Identification of two functional nuclear localization signals in DNase γ and their roles in its apoptotic DNase activity

Daisuke SHIOKAWA*, Yukari SHIKA* and Sei-ichi TANUMA*†[1]

*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan, and †Genome and Drug Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

Among DNase I family members, only DNase γ causes DNA fragmentation during apoptosis. However, the molecular basis for this functional feature of DNase γ is poorly understood. Here we describe the identification of functional NLSs (nuclear localization signals) in DNase γ and their roles in its apoptotic function. DNase γ contains two NLSs: a classical bipartite-type NLS (NLS1) located in the N-terminal half, and a short basic domain (NLS2) at the C-terminus. No potential NLSs are found in the primary structures of other DNase I family DNases. Inactivation of either NLS1 or NLS2 causes reduced DNA ladder-producing activity in DNase γ. Disruption of NLS2 suppresses ladder formation more effectively than disruption of NLS1. DNase γ doubly mutated in both NLSs is enzymically active, but no longer catalyses apoptotic DNA fragmentation. Although DNase I fails to produce ladder formation during apoptosis, DNase I fused to NLS2 of DNase γ through its C-terminus is able to catalyse DNA fragmentation in apoptotic cells. These results indicate that the presence of either NLS1 or NLS2 is necessary for the apoptotic function of DNase γ, and that the most important domain for this function is NLS2. These findings also explain the lack of apoptotic DNase activity in the other DNase I family DNases.

Key words: apoptosis, DNA fragmentation, DNase, green fluorescent protein, nuclear localization signal.

INTRODUCTION

DNase γ, a member of the DNase I family of DNases, is a 33 kDa Ca^{2+}/Mg^{2+}-dependent endonuclease whose activity is detected at high levels in some lymphoid organs, such as spleen, lymph node and thymus [1–4]. To date, four distinct, but highly related, DNase I-like genes have been identified in humans [4–8], and the enzymic properties of their coded proteins, DNase I, DNase X/Xib, DNase γ/DNAS1L3 and DNAS1L2, have been characterized in detail [9]. Consistent with their well conserved primary structures, the four DNases are found to share many common features: (i) they are activated by Ca^{2+} and Mg^{2+} in a synergistic manner, (ii) Zn^{2+} inhibits their activities with almost the same efficiency, and (iii) they hydrolyse DNA endonucleolytically to produce 3′-OH/5′-P ends [9].

In contrast with these similar physical and enzymic properties, the expression profiles of DNase I family genes are quite distinct among human tissues: *DNASE1* is highly expressed in pancreas, *DNASEX* in heart and skeletal muscle, and *DNASE*γ in liver, spleen and bone marrow. The expression level of *DNAS1L2* is quite low compared with those of the other members; however, its transcript is detected widely in human tissues [9]. These observations suggest unique physiological roles *in vivo*.

Recent experimental progress has provided some important clues to understanding the physiological importance of the DNase I family DNases. Targeted disruption of *dnase1* causes increased generation of anti-nuclear antibodies directed against naked DNA and nucleosomes, and results in the development of a systemic lupus erythematosus-like syndrome in mice [10]. Thus an important role of DNase I is the elimination of harmful extracellular DNA from animal bodies. DNase γ has been suggested to catalyse nucleosomal DNA fragmentation during cell death [9,11–13], an outstanding hallmark of apoptosis [14]. Although the apoptotic situations under which DNase γ is indispensable for DNA fragmentation are not yet fully understood, one such situation has been identified as an apoptotic process associated with myogenic differentiation [15].

Interestingly, forced expression of DNase γ causes enhanced DNA ladder formation when cells are exposed to apoptotic stimuli [3,9,12,13], whereas the expressions of other DNase I-like DNases has no such effect [9]. Thus DNase γ is shown to have an ability to produce apoptotic DNA fragmentation at the cellular level. However, the reason why DNase γ, but not other DNase I family members, catalyses apoptotic ladder formation remains unknown.

In the present study, our goal is to identify the domain(s) required for the apoptotic function of DNase γ, and to clarify the molecular basis by which DNase γ, but not the other DNase I family DNases, has apoptotic DNase activity.

