Involvement of DNase γ in Apoptosis Associated with Myogenic Differentiation of C2C12 Cells*

Daisuke Shiokawa¹, Takanobu Kobayashi¹, and Sei-ichi Tanuma¹, §, ¶

From the ¹Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 12 Funagawara-machi, Ichigaya, Shinjuku-ku, Tokyo 162-0826, Japan, and the § Genome and Drug Research Center, Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan

Running title: DNase γ is indispensable for DNA fragmentation in myoblast apoptosis

*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.

¶To whom correspondence should be addressed: Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 12 Funagawara-machi, Ichigaya, Shinjuku-ku, Tokyo 162-0826, Japan. Tel: +81-3-3260-6725, Fax: +81-3-3268-3045, e-mail: tanuma@ps.kagu.sut.ac.jp
The Abbreviations used are: GFP, green fluorescent protein; GM, growth medium; DM, differentiation medium; FITC, fluorescein isothiocyanate; DPBS; Dulbecco’s modified phosphate-buffered saline; STS, staurosporine; mAb, monoclonal antibody.
SUMMARY

Nucleosomal DNA fragmentation is detected in myoblasts only when apoptosis is induced under differentiating conditions. However, the molecular mechanisms and the DNase responsible for the differentiation-dependent apoptotic DNA laddering are poorly understood. Here we show that a Ca^{2+}/Mg^{2+}-dependent endonuclease, DNase $\gamma$, is induced in C2C12 myoblasts during myogenic differentiation and catalyzes apoptotic DNA fragmentation in differentiating myoblasts. A Ca^{2+}/Mg^{2+}-dependent, Zn^{2+}-sensitive endonuclease activity appears in C2C12 myoblasts during myogenic differentiation. The enzymatic properties of the inducible DNase were found to be quite similar to those of DNase I-family DNases. RT-PCR analysis revealed that the induction of DNase $\gamma$, a member of the DNase I-family DNases, is correlated with the appearance of inducible DNase activity. The induction of DNase $\gamma$ occurs simultaneously with myogenin induction, but precedes the up-regulation of p21. A high level of DNase $\gamma$ expression was also detected in differentiated myotubes, but not in skeletal muscle fibers in which DNase X is highly expressed. The role of DNase $\gamma$ in myoblast apoptosis was evaluated in the following experiments. Proliferating myoblasts acquire DNA ladder producing ability by the ectopic expression of DNase $\gamma$, but not DNase X, suggesting that the expression level of DNase $\gamma$ is the determinant of the differentiation-dependent apoptotic DNA laddering observed in myoblasts. DNA fragmentation during differentiation-induced apoptosis is strongly suppressed by the antisense-mediated down-regulation of DNase $\gamma$. Importantly, the extent of DNA laddering is well correlated with the level of endogenous DNase $\gamma$ activity. Our data demonstrate that DNase $\gamma$ is the endonuclease responsible for DNA fragmentation in apoptosis associated with myogenic differentiation.
INTRODUCTION

In developing vertebrates, the process of skeletal muscle formation proceeds through several distinct stages. Muscle precursor cells give rise to myoblasts that subsequently withdraw from the cell cycle, express muscle-specific genes, and fuse into multinucleated myotubes (1-3). It has been noted that skeletal muscle development is accompanied by the death of muscle cells via a physiological process recently referred to as programmed cell death, or apoptosis (3-5).

Apoptosis is a cellular suicidal program by which damaged or no longer needed cells are individually eliminated to maintain healthy homeostasis in multicellular organisms (6-8). Thus, apoptosis during muscle development is considered to be essential for normal skeletal muscle development by eliminating cells suffering trouble or undergoing damage during differentiation.

Apoptosis is evidenced by its specific morphologies, such as loss of cell volume and chromatin condensation, and biochemical markers, including the externalization of membrane phosphatidylyserine, the cleavage of death substrates by activated caspases, and the degradation of genomic DNA into nucleosomal fragments (8-11). The occurrence of the nucleosomal DNA fragmentation has long been recognized as an important feature of apoptosis, and the ladder configuration visualized in agarose gel electrophoresis is frequently used as reliable evidence of apoptosis (12).

Murine C2C12 myoblasts have been widely used as a model system in which to study apoptosis in developing muscle, because extensive cell death occurs during myogenic differentiation (13, 14). Interestingly, the patterns of DNA degradation in C2C12 apoptosis differ depending on the degree of differentiation; nucleosomal ladder formation is clearly detected in cells when apoptosis is induced under differentiating conditions, however, apoptosis under proliferating conditions produces no such DNA laddering (15, 16). Furthermore, C3H-10T1/2 cells, which are unable to degrade genomic DNA into nucleosomal fragments, acquire ladder producing ability when myogenic differentiation is induced by the forced expression of MyoD (15). The elucidation of the mechanism of
differentiation-dependent DNA fragmentation is important for understanding apoptosis in developing muscles; however, the molecular basis for the interesting observations is as yet not well understood.

