Molecular cloning and expression of a cDNA encoding an apoptotic endonuclease DNase γ

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An endonuclease named DNase γ has been purified from the nuclei of apoptotic rat thymocytes [Shiokawa, Ohyama, Yamada and Tanuma (1997) Biochem. J. **326**, 675–681]. Here we report the molecular cloning of a cDNA encoding a 35 kDa precursor protein for rat DNase γ. A 1.6 kb mRNA coding for the DNase γ precursor is detected at high levels in spleen, lymph nodes, thymus and liver. By using reverse transcriptase-mediated PCR, expression of DNase γ mRNA is observed in kidney and testis but not in brain or heart. Analysis of recombinant DNase γ reveals that full-length DNase γ, including the N-terminal precursor, is an inactive proenzyme. The mature form of recombinant DNase γ, from which the N-terminal precursor has been removed, has the same properties as purified DNase γ: requirement for divalent cations, dependence on pH, sensitivity to Zn^{2+}, and cleavage of chromosome DNA to nucleosomal units. In HeLa S3 cells stably transfected with the DNase γ cDNA, exogenously introduced DNase γ is activated by apoptotic stimuli; enhancement of DNA fragmentation, chromatin condensation and nuclear collapse are observed. These findings provide evidence for the involvement of DNase γ in DNA fragmentation and nuclear structural changes during apoptosis.

INTRODUCTION

Apoptosis is a mechanism for eliminating overproliferating or harmful cells from the cell community in multicellular organisms [1–5]. One of the hallmarks of apoptosis is the enzymic cleavage of genomic DNA into nucleosomal oligomers [6–8]. The internucleosomal cleavage of chromosomal DNA is the biochemical feature most commonly associated with apoptotic processes leading to cell death [6,7]. Thus the identification of the apoptotic endonuclease is critical in understanding the molecular mechanisms of apoptosis. The endonuclease(s) responsible for apoptotic DNA fragmentation are a current research focus; several Ca^{2+}/Mg^{2+}-dependent neutral endonucleases [9–17] and divalent cation-independent acidic endonucleases [18–20] have been suggested as candidates for the apoptotic endonuclease.

In our search for the apoptotic DNase we found a novel endonuclease, named DNase γ, in the nuclei of apoptotic rat thymocytes [9,10] and characterized its properties after purification [11]. DNase γ is a Ca^{2+}/Mg^{2+}-dependent neutral endonuclease whose molecular mass is 33 kDa [9–11]. This enzyme produces 3′-OH/5′-phosphate ends of nucleosomal DNA fragments. The DNA ends formed by cleavage with DNase γ are the same as those produced in apoptotic rat thymocytes [10]. Among divalent metal ions tested, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} inhibit DNase γ activity. These divalent cations also suppress apoptotic DNA fragmentation in rat thymocytes irradiated with X-rays. The inhibition abilities observed for the divalent metal ions *in vivo* (in intact cells) and *in vitro* are of the same order, suggesting that the suppression of apoptotic DNA fragmentation at the cellular level is due to the inhibition of DNase γ [11]. These observations have reinforced the role of DNase γ in nucleosomal DNA fragmentation during thymic apoptosis [9–11].

Here we describe the isolation, characterization and expression of a cDNA encoding rat DNase γ. Characterization of a glutathione S-transferase (GST)–DNase γ fusion protein reveals that DNase γ is synthesized as an inactive precursor protein and converted into an active enzyme by the removal of an N-terminal precursor peptide. The mature recombinant enzyme has the same enzymic properties as purified DNase γ. Importantly, overexpression of DNase γ causes great enhancement of apoptotic DNA fragmentation, chromatin condensation and nuclear collapse, in response to apoptotic stimuli. These results suggest an important role of DNA fragmentation catalysed by DNase γ in eliminating unwanted cells by apoptosis. Furthermore the characterization of the DNase γ gene and protein will represent an important step towards identifying other essential components in this cell-eliminating process.

EXPERIMENTAL

cDNA cloning of rat DNase γ

Plaques (3×10^6) of a λgt11 rat spleen cDNA library (Clontech) were screened with ^{32}P-labelled oligonucleotide probes. The sequences of the oligonucleotides used were 5′-AARGARAAY-CAYAAYGC-3′, 5′-AARGARCARTAYGCXTTYCT-3′ and 5′-AARGAYTTYGTNATHGT-3′. These sequences were based on partial amino acid sequences of DNase γ (KEHNA, KEQYAFL and KDFVIV respectively) [12]. Filters were hybridized in hybridization buffer ($6 \times$ SSPE/$5 \times$ Denhardt's solution/0.25% SDS/100 μg/ml heat-denatured salmon sperm DNA) at 37 °C overnight and the resulting filters were washed in 1x SSC/0.1% SDS at 37 °C and autoradiographed. Inserts of the positive clones were subcloned into pBluescript KS+ (Stratagene) and sequenced on both strands by cycle sequencing with a DSQ1000 DNA sequencer (Shimadzu).

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; PARP, poly(ADP-ribose) polymerase; RT–PCR, reverse transcriptase-mediated PCR.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U75689.

Northern blot analysis

Total RNA (20 μg) extracted from rat organs with TRIzol reagent (Gibco BRL) was separated in a 1 % (w/v) agarose/formamide gel and blotted on a Biodyne-A membrane (Paul). The blot was hybridized with a ^{32}P-labelled probe generated by random priming of an *Rsa*I–*Avr*II fragment of rγ 3.9 in hybridization buffer [5 × SSPE/5 × Denhardt's solution/50 % (v/v) formamide/0.1 % SDS/100 μg/ml heat-denatured salmon sperm DNA] at 42 °C overnight. The resulting filter was washed in 0.1 × SSC/0.1 % SDS at 50 °C and exposed to X-ray film at −80 °C with intensifying screens for 10 days.