EXPERIMENTAL

Construction of expression vectors

cDNA fragments containing open reading frames of the indicated human DNases were generated by PCR and subcloned into pBluescript KS+ (Stratagene). The primers used were: DNase I, 5′-**CTCGAG**CCACCATGAGGGGCATGAAGCTG-CTG-3′ (sense) and 5′-**CTCGAG**ACTTCAGCATCACCTCCA-CTG-3′ (antisense); DNase γ, 5′-**CTCGAG**CCACCATGTCA-CGGGAGCTGGCCCCA-3′ (sense) and 5′-**CTCGAG**AGGAG-CGTTTGCTCTTTGTTTTC-3′ (antisense); DNase γ/ΔNLS2, 5′-**CTCGAG**CCACCATGTCACGGGAGCTGGCCCCA-3′

Abbreviations used: BD, basic domain; GFP, green fluorescent protein; NLS, nuclear localization signal.

[1] To whom correspondence should be addressed, at Department of Biochemistry, Faculty of Pharmaceutical Sciences (e-mail tanuma@rs.noda.tus.ac.jp).

(sense) and 5′-**CTCGAG**TGCTGTTGGTGAAGGCCCTTGA-3′ (antisense). *Xho*I sites flanking the coding sequences are shown in bold face. Point mutations in the NLS1 (nuclear localization signal 1) sequence were introduced as described in the following section. After confirming the sequences, the inserts were excised by *Xho*I digestion and recloned into the *Xho*I site of pcDNA3-Myc-His C (Invitrogen) to generate expression vectors for C-terminal Myc- and His-tagged forms of DNase γ (phDNase γ-Myc-His), DNase I (phDNase I-Myc-His), DNase γ/ΔNLS2 (phDNase γ/ΔNLS2-Myc-His), DNase γ/NLS1mut (phDNas γ/NLS1mut-Myc-His), DNase γ/NLS1mut/ΔNLS2 (phDNase γ/NLS1mut/ΔNLS2-Myc-His) and DNase I-NLS2 (phDNase I-NLS2-Myc-His). In the case of GFP (green fluorescent protein) vector constructions, PCR fragments for NLS1, NLS1mut and NLS2 were subcloned into the *Sma*I site of pEGFP-C1 (Clontech) to generate the vectors pEGFP-NLS1, pEGFP-NLS1mut and pEGFP-NLS2 respectively. The plasmids were verified to have the correct nucleotide sequences. The primers used were: NLS1, 5′-AAGGAGAGGCATAACGTACAAC-3′ (sense) and 5′-TTTT-CTTCCAAGCCGAGAGCT-3′ (antisense); NLS1mut, 5′-AGC-GAGAGGCATAACGTACAAC-3′ (sense) and 5′-TGCTGCTCC-AAGCCGAGAGCT-3′ (antisense); NLS2, 5′-CAAAAAATCT-GTCACTCT-3′ (sense) and 5′-GAGCGTTTGCTCTTTGTTT-TC-3′ (antisense). Mutated bases are underlined.

Site-directed mutagenesis

The cDNA insert for DNase γ was excised from pBluescript KS+ by *Kpn*I and *Xba*I digestion, and the fragments were recloned into the same site of the pUC19 vector. These plasmids were used for oligonucleotide-directed mutagenesis using an LA PCR *in vitro* Mutagenesis Kit (Takara) according to the manufacturer's protocol. Residues Arg^{60} and Arg^{75}/Lys^{76} of human DNase γ were converted into alanines using the following oligonucleotides: R60A, 5′-AGAAATTCAGCGAGAGGCATA-3′; R75A/K76A, 5′-GGC-TTGGAGCAGCAACATATA-3′. Mutated bases are underlined.

Transfection and recombinant protein analyses

C2C12 cells (2×10^5), cultured in Dulbecco's modified Eagle's medium supplemented with 20 % (v/v) fetal calf serum, were transfected with 1 μg of expression vector using FuGENE 6 transfection reagent (Roche). Under optimized conditions, transfection efficiencies of approx. 70 % for C2C12 cells and 45 % for HeLa S3 cells were achieved. At 24 h post-transfection, the cells were harvested by treatment with 0.05 % trypsin, divided among three culture dishes, and allowed to attach for 6 h. The three resulting cultures were subjected to the induction of apoptosis, Western blot and activity gel analysis respectively. Apoptosis was induced by adding staurosporine at a concentration of 0.3 μM to the culture. The resulting cells were collected at the indicated time, and DNA fragmentation was analysed by 1.8 % (w/v) agarose gel electrophoresis as described previously [15]. The expression of Myc/His-tagged recombinant DNases was detected by Western blot using an anti-Myc antibody (Invitrogen) as described previously [9], and DNase activities were analysed by an activity gel assay as described previously [3].