To date, several mammalian DNases, such as DNase I, DNase X, DNase γ, DNase II, CAD/DFF40, and endonuclease G, have been suggested to be responsible for apoptotic DNA fragmentation (17-26). Among these candidates, the involvement of CAD in some apoptotic conditions, such as Fas-induced apoptosis in Jurcat cells and dexametasone-induced thymic apoptosis, is well documented (27, 28). However, it remains still controversial whether the apoptotic DNase is always the same or differs depending on cell type, differentiation stage, and/or apoptotic stimulus.

It has long been suggested that apoptotic DNA laddering is produced by a nuclear Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease (27, 29), and we identified a novel 33 kDa Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease, named DNase γ, in the nuclei of apoptotic rat thymocytes (30). DNase γ, also known as DNAS1L3 (31), is a member of the mammalian DNase I family of DNases. It shows high activity under neutral pH conditions and is strongly inhibited by certain metal ions, such as Zn\(^{2+}\) and Ni\(^{2+}\) (25). A previous study, performed using a DNase γ-green fluorescent protein (GFP) fusion protein, revealed that DNase γ is stored within the inner space of the nuclear envelope. When cells are induced to undergo apoptosis, DNase γ translocates into the nucleus and hydrolyzes genomic DNA into nucleosomal fragments (25). Thus, DNase γ is shown to have the ability to produce apoptotic DNA fragmentation; however, the apoptotic situations under which DNase γ is indispensable for DNA laddering have yet to be identified.

In this study, we have found that DNase γ is newly synthesized via transcriptional activation in C2C12 cells during myogenic differentiation, and that interference in DNase γ induction by the expression of its antisense RNA markedly suppresses apoptotic DNA fragmentation in differentiating myoblasts. Our results provide the first direct evidence for the involvement of DNase γ in the apoptosis of mammalian cells, and provides a clear explanation for the differentiation-dependent DNA laddering of apoptosis in C2C12.
myoblasts.

MATERIALS AND METHODS

Cell culture. Mouse C2C12 myoblasts were cultured in growth medium (GM) [Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum (FCS)]. To induce myogenic differentiation, subconfluent cultures were shifted to differentiation medium (DM) (DMEM supplemented with 2% horse serum). Apoptosis of proliferating C2C12 cells was induced by the addition of staurosporine (STS) at a concentration of 0.5 µM.

Microscopic analysis. Cells grown on a sterile collagen-coated cover slips or fronting dead cells were stained with FITC-conjugated annexin V using an Annexin V-FITC Apoptosis Detection kit (MBL) according to the manufacturer’s protocol. The resulting cells were then fixed in Dulbecco’s modified phosphate-buffered saline (DPBS) containing 2% paraformaldehyde, stained with 1 mM Hoechst 33258 in DPBS, and observed by fluorescence microscopy.

Detection of apoptotic DNA fragmentation. Equal numbers of cells were seeded in 35 mm diameter dishes, and apoptosis was induced by shifting the cells to DM or STS-treatment as described above. At the indicated times after the induction of apoptosis, both attached and detached cells were harvested and DNA fragmentation was analyzed as described previously with some modifications (30). In brief, the resulting cells were collected and washed twice with DPBS, lysed in lysis buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium N-lauroylsarcosinate, 0.5 mg/ml proteinase K], and incubated overnight at 50 °C. Then 0.5 mg/ml RNase A was added and the lysates were and incubated for additional 30 min. The same aliquots of the resulting solutions were then subjected to 1.8% agarose gel
electrophoresis. The DNA was visualized by UV illumination after Cyber Gold staining (Molecular Probes).

RT-PCR analysis. RT-PCR analysis was performed using total RNA extracted from the indicated cells or adult skeletal muscle (C57/black 6 mouse) as described previously (25). PCR amplification was carried out in 30 cycles, each cycle consisting of denaturation at 94 °C for 30 sec, annealing at 62 °C for 30 sec, and extension at 72 °C for 1 min. The primers used were: DNase I (551 bp), (sense) 5’-ATCCAAGAGGTCAGAGACTCC-3’ and (antisense) 5’-TCAGACACCAGAGTGCAAGAC-3’; DNase X (396 bp), (sense) 5’-GATATCATGGTGCTTCAGGAG-3’ and (antisense) 5’-TGCATTGAAGTCTCCAAGCAG-3’; DNase γ (330 bp), (sense) 5’-CACGTACAAAGAGCAGTATGC-3’ and (antisense) 5’-CGAATGTTCCTGCCAGGCGCTTC-3’; DNAS1L2 (431 bp), (sense) 5’-AGATGTAGCGTCAGTGAGTGAGAC-3’ and (antisense) 5’-AGTTGTGAACTGAGGCTGAGTGGG-3’; myogenin (504 bp), (sense) 5’-GCGGACTGAGCTCAGCTTAAG-3’ and (antisense) 5’-CGGGACTGAGCTCAGCTTAAG-3’; p21 (358 bp), (sense) 5’-TGTCCAATCTGGTGATGTCC-3’ and (antisense) 5’-TGTCCAATCTGGTGATGTCC-3’; and ICAD (772 bp), (sense) 5’-GGCCATTGATAAGTCCCTGAC-3’ and (antisense) 5’-CCTGAGTGAATGCAAGGCTCGTCTG-3’.