Reverse transcriptase-mediated PCR (RT–PCR)

RT–PCR analysis of DNase γ mRNA was performed by using RT–PCR high kit (Takara) in accordance with the manufacturer's protocol. Reverse transcription was performed by using 0.5 μg of total RNA and random primer (9-mers) at 42 °C for 30 min. PCR amplification was performed for 30 cycles in a Thermal Cycler (Perkin Elmer) with EX *Taq* DNA polymerase (Takara); each cycle consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The DNase γ primer sequences were 5′-CCCATGCTGATGGAGAAGCTG-3′ (sense) and 5′-CATCCAGGGCCTCCTCTTCAG-3′ (anti-sense), and they yielded a product of 607 bp. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pairs were 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (anti-sense), yielding a product of 452 bp. Aliquots (4 μl) of the PCR product were separated by 1 % (w/v) agarose gel electrophoresis, transferred onto a Biodyne-A membrane (Paul), and followed by Southern hybridization with a ^{32}P-labelled oligonucleotide probe (5′-AAGAAGGCCTGGAAGAACATC-3′) specific for the DNase γ sequence. Hybridization was performed in hybridization buffer (6 × SSPE/5 × Denhardt's solution/0.25 % SDS/100 μg/ml heat-denatured salmon sperm DNA) at 42 °C overnight and the resulting blot was washed in 1 × SSC/0.1 % SDS at 37 °C. Specific amplification of the DNase γ cDNA fragment was detected by autoradiography. For GAPDH, 4 μl aliquots of the PCR product were separated by 1 % (w/v) agarose gel electrophoresis and revealed by UV illumination after staining with ethidium bromide.

Preparation of GST–DNase γ fusion proteins

The GST–DNase γ M and the GST–DNase γ P fusion proteins were made by using pGEX-3X vector (Pharmacia). The cDNA fragments encoding the mature (nt 278–1135) and precursor (nt 203–1135) DNase γ proteins were obtained by PCR with 5′-C**GAATTC**TGAGGCTCTGCTCCTTCAATGTG-3′ (sense) and 5′-C**GAATTC**GCACATGAGACCTAGGAGCGACTGCC-3′ (anti-sense) primers and 5′-C**GAATTC**AGATGTCCCTGTACCCAGCTTCC-3′ (sense) and 5′-C**GAATTC**GCACATGAGACCTAGGAGCGACTGCC-3′ (anti-sense) primers respectively and subcloned into the *Eco*RI site of pGEX-3X. *Eco*RI sites flanking the coding sequences are shown in bold type. The fusion proteins were expressed in XL1-blue cells and purified in accordance with the manufacturer's protocol.

Assay of endonuclease activity

DNase γ activity was assayed by two methods, namely a DNA fragmentation assay [9–12] and a plasmid assay. In the DNA fragmentation assay, 5×10^5 HeLa S3 cell nuclei were resuspended in 30 μl of 50 mM Mops/NaOH, pH 7.2, containing 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 3 mM $CaCl_2$ and 3 mM $MgCl_2$ unless otherwise indicated. Recombinant DNase γ (10 ng) was then added and the mixtures were incubated at 37 °C for 30 min. The DNA was prepared from the reaction mixture by successive treatments with 0.5 μg/ml RNase A at 50 °C for 20 min and 0.5 μg/ml proteinase K at 50 °C for 30 min, then loaded on a 2 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. DNase activity detected as an apoptosis-like DNA ladder was quantified by densitometry as described previously [9–12]. In the plasmid assay, 1 ng of recombinant DNase γ was added to 10 μl of the reaction buffer [50 mM Mops/NaOH (pH 7.2)/1 mM 2-mercaptoethanol/0.1 mM PMSF/50 ng/ml pBluescript KS+] containing the indicated concentrations of $CaCl_2$, $MgCl_2$ and/or $ZnCl_2$. The enzyme reaction was performed by incubation at 37 °C for 10 min and terminated by extraction with phenol/chloroform. The resulting mixture was analysed by 1 % (w/v) agarose gel electrophoresis.

DNase activity gel system

Recombinant DNase γ (100 ng) was separated by electrophoresis in Laemmli SDS/polyacrylamide gels containing 200 μg/ml of native calf-thymus DNA. In the radiolabelled activity gel assay, pBluescript KS+ was labelled with ^{32}P by nick-translation and 5×10^5 c.p.m. of the labelled plasmid was additionally incorporated into the gel. After electrophoresis, the gels were washed with 10 mM Tris/HCl (pH 7.8)/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. The gels were then incubated in 10 mM Tris/HCl, pH 7.8, containing 1 mM 2-mercaptoethanol and indicated concentrations of $CaCl_2$, $MgCl_2$ and/or $ZnCl_2$ at 37 °C for 3 h. After the gels had been stained with 0.5 μg/ml ethidium bromide, apparent endonuclease activities were detected as dark areas on a fluorescent background by UV transillumination of the gels. In the radiolabelled activity gel, endonuclease activities were detected by autoradiography after exposure overnight at room temperature. To detect DNase γ activity in HeLa S3/γ cells, nuclei were isolated from 10^6 C2-ceramide-treated cells as described previously [10], and subjected to activity gel assay after the elimination of histone H1 by extraction with 2.5 % $HClO_4$. Enzyme reaction was performed by incubation of the gel in 10 mM Tris/HCl, pH 7.8, containing 1 mM 2-mercaptoethanol, 3 mM $CaCl_2$ and 3 mM $MgCl_2$ at 37 °C for 24 h.

