 337–385
30 Yamamoto, M. (1971) Biochim. Biophys. Acta **228**, 95–104
31 Hashida, T., Tanaka, Y., Matsunami, N., Yoshihara, K., Kamiya, T., Tanigawa, Y. and Koide, S. S. (1982) J. Biol. Chem. **257**, 13114–13119
32 Campbell, V. W. and Jackson, D. A. (1980) J. Biol. Chem. **225**, 3726–3735
33 Peitch, M. C., Irmer, M., French, L. E. and Tschopp, J. (1995) Biochem. Biophys. Res. Commun. **207**, 62–68
34 Kreuder, V., Dieckhoff, J., Sittig, M. and Mannherz, H. G. (1984) Eur. J. Biochem. **139**, 389–400
35 Oefner, C. and Suck, D. (1986) Nature (London) **321**, 620–625
36 Lahm, A. and Suck, D. (1991) J. Mol. Biol. **221**, 645–667
37 Yoshihara, K., Tanigawa, Y., Burzio, L. and Koide, S. S. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 289–293
38 Nelipovich, P. A., Nikonova, L. V. and Umansky, S. R. (1988) Int. J. Radiat. Biol. **53**, 749–765