Assay of DNase activity. Subconfluent cultures in 35 mm diameter dishes were shifted to DM and cultured for the indicated numbers of days. An equal number of cells grown in GM (up to 40% confluent in 60 mm diameter dishes) were used as a control (GM). Cells were collected, washed twice with DPBS, and lysed in 100 µl of extraction buffer [20 mM Tris-HCl (pH 7.8), 1 mM 2-mercaptoethanol, 300 mM NaCl, 3 mM MgCl2, 0.5% Triton X-100, and 1 protease inhibitor mix (Boelinger)], and kept on ice for 30 min. After removing the
debris by centrifugation at 10000 \text{ g} for 10 min, the supernatants were collected and used as cell extracts. The DNase activity of each extract was assayed by the plasmid assay as described previously (25). In brief, 1 \mu l of total cell extract was added to 19 \mu l of reaction buffer [50 mM Mops-NaOH (pH 7.2), 1 mM 2-mercaptoethanol, and 500 ng supercoiled pBluescriptIIKS+] containing the indicated divalent cations. The mixtures were then incubated for 30 min at 37\text{\degree}C, and equal aliquots were subjected to 1% agarose gel electrophoresis. Alternatively, the activity of DNase \gamma in C2C12 cells was analyzed by activity gel assay as described previously (32). DNase \gamma, purified from mouse spleen as described previously (33), was used as a positive control. The in-gel reaction was performed in reaction buffer [10 mM Tris-HCl (pH 7.8), 1 mM 2-mercaptoethanol, 3 mM CaCl\textsubscript{2}, 3 mM MgCl\textsubscript{2}] overnight at 50 \text{ C}.

Ectopic expression of DNase \gamma. C2C12 cells (2 \times 10^5) grown in GM were transfected with 1 \mu g of expression vector using a Superfect transfection reagent (Qiagen) according to the manufacturer’s protocol. At 24 h post transfection, the cultures were placed in fresh medium and subjected experiments. Cells co-transfected with pEGFP-C1 (Clontech) and phDNase \gamma-Myc-His or phDNase X-Myc-His (25) were fixed with 2% paraformaldehyde, stained with 1 mM Hoechst33258, and observed under fluorescence microscopy. Cells transfected with pcDNA-myc-his C (Invtrogen), phDNase \gamma-Myc-His or phDNase X-Myc-His were cultured for an additional 24 h in the presence or absence of 0.5 \mu M STS. DNA fragmentation in the resulting cells was analyzed as described above. The expressions of the DNase \gamma-Myc-His and DNase X-Myc-His proteins were detected by western blot using an anti-Myc antibody (Invtrogen) as described previously (25).

Indirect immunofluorescence analysis. Activated DNase \gamma located in apoptotic nuclei was detected \textit{in situ} using hg 302 anti-DNase \gamma mAb (34). Proliferating C2C12 cells, grown on collagen-coated coverslips, were cultured in DM for 2 days or treated with 0.5 \mu M STS for 24 h. The resulting cells were fixed with 3.5% formaldehyde, soaked in ethanol, and, after
blocking with DPBS containing 5% FCS, the resulting cells were incubated with hg302 (2µg/ml) at room temperature for 1h in a humid sealed chamber. After incubation, the cells were washed with DPBS and incubated with FITC-conjugated-anti mouse IgG (1:300 dilution, Vector Laboratory) under the same conditions. After washing with DPBS, the morphology of the cells and fluorescence of FITC were observed under fluorescence microscopy (Olympus).

Antisense RNA-mediated suppression of DNase γ. A cDNA fragment containing the entire open reading frame of mouse DNase γ was excised from pmDNase γ-Myc-His vector (34) by Xho I digestion. The fragment was then subcloned into a mammalian expression vector, pCAG/puro, a kind gift from Dr. D. Kitamura, in the reverse orientation and an antisense RNA expression vector for DNase γ (pCAG-mDNase γAS) was obtained. Proliferating C2C12 cells were transfected with pCAG-mDNase γAS as described above, and the cells stably integrated by the transgene were selected by puromycin resistance (10 µg/ml). The resulting cells were cloned by two cycles of colony isolation, and several independent clones of C2C12-DNase γ antisense cells were obtained. Subcultures of the clones were differentiated in DM for 3 days, and subjected to activity gel assay to select clones whose DNase γ activity was efficiently suppressed. As a result, we established two independent DNase γ-knockdown cells, C2C12/γAS1.34 and C2C12/γAS2.12. A mock transfectant, C2C12/puro cells, was obtained by the same method, except for the selection by DNase γ activity, and used in control experiments.