Transfection of HeLa S3 cells with DNase γ expression vector

The pOPRSVrγ vector was constructed by subcloning a PCR fragment encoding DNase γ (nt 203–1135) into the *Not*I site of pOPRSV vector (Stratagene). The primers used for PCR were 5′-CGAATTCTGCA**GCGGCCGC**ATGTCCCTGTACCCAGCTTCC-3′ (sense) and 5′-CGAATTCTGCA**GCGGCCGC**GCACATGAGACCTAGGAGCGACTGCC-3′ (anti-sense). *Not*I sites flanking the coding sequences are shown in bold face. HeLa S3 cells (5×10^6) were transfected with 2 μg of pOPRSVrγ (HeLa S3/γ) or empty vector (HeLa S3/neo) by using lipofectin (Gibco BRL). Stable HeLa S3 transfectants were selected by the addition of 800 μg/ml G418 to the culture medium (RPMI 1640 supplemented with 10 % fetal calf serum).

Induction of apoptosis

Apoptosis was induced with 30 μM C2-ceramide in the presence of 0.3 μg/ml actinomycin D. Cells were harvested at the indicated times and fixed with 1 % (w/v) glutaraldehyde; the frequencies of apoptotic cells were then determined by fluorescence microscopy after cell staining with Hoechst 33258. Cells with

condensed chromatin or fragmented nuclei were counted as apoptotic cells. The results are averages for three separate experiments. DNA fragmentation was analysed by 2% (w/v) agarose gel electrophoresis as described previously [9,10].

Western blot analysis

Total proteins from 2×10^5 cells were separated by SDS/PAGE [10% (w/v) gel] and transferred to Immobilon-P membrane (Millipore). Blots were blocked in TBST [20 mM Tris/HCl (pH 8.0)/400 mM NaCl/0.05% (w/v) Triton X-100] containing 2.5% (w/v) BSA for 1 h and probed with the anti-[poly(ADP-ribose) polymerase] (anti-PARP) antibody. After the membrane had been washed with TBST, retained antibody was detected with peroxidase-conjugated anti-(guinea pig) IgG (Jackson Immuno Research) and a Proto Blot Western detection kit (Promega).

RESULTS

Isolation and sequence analysis of a cDNA encoding rat DNase γ

To determine the primary structure of DNase γ by cDNA cloning of the DNase γ mRNA, degenerate oligonucleotide probes were designed from partial amino acid sequences of purified rat splenocyte DNase γ [12]. A rat spleen cDNA library (3×10^6 plaques) was screened with the resulting isolation of three positive clones with the same open reading frame. The longest clone, named rγ 3.9, was subjected to further characterization. Sequence analysis revealed this clone (Figure 1A) to have an open reading frame of 933 bp encoding 310 amino acid residues with a calculated molecular mass of 35685 Da. The existence of an in-frame stop codon in the 5′-untranslated region confirms the correct assignment of the first ATG.

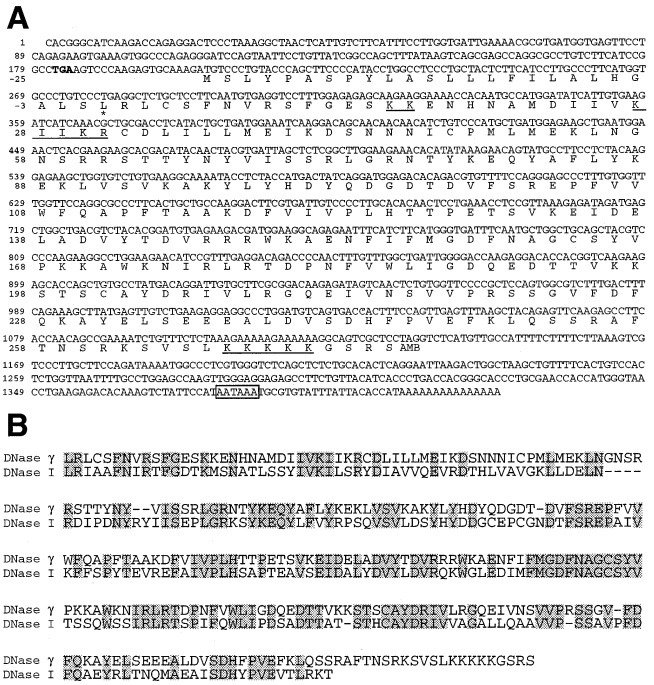

Figure 1 Nucleotide and deduced protein sequences of rat DNase γ cDNA

(**A**) The longest cDNA clone, rγ 3.9, was used to determine the nucleotide sequence. The predicted amino acid sequence is shown from the first ATG codon in the open reading frame. An in-frame stop codon upstream of the first ATG is indicated in bold type. Nucleotide and amino acid numbers are shown at the left. Amino acid numbering begins (marked by an asterisk) at the N-terminal sequence postulated for the mature enzyme with negative numbers for the precursor peptide. The polyadenylation signal is boxed. The putative nuclear localization signals are underlined. (**B**) Comparison of the amino acid sequences of rat DNase γ and DNase I. These amino acid sequences represent the mature enzyme regions. A minimum number of gaps (shown by dashes) were introduced to give maximum similarity. Shaded areas indicate amino acid identity between the proteins.

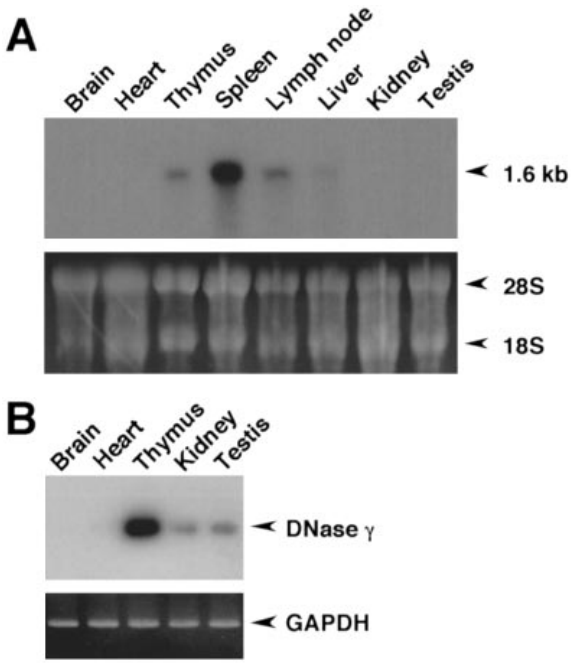

Figure 2 Tissue distribution of DNase γ mRNA

(**A**) Northern blot analysis of total RNA species from rat tissues. The identities of the RNA species are indicated at the top of each lane. Staining of the gel with ethidium bromide is shown in the lower panel, illustrating that equal amounts of RNA were loaded in each lane. (**B**) RT–PCR analysis of mRNA expression of DNase γ in rat tissues. The identities of the RNA species are indicated at the top of each lane (upper panel). GAPDH signals shown in the lower panel serve as an internal control.