Fluorescence microscopy

HeLa S3 cells (2×10^5), grown on a coverslip in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum, were transfected with 1 μg of GFP vector as described above. At 24 h post-transfection, the cells were fixed, the nuclei were counterstained with Hoechst 33258, and the cells were examined under fluorescence microscopy as described previously [16]. Digital images were processed with Photoshop 7.0 software (Adobe).

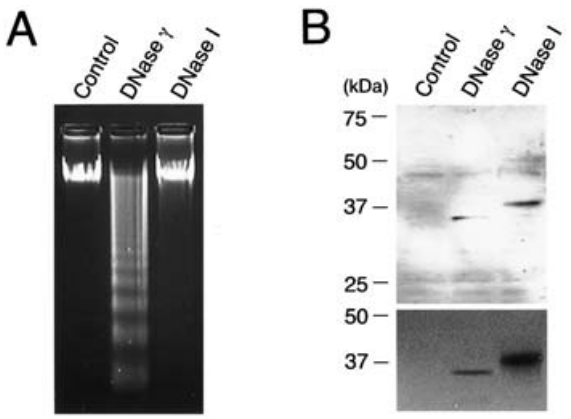

Figure 1 Apoptotic DNA fragmentation in C2C12 cells transfected with DNase γ or DNase I expression vectors

C2C12 cells were transfected with the indicated vectors and cultured for 24 h. The resulting cultures were divided by replating and subjected to the induction of apoptosis (**A**), Western blot (**B**, upper panel) and activity gel analysis (**B**, lower panel), as described in the Experimental section. Apoptosis was induced by staurosporine treatment at a dose of 0.3 μM for 16 h.

RESULTS AND DISCUSSION

DNase γ has a specific functional role in apoptosis

Figure 1 shows the ability of DNase γ to produce DNA fragmentation during apoptosis. Proliferating C2C12 cells are known to undergo apoptosis with no DNA ladder formation [17], and the expression of endogenous *dnase*γ and *dnase1* is scarcely detected unless myogenic differentiation is induced by shifting the culture to low mitogen conditions [15]. Thus undifferentiated C2C12 cells are considered to be an ideal system in which to examine the apoptotic DNase activity of DNase γ and DNase I. The ectopic expression of DNase γ or DNase I by itself caused no spontaneous apoptosis or DNA laddering in the host cells (results not shown). After 16 h of continuous treatment with staurosporine, nucleosomal DNA fragmentation appeared in cells expressing exogenous DNase γ (Figure 1A). Although slight smearing was detected, no clear ladder configuration could be seen in cells transfected with DNase I (Figure 1A). This was not due to differing transfection efficiencies of the DNases, because no obvious difference could be seen in the levels of their expression (Figure 1B, upper panel) or in their activities (Figure 1B, lower panel) in the host cells.

The apparent molecular mass of DNase I (37 kDa) was higher than that of DNase γ (36 kDa) (Figure 1B). This is likely to be caused by multiple glycosylation of DNase I observed when it is expressed in mammalian cells [18].

Identification of functional NLS domains of DNase γ

The primary structures of the DNase I family DNases are shown schematically in Figure 2. DNase γ contains two potential NLSs: a bipartite sequence located in the N-terminal half, and a Lys- and Arg-rich basic domain (BD) at the C-terminus. These two domains conform well to the criteria proposed for a classical bipartite

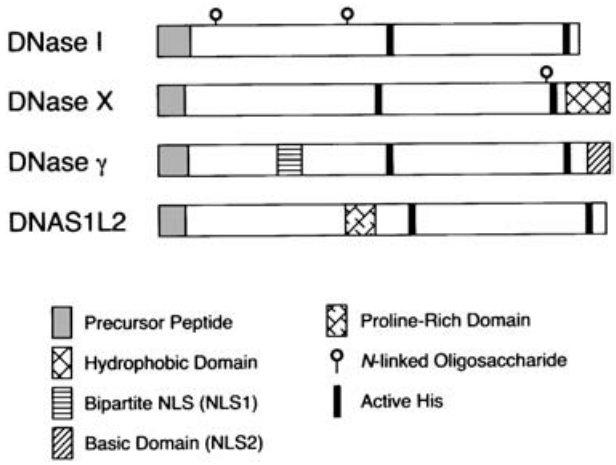

Figure 2 Schematic representation of the primary structures of human DNase I family DNases

Symbols for each characteristic domain are shown.