RESULTS

Apoptosis in C2C12 cells under differentiating and proliferating conditions. Myogenic differentiation is induced in C2C12 cells by shifting the culture to low mitogen conditions. Two days after the induction of differentiation, both differentiating and apoptotic cells could be seen in the same field (Fig. 1A, center panels). Floating dead cells were most
frequently observed at this time. After 4 days, the appearance of newly dead cells was only slight, and most living myoblasts were fused to multinucleated myotubes (Fig. 1A, right panels). The morphological features of apoptosis that occur naturally during myogenic differentiation were found to be almost the same as those induced by staurosporine (STS) treatment under proliferating conditions (Fig. 1B). In both cases, a loss of cell volume and chromatin condensation could be observed (Fig. 1A, center panels, and B). The positive staining with fluorescein isothiocyanate (FITC)-annexin V indicates the externalization of membrane phosphatidylserine, a biochemical marker of apoptosis (Fig. 1B, lower panels). Thus, apoptotic cells induced under two distinct conditions are shown to be morphologically indistinguishable. However, their patterns of DNA degradation are markedly different. As shown in Fig. 2, nucleosomal DNA fragmentation was observed only in differentiation-induced apoptosis (Fig. 2A), but not in that induced by STS treatment (Fig. 2B).

These results strongly suggest that part of the death mechanism regulating the production of DNA fragmentation must differ depending on the differentiation state of C2C12 cells. In this study, our goal was to identify the DNase responsible for apoptotic DNA fragmentation in C2C12 cells, and to clarify the reason that DNA laddering could be seen in myoblasts only under differentiating conditions.

**Induction of a Ca\(^{2+}\)/Mg\(^{2+}\)-dependent DNase activity in C2C12 cells during myogenic differentiation.** In order to examine whether some DNase might be newly synthesized or activated during differentiation, we analyzed cellular DNase activities in proliferating and differentiating C2C12 cells. Total cell extracts were prepared from cells cultured in GM (Fig. 3A, left panel) or differentiated in DM for 3 days (Fig. 3A, right panel), and their DNase activities were assayed using supercoiled plasmid DNA as a substrate. Little difference could be detected between their DNase activities in the absence or presence of Ca\(^{2+}\) or Mg\(^{2+}\) alone. However, strong DNase activity was observed in differentiating, but not in proliferating C2C12 cells in the presence of both Ca\(^{2+}\) and Mg\(^{2+}\) (Fig. 3A).
maximum activity was observed under neutral pH conditions (data not shown), and the 
activity was strongly inhibited by the addition of Zn\textsuperscript{2+} ion (Fig. 3A). Up to 100 µg/ml of 
G-actin, a specific inhibitor of DNase I, had no inhibitory effect on the DNase activity 
(data not shown).

Changes in the DNase activity during myogenic differentiation are shown in Fig. 3B. The 
results indicate that the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent DNase activity is induced early in myogenic differentiation and remains high in differentiated myotubes.

**Induction of DNase \(\gamma\) during myogenic differentiation in C2C12 cells.** To date, four 
distinct mammalian genes encoding DNase I-like DNase, DNase I, DNase X, DNase \(\gamma\), 
and DNAS1L2 have been identified (31, 35-39). Although the DNase I family genes are 
expressed with distinct tissue specificities, their coding proteins are shown to have similar 
Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent, Zn\textsuperscript{2+} sensitive DNase activities (25). On the basis of their properties, 
we assumed that one of them is likely to be responsible for the inducible DNase activity. 
Thus, we performed RT-PCR to examine changes in their expression during myogenic 
differentiation. The mRNA for DNase X was strongly detected in terminally-
differentiated adult skeletal muscle. The expression of DNase X was also observed in 
C2C12 cells, however, the level was quite low compared with that observed in skeletal 
muscle, and remain unchanged during differentiation (Fig. 4A). In contrast to DNase X, no 
signs of DNase \(\gamma\) expression could be detected in adult skeletal muscle or proliferating 
C2C12 cells, however, DNase \(\gamma\) expression was induced in C2C12 cells during myogenic 
differentiation. A time course study revealed that the induction of DNase \(\gamma\) occurs almost simultaneously with myogenin induction, but preceding the up-regulation of p21 (Fig. 
4A). No mRNAs for DNase I or DNAS1L2 could be detected in C2C12 cells throughout 
the time course of differentiation (data not shown). We also investigated the expression of 
a Mg\textsuperscript{2+}-dependent endonuclease, CAD, and its inhibitor, ICAD. The expression of CAD 
could not be detected in C2C12 cells at any time during differentiation or in terminally 
differentiated adult skeletal muscle (data not shown), suggesting that the differentiation-
dependent DNA laddering seen in C2C12 apoptosis can not be explained by the CAD/ICAD system. In contrast, ICAD is ubiquitously expressed in all cells or tissues tested, and the level was unchanged during the differentiation of C2C12 cells. Thus, the cDNAs generated in the indicated cells or tissues were normalized for ICAD and used for RT-PCR.