The N-terminal sequence of purified DNase γ from rat thymus and spleen [11,12] was found to start from Leu 26, showing the 35 kDa protein to be a DNase γ precursor with a 25-residue precursor peptide. The molecular mass of 33042 Da calculated from 285 residues in the mature form of DNase γ is consistent with the value (33 kDa) estimated for the purified DNase γ by SDS/PAGE [9–12]. The sequence contains two nuclear localization signals satisfying the bipartite and SV40 consensus types [21], one in the N-terminal half and the other in the C-terminal half (Figure 1A). This suggests the location of DNase γ to be in the nucleus, consistent with our previous observation that DNase γ is tightly bound to the nuclear structure [9,10].

There is no identical gene or protein listed in the GenBank/EMBL/DDBJ nucleotide or SWISSPROT protein databases. Comparison of the deduced amino acid sequence of DNase γ with previously reported cDNA species and proteins reveals that rat DNase γ is homologous to human DNAS1L3/nhDNase [22,23], which has been cloned as a DNase I-like protein whose physiological significance is unknown. DNase γ protein shows 88% identity with DNAS1L3, indicating that DNAS1L3 is a human homologue of rat DNase γ. DNase γ also has sequence similarities to DNase I [24] and DNase X [25,26]. The mature rat DNase γ protein shows 45% identity with rat DNase I (mature form) (Figure 1B).

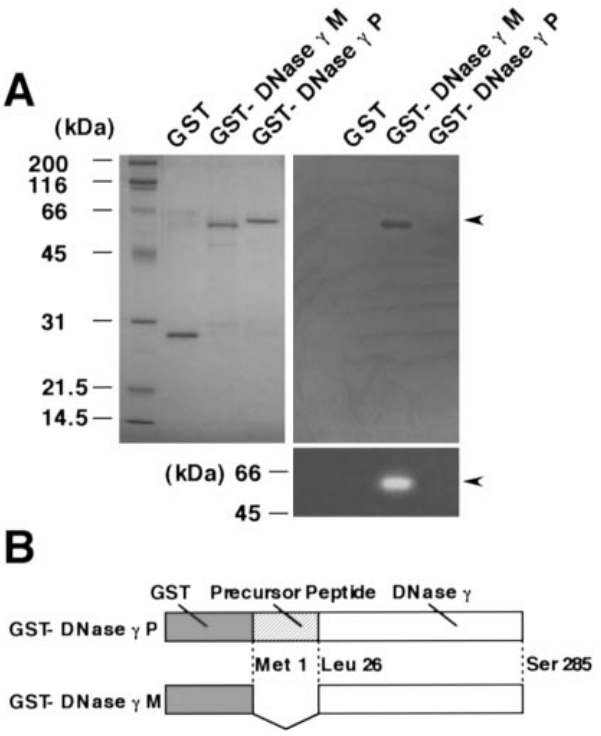

Figure 3 Preparation of GST–DNase γ fusion proteins

(**A**) SDS/PAGE (left panel) and activity gel analyses (right panels) of GST–DNase γ M and GST–DNase γ P. In the activity gel analyses, native calf thymus DNA (upper panel) or native calf thymus DNA plus ^{32}P-labelled plasmid DNA (lower panel) are incorporated in the gels as described in the Experimental section. After incubation of the gels, DNase activities were revealed by staining with ethidium bromide as a dark band (upper panel) or by autoradiography as a 'hole' band (lower panel). Positions representing the GST–DNase γ M activity are marked by arrowheads. (**B**) Schematic illustrations of the construction of GST–DNase γ M and GST–DNase γ P.

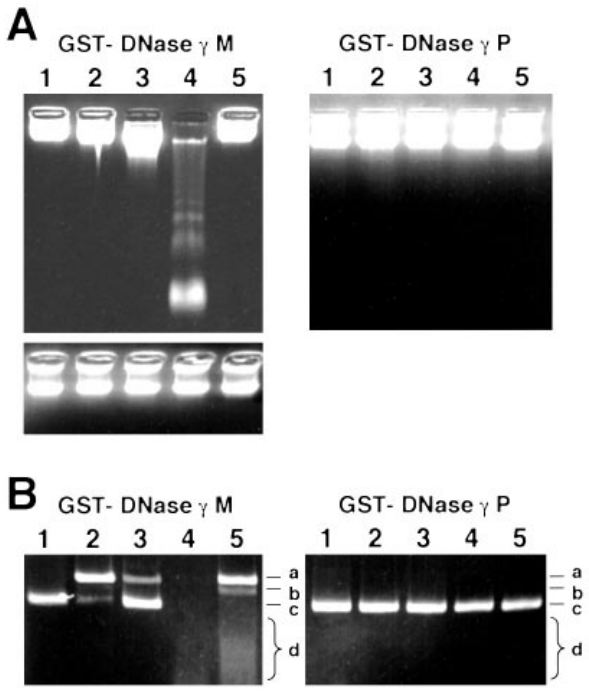

Figure 4 Endonuclease activity of the recombinant DNase γ

Endonuclease activities of GST–DNase γ M (left panels) and GST–DNase γ P (right panels) were analysed by the DNA fragmentation assay (**A**) and the plasmid assay (**B**). Activities were measured in the absence of $CaCl_2/MgCl_2$ (lane 1), in the presence of 3 mM $CaCl_2$ (lane 2), 3 mM $MgCl_2$ (lane 3), 3 mM $CaCl_2$/3 mM $MgCl_2$ (lane 4) or 3 mM $CaCl_2$/3 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ (lane 5), as described in the Experimental section. The result of a control assay performed in the absence of GST–DNase γ M shows that the activity detected by this assay was not due to the artificial activation of an endogenous endonuclease present in HeLa S3 cell nuclei (**A**, lower left panel). Forms of plasmid DNA are indicated at the right sides of (**B**): open circular (a), linear (b), supercoiled (c) and degraded (d) fragments.