NLS [19] and a monopartite NLS [20] respectively. DNase X and DNAS1L2 are also found to have their own unique domains, a Leu-rich hydrophobic domain and a proline-rich domain respectively. However, we could not find any NLS-like domains in DNase I-like members other than DNase γ. Therefore we postulated that these two potential NLSs might be the key domains in the apoptotic function of DNase γ.

The NLS activity of each domain was examined in HeLa S3 cells using GFP as the cargo. In contrast with the uniform cellular distribution of GFP alone (Figure 3A), the fluorescent image of GFP containing the putative bipartite NLS shows a nuclear-specific localization (Figure 3B, upper panels). The nuclear localization of GFP–NLS1 was abolished by point mutations introduced to void the consensus for a bipartite NLS (Figure 3B, lower panels). GFP fused to the BD also accumulated within the nucleus (Figure 3C). On the basis of these results, both of the putative NLSs, the bipartite type and the BD, are shown to be functional, and named as NLS1 and NLS2 respectively.

Similar results were obtained in C2C12 cells (not shown). Only the data obtained using HeLa S3 cells are shown in Figure 3, because the cellular morphology of HeLa S3 cells is clearer and more uniform than that of C2C12 cells.

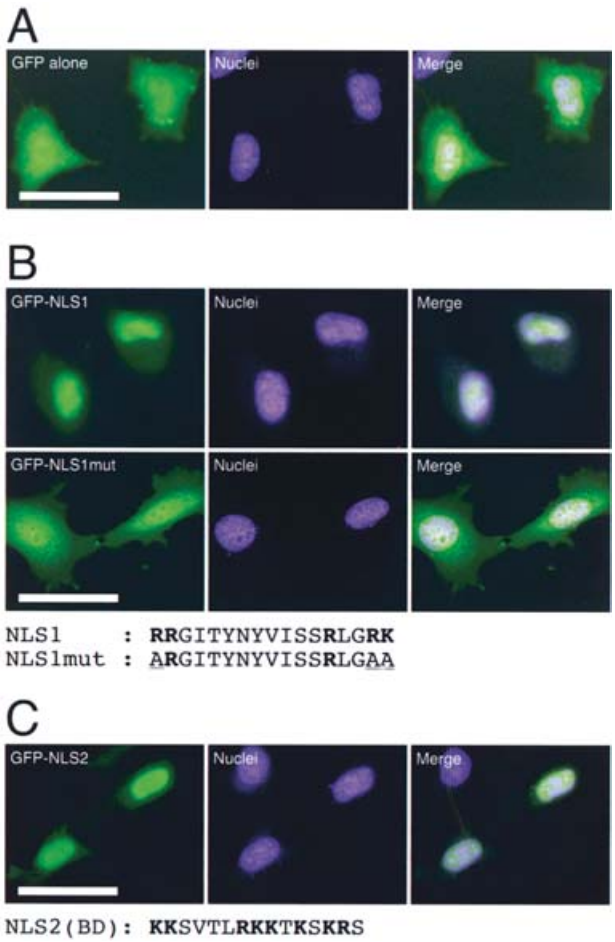

Figure 3 Subcellular localization of GFP fused to putative NLS sequences of DNase γ

HeLa S3 cells transfected with pEGFP-C1 (**A**), pEGFP-NLS1 (**B**, upper panel), pEGFP-NSL1mut (**B**, lower panel) or pEGFP-NLS2 (**C**) were fixed and stained with Hoechst 33258, and the images of GFP (green; left panels) and nuclei (magenta; centre panels) were monitored by fluorescence microscopy as described in the Experimental section. Merged images are shown in the right panels (white). The amino acid sequences of the GFP-fused NLSs are shown below the panels. Basic amino acids (Lys and Arg) are in bold. Mutated residues in NLS1mut are underlined. The white bars represent 50 μm.

Roles of NLS1 and NLS2 in the apoptotic function of DNase γ

To evaluate the importance of the two NLSs for the apoptotic function of DNase γ, we constructed a series of DNase γ expression vectors in which either or both NLS1 and NLS2 were inactivated, by point mutations and deletion mutation respectively (Figure 4, below the panels).

