Identification of two functional nuclear localization signals in DNase \(\gamma\) and their roles in its apoptotic DNase activity

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Among DNase I family members, only DNase \(\gamma\) causes DNA fragmentation during apoptosis. However, the molecular basis for this functional feature of DNase \(\gamma\) is poorly understood. Here we describe the identification of functional NLSs (nuclear localization signals) in DNase \(\gamma\) and their roles in its apoptotic function. DNase \(\gamma\) 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 \(\gamma\). Disruption of NLS2 suppresses ladder formation more effectively than disruption of NLS1. DNase \(\gamma\) 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 \(\gamma\) 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 \(\gamma\), 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 \(\gamma\), 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 \(\gamma\)/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\(\gamma\) 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 \(\gamma\) 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 \(\gamma\) 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 \(\gamma\) 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 \(\gamma\) is shown to have an ability to produce apoptotic DNA fragmentation at the cellular level. However, the reason why DNase \(\gamma\), 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 \(\gamma\), and to clarify the molecular basis by which DNase \(\gamma\), 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'-CTCGAGCTCAGCTGCACTCAGCATCACCTCA-CTG-3' (sense) and 5'-CTCGAGACTTCAGCATCACCTCA-CTG-3' (antisense); DNase \(\gamma\), 5'-CTCGAGCTCAGCTGCACTCAGCATCACCTCA-CTG-3' (antisense); DNase \(\gamma\), 5'-CTCGAGCTCAGCTGCACTCAGCATCACCTCA-CTG-3' (antisense); DNase \(\gamma\), 5'-CTCGAGCTCAGCTGCACTCAGCATCACCTCA-CTG-3' (antisense).

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

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above. At 24 h post-transfection, the cells were fixed, the nuclei by an activity gel assay as described previously [3].

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Site-directed mutagenesis

The cDNA insert for DNase $\gamma$ was excised from pBluescript KS+ by KpnI and XhoI digestion, and the fragments were recloned into the same site of the pUC19 vector. These plasmids were used for oligonucleotide-directed mutagenesis using an ALR PCR in vitro Mutagenesis Kit (Takara) according to the manufacturer's protocol. Residues Arg60 and Arg75/Lys76 of human DNase $\gamma$ were converted into alanines using the following oligonucleotides: R60A, 5'-AGGAGAGCCATAGTACAAC-3' (sense) and 5'-TTTTCTTCCAAGCCGAGACT-3' (antisense); NLS1mut, 5'-AGCCAGGGTACATACAAC-3' (sense) and 5'-TGTCTGCCAACGCGAGACT-3' (antisense); NLS2, 5'-CAAAAGATCTGTACCTCT-3' (sense) and 5'-GAGGGTTTCCGCTTTTTTC-3' (antisense). 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 for 16 h. After 16 h of continuous treatment with staurosporine, nucleiosomal DNA fragmentation appeared in cells expressing exogenous DNase $\gamma$ (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 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 $\gamma$ (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 $\gamma$

The primary structures of the DNase I family DNases are shown schematically in Figure 2. DNase I 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 1 Apoptotic DNA fragmentation in C2C12 cells transfected with DNase $\gamma$ 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.
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.

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 DNASE1L2, 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.

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.

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Apoptosis-related domains in DNase γ


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