The induction of DNase \( \gamma \) was also confirmed by activity gel assay (Fig. 4B). Dark bands representing DNase \( \gamma \) activity appeared to correlate well with the expression of the DNase \( \gamma \) mRNA. On the basis of these results, DNase \( \gamma \) is shown to be induced in C2C12 cells during myogenic differentiation via the transcriptional activation of its coding gene. This finding provides a good explanation for the appearance of a \( \text{Ca}^{2+}/\text{Mg}^{2+} \)-dependent, \( \text{Zn}^{2+} \)-sensitive DNase activity in differentiated C2C12 cells. Furthermore, these results suggest the involvement of DNase \( \gamma \) in apoptosis associated with the differentiation of C2C12 cells.

**Proliferating C2C12 cells acquire DNA ladder producing activity by the ectopic expression of DNase \( \gamma \).** To evaluate the physiological consequence of DNase \( \gamma \) induction, we examined whether proliferating C2C12 cells acquire ladder producing ability upon the ectopic expression of DNase \( \gamma \). The transient expression of DNase \( \gamma \) causes no apoptotic phenotypes, such as cell shrinkage or nuclear condensation, in transfected (GFP positive) cells (Fig. 5A and B), indicating that DNase \( \gamma \) expression itself causes no toxic or apoptosis inducing effects in proliferating myoblasts. We next examined the effects of DNase \( \gamma \) expression under apoptotic conditions. Cultures individually transfected with DNase \( \gamma \) or DNase X expression vectors, or an empty vector, were maintained in the presence or absence of 0.5 \( \mu \text{M} \) STS. After 24 hours of continuous treatment, genomic DNA was extracted from each culture and analyzed by agarose gel electrophoresis. As shown in Fig. 5C, no spontaneous DNA fragmentation could be observed in cells cultured without STS. However, STS-treatment caused extensive DNA fragmentation only in cells transfected with DNase \( \gamma \). Western blot confirmed the same expression levels of DNase \( \gamma \) and DNase X in each transfectant (Fig. 5D). These results clearly indicate that the ectopic expression
of DNase \( \gamma \) enables proliferating C2C12 cells to undergo DNA ladder formation, and suggest that the expression of DNase \( \gamma \) is the cause of the differentiation-dependent DNA fragmentation observed in C2C12 apoptosis. Importantly, little DNA fragmentation was produced by STS-treatment in cells expressing exogenous DNase X. This indicates that DNase \( \gamma \)-mediated DNA fragmentation is not a nonspecific result caused by the expression of an active DNase, but reflects a feature specific for DNase \( \gamma \).

**Nuclear localization of DNase \( \gamma \) in apoptotic myoblasts under differentiating conditions.**

In a previous study, the nuclear translocation of DNase \( \gamma \) was suggested to be an important process for its apoptotic function (25). To examine whether DNase \( \gamma \) is located in the nuclei of apoptotic myoblasts, we performed indirect immunofluorescence analysis for DNase \( \gamma \). For this purpose, we used an anti-DNase \( \gamma \) monoclonal antibody (mAb), hg302, which has been shown to recognize activated DNase \( \gamma \) located within apoptotic nuclei (34).

Fig. 6A and B show the results of the *in situ* detection performed on differentiating C2C12 cells cultured in DM for 2 days. Positive stainings for DNase \( \gamma \) (white arrows) were observed in the nuclei of apoptotic, but not living, myoblasts. In contrast, nuclear staining by hg302 mAb could scarcely be detected when apoptosis was induced by STS under proliferating conditions (Fig. 6C and D). Although a small fraction of apoptotic nuclei was stained by the mAb (Fig. 6B and B, white arrow), this is considered to reflect spontaneously differentiated cells included in the proliferating culture. These results are consistent with the expression profile of DNase \( \gamma \) mRNA in C2C12 cells, and indicate that the nuclear localization of activated DNase \( \gamma \) is correlated with the occurrence of nucleosomal DNA fragmentation in C2C12 cells (Fig. 2).