Deletion mutation of the C-terminal NLS2 significantly reduced the apoptotic DNase activity of DNase γ (Figure 4A, lane 2). DNase γ disrupted at NLS1 alone exerted a slightly suppressed ladder-producing activity (Figure 4A, lane 3). However, when both NLSs were inactivated simultaneously, DNase γ no longer catalysed apoptotic DNA fragmentation in staurosporine-treated C2C12 cells (Figure 4A, lane 4). These observations are not due to the inactivation or reduced specific activities of the mutant DNase γ proteins, because they showed almost the same expression levels and activities by Western blot (Figure 4B, upper panel) and activity gel assay (Figure 4B, lower panel) respectively.

These results indicate that the presence of either one of the two NLSs is essential for the apoptotic DNase activity of DNase γ, and that both are required to exert maximal activity. As judged by the ladder intensities produced by the mutant DNases, NLS2 is suggested to be the primary domain for the apoptotic function of DNase γ.

C-terminal fusion of NLS2 allows DNase I to have apoptotic DNase activity

To verify the above prediction, we hypothesized that the C-terminal fusion of NLS2 would allow DNase I to catalyse apoptotic DNA fragmentation. DNase I alone produced almost no nucleosomal ladders in C2C12 cells, and the extent of DNA degradation was almost the same as that observed in mock transfectants (Figure 5A). In clear contrast, extensive DNA fragmentation appeared in cells transfected with DNase I–NLS2 (Figure 5A). Thus the C-terminal fusion of NLS2 is found to be sufficient to confer apoptotic DNase activity on DNase I in

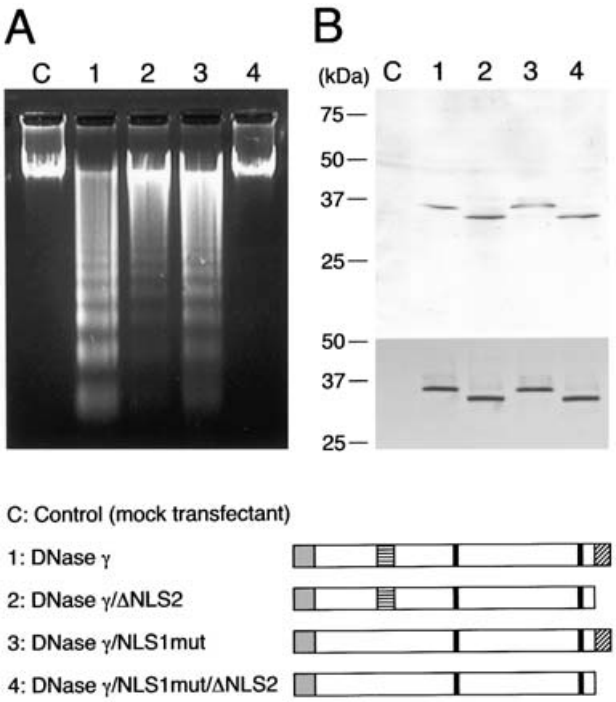

Figure 4 Roles of NLS1 and NLS2 in the apoptotic DNase activity of DNase γ

C2C12 cells were transfected with expression vectors for the indicated forms of DNase γ. Each culture was divided by replating, and subjected to the induction of apoptosis (**A**), Western blot (**B**, upper panel) and activity gel analysis (**B**, lower panel), as described in the Experimental section. Apoptosis was induced by staurosporine treatment at a dose of 0.3 μM for 16 h. The DNase γ mutants are shown schematically below the panels. Details of each domain are shown in Figure 2.

C2C12 cells. This provides further evidence of the importance of NLS2 in the apoptotic function of DNase γ.

As compared with the DNA ladders produced by DNase γ, those detected in DNase I–NLS2-transfected cells were found to be smeared and more extensively degraded. This is probably caused by their distinct catalytic features: DNase γ hydrolyses chromosomal DNA only at linker sequences, whereas DNase I also attacks the region included in the nucleosome core [21].

In a previous study, DNase γ fused to GFP through its C-terminus was expressed in HeLa S3 cells, and its subcellular distribution was determined; GFP–DNase γ was found to locate in the perinuclear region in living cells, and to translocate into the nucleus during apoptosis [9]. Importantly, this apoptosis-dependent nuclear translocation is not seen for DNAS1L2, which has no NLS and no ability to produce apoptotic DNA fragmentation [9]. Furthermore, apoptosis-dependent nuclear localization has also been observed for endogenous DNase γ in differentiating C2C12 myoblasts and X-ray-irradiated thymic cells *in vivo* [15,22]. Thus the nuclear translocation of DNase γ is considered to be an important process for its apoptotic function.