Expression of DNase γ mRNA in rat tissues

The distribution of the DNase γ gene transcript was determined by Northern blot analysis of total RNA species derived from various adult rat tissues. The expression of a 1.6 kb DNase γ mRNA was detected at high levels in spleen, thymus, lymph nodes and liver. Little hybridization was seen in brain, heart, kidney or testis (Figure 2A). For further analysis of the DNase γ gene expression in these tissues we performed RT–PCR to detect DNase γ mRNA. As shown in Figure 2(B), expression of DNase γ mRNA was detected in kidney and testis, but minimally in brain or heart.

Properties of recombinant DNase γ proteins

To confirm that the protein encoded by this clone had endonuclease activity, we made and characterized two forms of recombinant DNase γ as fusion proteins with GST. One was full-length DNase γ (GST–DNase γ P) including the N-terminal 25 residues of precursor peptide; the other was mature DNase γ (GST–DNase γ M) starting from Leu 26 (Figure 3B). The GST–DNase γ M and P fusion proteins were each detected as a single protein band (60 and 62.5 kDa respectively) by SDS/PAGE (Figure 3A, left panel). To examine whether these recombinant enzymes had DNase activities, we subjected them to activity gel analysis (Figure 3A, right panel). To avoid the problem of detecting false positive signals generated by DNA-binding proteins [27], we performed radiolabelled activity gel analysis (Figure 3A, lower right panel) along with ethidium bromide staining (Figure 3A, upper right panel). In both assays a Ca^{2+}/Mg^{2+}-dependent endonuclease activity was detected in GST–DNase γ M but not in GST–DNase γ P. The GST–DNase γ M activity was detected at the position corresponding to the molecular mass of GST–DNase γ M estimated by SDS/PAGE (Figure 3A, left panel). These results reveal that GST–DNase γ M is an active endonuclease, whereas GST–DNase γ P has no DNase activity, and also indicate that the DNase activity is not due to bacterial contaminants but to the recombinant DNase γ.

The enzymic properties of GST–DNase γ were next examined in a DNA fragmentation assay with HeLa S3 nuclei as a substrate. As shown in Figure 4 (A, upper panels), GST–DNase γ M (Figure 4A, upper left panel) but not GST–DNase γ P (Figure 4A, right panel) catalysed the internucleosomal cleavage of chromatin DNA, reminiscent of apoptotic DNA fragmentation, in the presence of both 3 mM Ca^{2+} and 3 mM Mg^{2+} (Figure 4A, lanes 4). Furthermore this DNase activity was inhibited by the addition of 0.1 mM Zn^{2+} (Figure 4A, lanes 5). No DNA fragmentation was observed when HeLa S3 nuclei were incubated alone, indicating that the DNase activity was not due to the artificial activation of endogenous nuclease by $CaCl_2$ and $MgCl_2$ (Figure 4A, lower left panel). Further evidence for DNase activity of the recombinant protein was obtained by the plasmid assay (Figure 4B). Consistent with the result of Figure 4(A), GST–DNase γ M digested plasmid DNA in the presence of

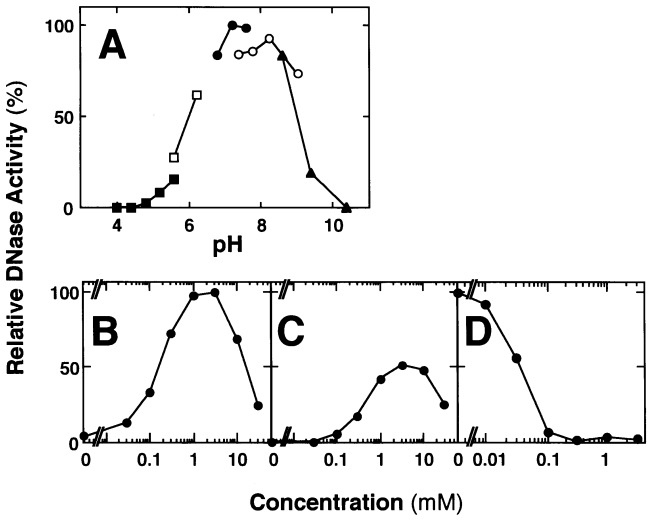

Figure 5 Enzymic properties of the GST–DNase γ M protein

(**A**) Effect of pH on GST–DNase γ M activity. The activity of GST–DNase γ M was measured by the DNA fragmentation assay 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 cyclohexylaminoethanesulphonic acid/NaOH (▲) (pH 8.6, 9.4 and 10.4). (**B**,**C**) Divalent cation requirements of GST–DNase γ M. The activity of GST–DNase γ M was measured by the DNA fragmentation assay. Reactions were performed in the presence of 3 mM $MgCl_2$ and increasing concentrations of $CaCl_2$ (**B**) or increasing concentrations of $MnCl_2$ alone (**C**). (**D**) Inhibition of GST–DNase γ M by Zn^{2+}. GST–DNase γ M activity was measured by the DNA fragmentation assay in the presence of the indicated concentrations of $ZnCl_2$.