**Suppression of apoptotic DNA fragmentation by the antisense RNA-mediated down-regulation of DNase \( \gamma \).** To obtain more direct evidence for the involvement of DNase \( \gamma \) in C2C12 apoptosis, we established DNase \( \gamma \) knockdown cells by the stable expression of its antisense RNA, and analyzed their phenotypes during differentiation-induced apoptosis.
Cells stably transfected with an antisense vector for DNase γ were screened for puromycin resistance, and further selected by activity gel assay to obtain cells with effectively suppressed DNase γ induction. As a consequence, we obtained two independent clones, C2C12/γAS1.34 and C2C12/γAS2.12 cells, with low and almost no DNase γ activity, respectively.

Activity gel analysis shows the DNase γ activity in each transfectant cultured in DM for 3 days (Fig. 7A). DNase γ activity in the mock transfectant (C2C12/puro) was detected normally at the same level as the parental cells (C2C12). In contrast, DNase γ activity in the antisense-expressed cells appeared to be effectively suppressed as compared with control cells. In clone 2.12, no DNase γ activity was detected, while a slight dark band could be seen in the lane loaded with clone 1.34. The normal induction of myogenin in these transfectants as shown by RT-PCR (Fig. 7B), indicates that the down-regulation of DNase γ is not due to defects in their ability to differentiate that arose artificially during the selection process.

Using these transfectants, we examined whether the DNA fragmentation in differentiation-induced apoptosis could be abolished by the down-regulation of DNase γ. Apoptotic DNA fragmentation was observed normally in C2C12/puro cells during myogenic differentiation (Fig. 7C, left panel). In contrast, apoptotic ladder formation in the antisense cells was found to be strongly suppressed. Although slight ladder formation was detected in clone 1.34 (Fig. 7C, middle panel), almost no DNA fragmentation could be seen in clone 2.12 during differentiation (Fig. 7C, right panel). Importantly, the magnitudes of DNA fragmentation in the two antisense-expressed cells correlated well with their DNase γ activities as detected in the activity gel assay. On the basis of these results, DNase γ is shown to be indispensable for the apoptotic ladder formation in C2C12 cells during myogenic differentiation.

**DISCUSSION**
DNase γ/DNAS1L3 has been suggested to play a role in apoptosis as an executioner of nucleosomal DNA fragmentation. Purified DNase γ attacks at the linker regions of chromosomal DNA, so that the resulting DNA fragments display the same nucleosomal ladder configuration in agarose gels as that produced during apoptosis (30). Furthermore, DNase γ exogenously expressed in mammalian cells is activated by apoptotic stimuli and produces DNA ladder formation (25, 26, 32, 40). Thus, DNase γ has been demonstrated to be capable of producing apoptotic DNA laddering both in vitro and at the cellular level, however, the apoptotic conditions under which DNA fragmentation is actually catalyzed by endogenous DNase γ have yet to be defined.

It is known that a large fraction of cells die by apoptosis during skeletal muscle development both in vivo and in vitro (3-5). Although the reason extensive cell death occurs during myogenesis is not yet fully understood, one major cause is thought to be the failure of differentiating myoblasts to establish the postmitotic state (14). Once proliferating myoblasts are committed to enter the differentiation program, cells irreversibly exit from the cell cycle and stabilize their postmitotic state depending on the up-regulation of a cyclin-dependent kinase inhibitor p21^{CIP1/WAF1} and the resulting hypophosphorylation of retinoblastoma protein Rb (3, 14).

A possible molecular sequence for myogenic differentiation and apoptosis, as suggested by recent experimental progress and the results of this study, is as follows. Differentiating myoblasts expressing both myogenin and p21 acquire an apoptosis-resistant phenotype, and cells positive in myogenin but that fail to induce p21 must be eliminated by apoptosis using pre-existing DNase γ in the cells. It is of note that the induction of DNase γ occurs in parallel with myogenin induction (Fig. 4). This indicates that the preparation for apoptosis and the commitment to differentiation are coordinated in myoblasts.

Myoblasts that escaped apoptosis continue to differentiate and eventually fuse into myotubes that retain the apoptosis-resistance. As mentioned above, one critical factor for this phenotype is the stabilization of the post mitotic state by p21 and Rb. In addition, recent studies have revealed the significance of a serine-threonine kinase, Akt, in the
promotion of myocyte survival (13, 41, 42). During myogenic differentiation, cell cycle withdrawal promotes the induction of Akt in C2C12 myoblasts. The over-expression of Akt protects myoblasts from apoptosis during myogenesis and the opposite results are obtained in its dominant negative mutant, indicating that Akt is a negative regulator of myoblast apoptosis (13). Furthermore, insulin and insulin-like growth factors, IGF I and IGF II, have been shown to have an anti-apoptotic effect on myogenic cells by activating Akt through a phosphatidyl inositol 3-kinase (PI3-kinase)-dependent pathway (41, 43). Akt has been demonstrated to phosphorylate some pro-apoptotic proteins, such as Bad, a member of Bcl-2 family, to promote the release of cytochrome c from mitochondria (44, 45). Importantly, the pro-apoptotic activity of Bad is inactivated by its phosphorylation, because the phosphorylated form has a reduced ability to interact with Bel-xL (46). Thus, one important step in the mitochondrial apoptosis pathway is suppressed in differentiated myotubes; however, previous studies have revealed that apoptosis with oligonucleosomal DNA fragmentation is still inducible in such myotubes when the cells are exposed to toxic stimuli (16). Although the way in which myotubes overcome Akt-mediated inhibition is presently unclear, DNase γ expressed in differentiated myotubes may provide an alternative apoptosis pathway by which myotubes undergo apoptosis if they suffer some unexpected trouble or damage during the maturation process into myofibers.