It has been shown that proteins carrying monopartite and/or bipartite NLSs are recognized by the importin/karyopherin/PTAC (nuclear pore-targeting complex) complex, and transported selectively into the nucleus through the nuclear pore complex [23]. Taking this into account, the nuclear translocation of DNase γ is suggested to be controlled by importins through NLS2 and, in part, NLS1. This also explains why other DNase I family DNases lack apoptotic DNase activity, because they have no canonical NLS and do not show nuclear localization.

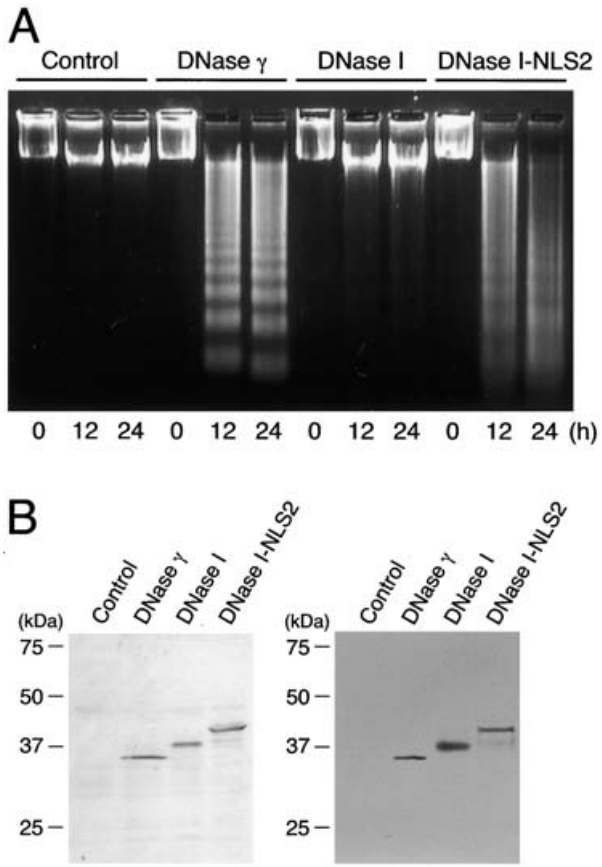

Figure 5 DNase I acquires apoptotic DNase activity by the C-terminal fusion of NLS2 from DNase γ

C2C12 cells were transfected with the expression vectors for the indicated DNases. 'Control' represents the results of mock transfection. Each culture was divided by replating, and subjected to the induction of apoptosis (**A**), Western blot (**B**, left panel) and activity gel analysis (**B**, right panel), as described in the Experimental section. Apoptosis was induced by staurosporine treatment at a dose of 0.3 μM for the indicated times.

In summary, we have identified two functional NLSs in DNase γ and clarified their roles in the apoptotic DNase function of DNase γ. Our results provide an important clue to understanding the regulatory mechanism of DNase γ during apoptosis.

We thank Miss Mana Kawabata and Miss Aya Maehara for their excellent technical assistance. This work was funded in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

1 Shiokawa, D., Iwamatsu, A. and Tanuma, S. (1997) Purification, characterization, and amino acid sequencing of DNase γ from rat spleen. Arch. Biochem. Biophys. **346**, 15–20
2 Shiokawa, D., Ohyama, H., Yamada, T. and Tanuma, S. (1997) Purification and properties of DNase γ from apoptotic rat thymocytes. Biochem. J. **326**, 675–681
3 Shiokawa, D. and Tanuma, S. (1998) Molecular cloning and expression of a cDNA encoding an apoptotic endonuclease DNase γ. Biochem. J. **332**, 713–720
4 Rodriguez, A. M., Rodin, D., Nomura, H., Morton, C. C., Weremowicz, S. and Schneider, M. C. (1997) Identification, localization, and expression of two novel human genes similar to deoxyribonuclease I. Genomics **42**, 507–513
5 Shak, S., Capon, D. J., Hellmiss, R., Marsters, S. A. and Baker, C. L. (1990) Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. Proc. Natl. Acad. Sci. U.S.A. **87**, 9188–9192
6 Pergolizzi, R., Appierto, V., Bosetti, A., DeBellis, G. L., Rovida, E. and Biunno, I. (1996) Cloning of a gene encoding a DNase I-like endonuclease in the human Xq28 region. Gene **168**, 267–270