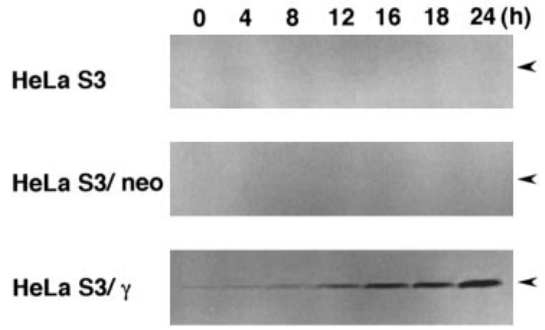

Figure 7 Changes in nuclear DNase γ activity during apoptosis in HeLa S3/γ cells

Parental HeLa S3 (top panel), HeLa S3/neo (middle panel), and HeLa S3/γ (bottom panel) cells were harvested at the indicated times after the addition of 30 μM C2-ceramide, and nuclear DNase γ activities were detected by activity gel analysis as described in the Experimental section. Positions corresponding to 33 kDa DNase γ are marked by arrowheads.

both Ca^{2+} and Mg^{2+}, and was inhibited by Zn^{2+} (Figure 4B, lanes 5). No endonuclease activity was detected by GST–DNase γ P under any conditions tested (Figures 4A and 4B, right panels), confirming the absence of DNase activity from the precursor protein.

Further characterizations of GST–DNase γ M were performed with the DNA fragmentation assay (Figure 5). The effect of pH on GST–DNase γ M activity is illustrated in Figure 5(A). The

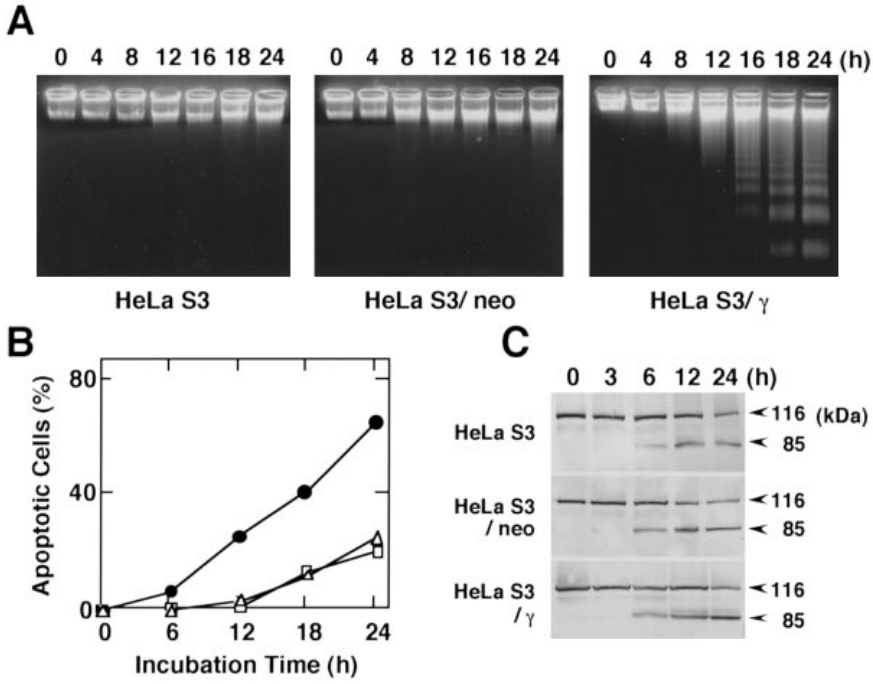

Figure 6 Induction of apoptosis in HeLa S3 cells stably transfected with DNase γ cDNA

(**A**) DNA fragmentation during C2-ceramide-induced apoptosis in parental HeLa S3 (left panel), HeLa S3/neo (middle panel) and HeLa S3/ γ (right panel) cells. (**B**) Appearance of apoptotic cells (chromatin condensation and nuclear fragmentation) in parental HeLa S3 (□), HeLa S3/neo (△) and HeLa S3/γ (●) cells after the addition of 30 μM C2-ceramide. DNA fragmentation and the frequencies of apoptotic cells were determined as described in the Experimental section. (**C**) Western blot analysis of PARP cleavages. Apoptosis was induced by 30 μM C2-ceramide and the cells were harvested at the indicated times after the induction of apoptosis, then subjected to Western blot analysis as described in the Experimental section. The 116 and 85 kDa bands represent intact and cleaved PARP respectively.

result shows that the DNase activity was observed in the neutral pH range with a maximum at pH 7.2 in Mops/NaOH buffer. GST–DNase γ M required both Ca^{2+} and Mg^{2+} for full activity, with the optimal concentrations for both of 1–3 mM (Figure 5B). Of the divalent cations tested, only Mn^{2+} could substitute for Ca^{2+} and Mg^{2+}. Approx. 50% of full activity was achieved at 1–3 mM Mn^{2+} (Figure 5C). Zn^{2+} strongly inhibited GST–DNase γ M activity with an IC_{50} of 35 μM (Figure 5D). These properties of GST–DNase γ M are identical with those of purified DNase γ [11,12]. These results indicate that no post-transcriptional modification such as phosphorylation, poly(ADP-ribosyl)ation or glycosylation is required for DNase γ activity.