The stage specific expression patterns of DNase γ and DNase X are also interesting. In adult animals, the major DNase detected in skeletal muscle is DNase X, whereas no expression of DNase γ is observed in such myogenic tissues. Thus, the finding that DNase γ is induced in C2C12 myoblasts during myogenesis is an unexpected observation. In contrast to these DNases, no expression of DNase I or DNAS1L2 could be detected in C2C12 cells at any point in the time course of myogenic differentiation. This is in agreement with the following observations that the DNase activity induced in C2C12 cells is insensitive to G-actin, a specific inhibitor of DNase I, and that a high activity of inducible DNase is detected at neutral pH, whereas DNAS1L2 is observed under acidic conditions (25).
Recent studies have revealed that the expressions of muscle-specific genes are regulated in large part by a family of muscle specific transcriptional factors, MyoD, Myf-5, myogenin, and MRF4/Myf-6, which share a common dimerization domain and the basic helix-loop-helix DNA binding motif (47, 48). At present, whether the myogenic expression of the DNase γ and DNase X genes is controlled by such transcriptional factors is unknown, however, elucidation of their regulatory mechanisms will provide important information for a better understanding of transcriptional control in the late phase of myogenesis.

In this report, we provide convincing evidence for the involvement of DNase γ in apoptosis associated with the myogenic differentiation of C2C12 myoblasts. Furthermore, our results strongly suggest that the expression level of DNase γ is the determinant for the differentiation-dependent ladder formation previously reported in myoblast apoptosis. In adult animals, a high expression of DNase γ mRNA is observed mainly in lymphoid tissues such as spleen, lymph node, thymus, and bone marrow (25, 32), suggesting that the apoptosis pathway(s) leading to the activation of DNase γ is also present in such non-myogenic cells. As mentioned in the introduction, the identification of the responsible DNase in each apoptotic case remains controversial and more studies are required to provide the answers. However, such information will provide important clues for understanding cell type-dependent apoptosis pathways and the physiological significance of DNA fragmentation in apoptosis.

ACKNOWLEDGMENTS

We thank Dr. Daisuke Kitamura for the kind gift of pCAG/puro vector and Dr. Tsutomu Aoki for helpful discussions.
REFERENCES


LEGENDS TO FIGURES

FIG. 1. Apoptosis of C2C12 cells occurring naturally during myogenic differentiation or induced by STS treatment under proliferating conditions. Cultures maintained in growth medium (GM) were shifted to differentiation medium (DM) for the indicated number of days, or treated with 0.5 µM STS for 24 h. The resulting cells, either attached to the cover slips (A) or detached from the culture dishes (B), were stained with FITC-annexin V and Hoechst dye as described under MATERIALS AND METHODS. Differential interference contrast microscopy (DIC) revealed their morphological changes (upper panels). Nuclear condensation and externalization of phosphatidylserine were detected by Hoechst (middle panels) and FITC-annexin V (lower panels) staining, respectively. Original magnification 200 (A) or 400 (B).

FIG. 2. Patterns of DNA fragmentation during differentiation- or STS-induced apoptosis in C2C12 cells. Proliferating cells were shifted to DM and cultured for 0~4 days (A), or treated with 0.5 µM STS for 0~24 h (B). The resulting cells were harvested at the indicated time and genomic DNA extracted from the cells was analyzed by 1.8% agarose gel electrophoresis as described under MATERIALS AND METHODS.

FIG. 3. Induction of a Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent DNase activity during myogenic differentiation of C2C12 cells. (A) Total cell extracts were prepared from C2C12 cells grown in GM (left panel) or differentiated in DM for 3 days (right panel), and characterized for their DNase activities as described under MATERIALS AND METHODS. Assays were performed using supercoiled plasmid DNA as a substrate in the absence or presence of the indicated divalent cations. (B) Kinetic analysis of the induction of Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent DNase activity during myogenic differentiation. DNase activity was assayed by plasmid assay as described under MATERIALS AND METHODS. Lanes (from the left): GM, C2C12 cells grown in GM; DM 0.5~4, C2C12 cells cultured in DM
for 0.5~4 days. The forms of plasmid DNA are indicated to the right of the panels; open coil (a), linear (b), supercoil (c), and degraded fragments (d).