7 Parrish, J. E., Ciccodicola, A., Wehhert, M., Cox, G. F., Chen, E. and Nelson, D. L. (1995) A muscle-specific DNase I-like gene in human Xq28. Hum. Mol. Genet. **4**, 1557–1564

8 Shiokawa, D., Hirai, M. and Tanuma, S. (1998) cDNA cloning of human DNase γ: chromosomal localization of its gene and enzymatic properties of recombinant protein. Apoptosis **3**, 89–95

9 Shiokawa, D. and Tanuma, S. (2001) Characterization of human DNase I family endonucleases and activation of DNase γ during apoptosis. Biochemistry **40**, 143–152

10 Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H. G. and Moroy, T. (2000) Features of systemic lupus erythematosus in Dnase1-deficient mice. Nat. Genet. **25**, 177–181

11 Yakovlev, A. G., Wang, G., Stoica, B. A., Simbulan-Rosenthal, C. M., Yoshihara, K. and Smulson, M. E. (1999) Role of DNAS1L3 in Ca^{2+}- and Mg^{2+}-dependent cleavage of DNA into oligonucleosomal and high molecular mass fragments. Nucleic Acids Res. **27**, 1999–2005

12 Boulares, A. H., Zoltoski, A. J., Contreras, F. J., Yakovlev, A. G., Yoshihara, K. and Smulson, M. E. (2002) Regulation of DNAS1L3 endonuclease activity by poly(ADP-ribosyl)ation during etoposide-induced apoptosis: role of PARP-1 cleavage in endonuclease activation. J. Biol. Chem. **277**, 372–378

13 Liu, Q. Y., Ribecco, M., Pandey, S., Walker, P. R. and Sikorska, M. (1999) Apoptosis-related functional features of the DNaseI-like family of nucleases. Ann. N.Y. Acad. Sci. **887**, 60–76

14 Arends, M. J., Morris, R. G. and Wyllie, A. H. (1990) Apoptosis. The role of the endonuclease. Am. J. Pathol. **136**, 593–608

15 Shiokawa, D., Kobayashi, T. and Tanuma, S. (2002) Involvement of DNase γ in apoptosis associated with myogenic differentiation of C2C12 cells. J. Biol. Chem. **277**, 31031–31037

16 Shiokawa, D. and Tanuma, S. (1999) DLAD, a novel mammalian divalent cation-independent endonuclease with homology to DNase II. Nucleic Acids Res. **27**, 4083–4089

17 Fimia, G. M., Gottifredi, V., Passananti, C. and Maione, R. (1996) Double-stranded internucleosomal cleavage of apoptotic DNA is dependent on the degree of differentiation in muscle cells. J. Biol. Chem. **271**, 15575–15579

18 Nishikawa, A., Gregory, W., Frenz, J., Cacia, J. and Kornfeld, S. (1997) The phosphorylation of bovine DNase I Asn-linked oligosaccharides is dependent on specific lysine and arginine residues. J. Biol. Chem. **272**, 19408–19412

19 Robbins, J., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell **64**, 615–623

20 Chelsky, D., Ralph, R. and Jonak, G. (1989) Sequence requirements for synthetic peptide-mediated translocation to the nucleus. Mol. Cell. Biol. **9**, 2487–2492

21 Prunell, A., Kornberg, R. D., Lutter, L., Klug, A., Levitt, M. and Crick, F. H. (1979) Periodicity of deoxyribonuclease I digestion of chromatin. Science **204**, 855–858

22 Shiokawa, D., Tanaka, M., Kimura, T., Hashizume, K., Takasawa, R., Ohyama, H., Fujita, K., Yamada, T. and Tanuma, S. (2000) Characterization of two DNase γ-specific monoclonal antibodies and the in situ detection of DNase γ in the nuclei of apoptotic rat thymocytes. Biochem. Biophys. Res. Commun. **275**, 343–349

23 Nigg, E. A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature (London) **386**, 779–787

Received 3 June 2003/30 June 2003; accepted 28 August 2003
Published as BJ Immediate Publication 28 August 2003, DOI 10.1042/BJ20030820