Enhancement of apoptotic DNA fragmentation by overexpression of DNase γ

We next examined the effect of the forced expression of the DNase γ on apoptosis in mammalian cells. Transfection with full-length DNase γ expression vector alone did not cause the death of host HeLa S3 cells. The numbers of dead cells in the transient DNase γ transfectants were as low as in empty vector cells (results not shown). This failure of the expression of DNase γ alone to induce apoptosis suggests the existence of one or more mechanisms by which DNase γ is suppressed under normal (non-apoptotic) conditions. Thus the activation of DNase γ was considered to require the exposure of the cells to apoptosis inducers. To test this possibility we established stable transfectants of full-length DNase γ (HeLa S3/γ cells). As judged by morphological changes, C2-ceramide treatment caused apoptosis in normal HeLa S3 and HeLa S3/neo cells (Figure 6B). In these cells, however, apoptotic DNA fragmentation scarcely occurred (Figure 6A, left and middle panels). In contrast, in HeLa S3/γ cells, extensive DNA fragmentation was accompanied by the morphological changes of apoptosis (Figure 6A, right panel). These results clearly show that DNase γ is activated by apoptotic stimuli and catalyses DNA fragmentation during apoptosis.

It is noteworthy that the appearance of apoptotic cells in HeLa S3/γ cells was enhanced compared with control cells (Figure 6B). The same results were obtained when tumour necrosis factor α was used as an inducer of apoptosis (results not shown). To determine whether the forced expression of DNase γ caused acceleration of the whole apoptotic process, we assessed the kinetics of cell death by using another marker for apoptosis, the cleavage of poly(ADP-ribose) polymerase (PARP). The cleavage of PARP (116 kDa) into 85 kDa and 30 kDa fragments has been reported to occur during apoptosis and has suggested to be catalysed by caspase-3 [28–32]. As shown in Figure 6(C), the kinetics and extent of the appearance of 85 kDa PARP fragments in HeLa S3/γ cells were almost the same as those in HeLa S3 and HeLa S3/neo cells. These results indicate that the overexpression of DNase γ does not always accelerate the overall processes of apoptosis. The observation that the cleavage of PARP occurred before DNA fragmentation in HeLa S3/γ cells suggests that the activation of DNase γ is regulated downstream or by an independent pathway of the caspase activation cascade.

Activation of DNase γ during apoptosis

To address the activation mechanism of DNase γ, we used activity gel analysis to examine the changes of DNase γ activity in HeLa S3/γ cells during apoptosis induced by C2-ceramide. At the start of induction, only slight DNase γ activity was detected in non-apoptotic HeLa S3/γ cells (Figure 7). However, the band corresponding to DNase γ activity increased in accordance with the appearance of apoptotic DNA fragmentation (compare Figure 7, bottom panel, with Figure 6B). In contrast, endogenous DNase γ activities in HeLa S3 and HeLa S3/neo cells were too low to be detected throughout the time course (Figure 7, upper and middle panels).

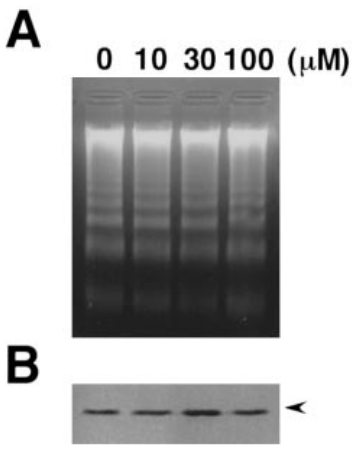

Figure 8 Effect of cycloheximide on DNA fragmentation and nuclear DNase γ activity during apoptosis in HeLa S3/γ cells

Apoptosis was induced by 30 μM C2-ceramide for 24 h in the presence of the indicated concentrations of cycloheximide. Apoptotic DNA fragmentation (**A**) and nuclear DNase γ activity (**B**) were analysed by agarose gel electrophoresis and activity gel analysis respectively, as described in the Experimental section. The arrowhead indicates the position of DNase γ.

This increase in DNase γ activity might be explained by two alternative mechanisms: (1) pre-existing DNase γ in an inactive state in non-apoptotic cells is activated in response to apoptotic stimuli; or (2) DNase γ is newly synthesized by transcriptional/translational activation during apoptosis. To define which model held true here, we examined the effect of cycloheximide, an inhibitor of protein synthesis, on DNase γ activity during C2-ceramide-induced apoptosis in HeLa S3/γ cells. Figure 8 shows that cycloheximide suppressed neither apoptotic DNA fragmentation nor DNase γ activation. This implies that no new protein synthesis is required for the up-regulation of DNase γ activity. These results support the post-translational activation model for the increase in DNase γ activity by apoptotic stimuli.

DISCUSSION

In this paper we describe the first isolation of a cDNA for DNase γ. Furthermore by using the stable transfectant of DNase γ we demonstrate that DNase γ is activated post-translationally in the cells by apoptotic stimuli and that its primary function is to cleave genomic DNA during apoptosis. The DNA fragmentation catalysed by DNase γ is shown to accelerate the morphological changes of the nucleus such as chromatin condensation and nuclear collapse.

Comparison of the deduced amino acid sequence of DNase γ with those of previously reported DNases reveals that DNase γ is a novel member of the DNase I family of endonucleases (Figure 1B). It has been demonstrated that the amino acid residues Glu-78, His-134, Asp-168, Asp-212 and His-252 of DNase I have crucial roles in the catalytic activity [33,34]. Interestingly these residues and their adjacent sequences are well conserved in DNase γ. This sequence similarity suggests that DNase γ has DNase I-like secondary and tertiary structures and catalyses DNA hydrolysis in a similar fashion to DNase I. We have previously observed some similarities in physical and

enzymic properties between DNase γ and DNase I [10–12]. The sequence similarity between DNase γ and DNase I demonstrated here might account for the resemblance in their properties.

Northern blot analysis reveals that the expression of DNase γ mRNA is predominantly observed in thymus, spleen, lymph node and liver (Figure 2A). Although little hybridization is seen in brain, heart, kidney or testis, expression of DNase γ can be detected in kidney and testis by RT–PCR (Figure 2B). Little detection even by RT–PCR in adult brain and heart might imply the stringent repression of DNase γ gene expression in these organs.