FIG. 4. Induction of DNase γ gene expression during myogenic differentiation of C2C12 cells. (A) Changes in DNase X and DNase γ gene expression during differentiation. RT-PCR analyses were performed on differentiating C2C12 cells as described under MATERIALS AND METHODS. cDNAs generated from the indicated cells or tissue were normalized for ICAD expression. Results of myogenin and p21 expression are given as markers for myogenic differentiation. Lanes (from the left): GM, C2C12 cells grown in GM; DM 0.5~4, C2C12 cells differentiated in DM for 0.5~4 days; SM, adult skeletal muscle. (B) Changes in DNase γ activity during myogenic differentiation. C2C12 cells grown in GM or cultured in DM for 0.5~4 days were harvested and their DNase γ activities were assayed by the activity gel method as described under MATERIALS AND METHODS. Lanes (from the left): C, DNase γ purified from mouse spleen (positive control); GM, C2C12 cells grown in GM; DM 0.5~4, C2C12 cells cultured in DM for 0.5~4 days. The closed arrowhead indicates the 33 kDa bands representing DNase γ activity.

FIG. 5. Acquisition of ladder producing ability of proliferating C2C12 cells by the ectopic expression of DNase γ. (A, B) Proliferating C2C12 cells were co-transfected with expression vectors for DNase γ-Myc-His and GFP. Twenty-four hours after transfection, the cells were fixed, stained with Hoechst dye, and observed under fluorescence microscopy as described under MATERIALS AND METHODS. (C) Effects of ectopic expression of DNase γ and DNase X on STS-induced apoptosis in proliferating C2C12 cells. Cultures of proliferating C2C12 cells were individually transfected with pcDNA- myc-his C (control), phDNase γ-myc-his (DNase γ), or phDNase X-myc-his (DNase X). At 24h post transfection, the medium was replaced with fresh medium, and the cells were cultured in the presence (+) or absence (-) of 0.5 µM STS for an additional 24 h. The
resulting cells were harvested and the genomic DNA extracted from the cells was separated in 1.8% agarose gels as described under MATERIALS AND METHODS. (D) Western blot analysis for DNases exogenously expressed in proliferating C2C12 cells. C2C12 cells were transfected with pcDNA-myc-his C (control), phDNase γ-myc-his (DNase γ), or phDNase X-myc-his (DNase X) and cultured for 24 h. The Myc-His-tagged DNases were detected by western blot using an anti-Myc antibody as described under MATERIALS AND METHODS.

FIG. 6. Detection of DNase γ by indirect immunofluorescence. C2C12 cells cultured in DM for 2 days (A, B) or treated with 0.5 µM STS for 24 h under proliferating conditions (C, D) were successively stained with hg302 mAb and FITC-conjugated secondary antibody, and observed by fluorescence microscopy as described in MATERIALS AND METHODS. DIC microscopy revealed the morphologies (A and C). The fluorescence of FITC indicates the nuclear localization of DNase γ in apoptotic nuclei (B and D, white arrows). Original magnification 400.

FIG. 7. Suppression of apoptotic DNA fragmentation by the antisense RNA-mediated down-regulation of DNase γ. (A) DNase γ activities in stable transfectants expressing the antisense RNA for DNase γ. Parental cells (C2C12), mock transfectants (C2C12/puro), clone 1.34 (C2C12/γAS1.34), and clone 2.12 (C2C12/γAS2.12) were cultured in DM for 3 days, and their DNase γ activities were assayed by the activity gel method as described under MATERIALS AND METHODS. The closed arrowhead indicates the band representing DNase γ activity. (B) Myogenin expressions in transfectants during myogenic differentiation. Cells maintained in GM, or cultures in DM for 1~4 days were harvested and subjected to RT-PCR analysis to determine their myogenin expression (upper panels) as described under MATERIALS AND METHODS. The expression of ICAD was unchanged among the clones and during differentiation, and the results are given for comparison (lower panels). (C) Apoptotic DNA fragmentation in the
transfectants caused during myogenic differentiation. Cells were allowed to differentiate in DM for 1~4 days, and genomic DNA extracted from the cells was subjected to 1.8% agarose gel electrophoresis as described under MATERIALS AND METHODS.
Figure 2. Shiokawa D. et al.
Figure 3. Shiokawa D. et al.
Figure 4. Shiokawa D. et al.
Figure 5. Shiokawa D. et al.
Figure 6. Shiokawa D. et al.
Involvement of DNase gamma in apoptosis associated with myogenic differentiation of C2C12 cells
Daisuke Shiokawa, Takanobu Kobayashi and Sei-ichi Tanuma

J. Biol. Chem. published online June 5, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204038200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2002/06/05/jbc.M204038200.citation.full.html#ref-list-1