DNase γ seems to be synthesized as an inactive precursor protein and converted into an active mature enzyme by removal of the N-terminal precursor peptide. This process is essentially the same as the maturation scheme of DNase I: that is, the mature DNase I is derived from its precursor protein, which has a hydrophobic precursor peptide at its N-terminus [24,35]. However, DNase I is a secretory protein and its precursor peptide has been suggested to work as a signal sequence for extracellular secretion [24,35]. When mammalian cells are transfected with the expression vector for full-length DNase I, the exogenous DNase I protein and activity are largely detected in their culture supernatant [15,35,36]. In contrast, DNase γ activity in HeLa S3/γ transfectant is predominantly found in the nuclear fraction (Figure 6A) and is barely detectable in the culture medium (results not shown). These results show the strict distribution of DNase γ in the nuclei and are consistent with the fact that DNase γ has two strong nuclear localization signals, which are not present in DNase I (Figure 1). The precursor region of DNase γ is therefore considered to be important for the suppression of DNase γ activity rather than as a signal sequence for secretion.

Until now the endonuclease responsible for apoptotic DNA fragmentation has been sought, and several endonucleases, including DNase γ, have been purified and proposed as candidates for the apoptotic DNase [9–20]. Our present results showing that the activation of DNase γ is initiated only by apoptotic stimuli, thereby bringing about the massive enhancement of apoptotic DNA fragmentation in the HeLa S3/γ transfectant, provide evidence for the involvement of DNase γ in apoptotic DNA fragmentation. Among the candidates, DNase I has been examined for its ability to fragment DNA by using nuclei isolated from COS cells transiently transfected with DNase I. Consequently degradation of chromatin DNA into oligonucleosomal fragments was observed in the presence of Ca^{2+} and Mg^{2+} [14]. The result indicates the ability of DNase I to produce apoptosis-like DNA fragmentation in the nuclear system *in vitro*. However, the activation of introduced DNase I by apoptotic stimuli and the production of apoptotic DNA fragments in intact cells remain to be determined.

The tissue distributions of DNase γ and DNase I are quite different [37,38]: DNase I tends to be present mainly in parotid gland, intestinal mucosa and submaxillary gland, locations in which DNase γ activities are barely detectable. Thus at present we cannot exclude the possibility that DNase I participates in the cleavage of DNA during apoptosis in these organs. It might also be that different endonucleases, such as DNase II or *Nuc*18, are involved in apoptotic DNA fragmentation in particular cell types under different statuses and apoptotic stimuli. A]t present it is hard to draw a conclusion that DNase γ exclusively catalyses the internucleosomal cleavage of DNA in all situations of apoptosis.

It is of note that the overexpression of DNase γ causes not only an enhancement of DNA fragmentation but also an acceleration of appearance of apoptotic cells in response to apoptotic stimuli. These results indicate a direct linkage of the DNA fragmentation catalysed by DNase γ to nuclear structural changes characteristic of apoptosis. This implies the physiological significance of apoptotic DNA fragmentation in the acceleration of cell suicide to allow the rapid elimination of unwanted cells *in vivo*.

Figure 8 shows that the activation of DNase γ occurs even in the presence of a protein synthesis inhibitor, cycloheximide. This result indicates that DNase γ pre-exists in an inactive form and is activated by one or more post-translational mechanisms during apoptosis. This is consistent with the fact that the expression of introduced DNase γ is driven by a constitutive promoter Rous sarcoma virus long terminal repeat. On the basis of these results, together with the results of Figure 3, one of the likely activation mechanisms of DNase γ is considered to be the conversion of the precursor form to the mature enzyme by the removal of the N-terminal precursor peptide. This includes the possibility that the hydrophobic sequence of the precursor peptide directs DNase γ to microsomes or anchors in the nuclear envelope, where it is stored until apoptotic signals arrive, and after removal of the precursor peptide the nuclear localization signals direct the mature active enzyme to the nucleus. This activation process might be performed by some peptidase/protease activated by apoptotic signals. Recent studies reveal the importance of interleukin 1β-converting enzyme-like proteases (caspases) as the agents of apoptosis [30,31]; some cellular proteins, such as PARP, α-fodrin, DNA-dependent protein kinase and lamins, are shown to be cleaved by the caspase cascade during apoptosis [28–32]. However, no target sequence of the caspase family proteases is present in the cleavage site of the precursor peptide of DNase γ, suggesting that caspases do not cleave the precursor peptide directly.

At present we consider it possible that the activity of DNase γ is further regulated by some other mechanisms, such as interaction with one or more protein inhibitors and activators, and/or one or more post-translational chemical modifications. The active form of DNase γ is present in non-apoptotic thymocytes and the activity is kept constant during apoptosis induced by X-ray irradiation or dexamethasone [9,10]. Furthermore the activities of Ca^{2+}/Mg^{2+}-dependent endonucleases present in rat thymus, liver and bull semen have been shown to be regulated by poly(ADP-ribosyl)ation [39–41]. These results indicate the different statuses of DNase γ in different cell types and the existence of multiple regulatory steps in DNase γ activation. In any case the cleavage of precursor peptide by a peptidase/protease must occur as the first step of the activation process of DNase γ.

On the basis of our results, we suggest that DNase γ is a central component of the apoptotic machinery that is activated in response to cellular signals (either external or internal) leading to apoptosis. These signals are probably transmitted to specific regulators for DNase γ, such as peptidase/protease, one or more protein inhibitors and one or more activators. We are now attempting to identify these regulators and to clarify the activation and/or suppression mechanisms of DNase γ. Further studies on the regulatory mechanisms of DNase γ will increase our understanding of the whole process of apoptosis.

While this manuscript was being reviewed, a paper appeared describing the purification and cDNA cloning of a murine apoptotic endonuclease CAD and its inhibitor ICAD [42]. A comparison of the amino acid sequences of DNase γ and CAD reveals that there is no identity in the primary structures. Thus DNase γ is apparently different from CAD.

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