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DLAD, a novel mammalian divalent cation-independent endonuclease with homology to DNase II

Daisuke Shiokawa[1] and Sei-ichi Tanuma[1,2,*]

[1]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Funagawara-machi, Ichigaya, Shinjuku-ku, Tokyo 162-0826, Japan and [2]Research Institute for Bioscience, Science University of Tokyo, 2669 Yamazaki, Noda, Chiba 278-0022, Japan

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ABSTRACT

In this report, we describe the molecular cloning and characterization of DLAD, a novel mammalian deoxyribonuclease homologous to DNase II. The full length cDNA for mouse DLAD has been cloned by polymerase chain reaction. The cDNA contains a 1065 bp open reading frame (ORF) encoding a 354 amino acid protein with a calculated molecular mass of 40 767. The predicted protein for DLAD shares 34.4% identity with DNase II. DLAD is also homologous to three predicted proteins, C07B5.5, F09G8.2 and K04H4.6, from the nematode *Caenorhabditis elegans*. Furthermore, the third ORF of the fowlpox virus genome is found to encode a DLAD homologue showing 37.1% identity at the amino acid level. Northern blot analysis reveals that expression of the DLAD mRNA is highly restricted to the liver. DLAD mainly exists as a cytoplasmic protein with divalent cation-independent endonuclease activity and cleaves DNA to produce 3′-phosphoryl/5′-hydroxyl ends. It is active under a wide range of pH with maximum activity at pH 5.2. Among known DNase inhibitors tested, aurintricarboxylic acid and Zn^{2+} are found to be effective inhibitors of the DLAD activity.

INTRODUCTION

Acid DNase is one of the best characterized enzymes that catalyze DNA hydrolysis in the absence of divalent cations at acidic pH (1). Its activities are found in a wide variety of animal tissues (2,3), and, to date, DNase II has been recognized as the sole enzyme responsible for these acid DNase activities. On the basis of its ubiquitous distribution, the physiological importance of DNase II in some fundamental biological phenomena such as DNA catabolism and apoptosis has been suggested (1,4,5).

Apoptosis is a form of cell death that plays important roles under a variety of physiological circumstances in multicellular organisms (6,7). Cleavage of chromosomal DNA into nucleosomal fragments is one of the most outstanding biochemical characteristics of apoptotic cell death (8,9). At present, several divalent cation-dependent endonucleases, including DNase I, Nuc-18, DNase γ and CAD, have been identified as apoptotic DNases responsible for the DNA fragmentation (10–14). DNase II has also been proposed to be involved in some specific cases of apoptosis (4,5,15–17). Thus, the physiological importance of DNase II is now being recognized again and has become a research focus.

The enzymatic properties of DNase II isolated from different organs are very similar (1), however, their physical properties and molecular structures are quite different. For instance, porcine DNase II is a complex of non-identical subunits derived from its precursor protein, whereas DNase II from other species are largely recognized to be single polypeptides (18–21). Furthermore, the apparent molecular masses of DNase II vary from 26.5 to 45 kDa (20–23). The diversity of DNase II is also recognized in their subcellular localizations; DNase II is localyzed intracellulaly in lysosomes (23,24), however, acid DNase activities are also found in nuclear fractions (4,25).

Although the reason for the molecular diversity of DNase II remains unclear, these observations imply the existence of another acid DNase(s) related to but distinct from DNase II. Nuclear acid endonucleases, DNases α and β, have been identified and partially purified from rat thymocytes (26,27). Furthermore, a ubiquitous serine protease inhibitor has recently been shown to have an acid DNase activity (28). In this study, we attempted to identify a novel acid DNase. As a result, we isolated a cDNA for DLAD, a protein with sequence homology to DNase II. On the basis of its enzymatic properties, DLAD appears to be a novel mammalian endonuclease that catalyzes DNA degradation at acidic pH in the absence of divalent cations.

MATERIALS AND METHODS

Molecular cloning of the cDNA for DLAD

The Expressed Sequence Tags (EST) subdivision of the NCBI GenBank database was searched with the deduced amino acid sequence of human DNase II (GenBank AF060222) using the tblastn program. As a result, we identified a mouse EST clone (GenBank AI048641) coding for DLAD. Two oligonucleotide primers, sense (GSP2/mD; 5′-AATGAATATGGTGAAGCTG-

*To whom correspondence should be addressed at: Department of Biochemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Funagawara-machi, Ichigaya, Shinjuku-ku, Tokyo 162-0826, Japan. Tel: +81 3 3260 6725; Fax: +81 3 3268 3045; Email: tanuma@ps.kagu.sut.ac.jp

TGGACTGG-3′) and antisense (GSP1/mD; 5′-CCATCGTTG-TATATTAGATAGGCTGTG-3′), were generated from the sequence and used to clone the full length cDNA by rapid amplification of cDNA ends (RACE) reactions as described previously (19). In brief, adaptor-ligated cDNA was generated from C57black/6 mouse liver polyA(+) RNA using a Marathon cDNA amplification kit (Clontech). 5′ and 3′ RACE reactions were performed with the gene-specific primers GSP1/mD and GSP2/mD, respectively, and a linker primer (AP1; 5′-CCATC-CTAATACGACTCACTATAGGGC-3′). The resulting PCR products were subcloned into pBluescript KS+ (Stratagene) and the nucleotide sequences were determined on both strands by cycle sequencing using a 7-deaza Thermo Sequenase kit (Amersham) and DSQ1000L DNA sequencer (Shimadzu).

Northern blot analysis

Total RNAs (15 μg) were subjected to 1% agarose–formamide gel electrophoresis and blotted onto a Biodyne-A membrane (Paul). The blot was hybridized with a ^{32}P-labeled probe, generated by random priming of a *Xho*I fragment of pDLAD-Myc-His (see below), as described previously (13).

Construction of expression vectors

A cDNA fragment containing the DLAD open reading frame (without a stop codon) was generated by PCR and subcloned into pBluescript KS+. The primers used were 5′-**CTCGAG**-CCACCATGACAGCAAAGCCTCTAAGAACA-3′ (sense) and 5′-**CTCGAG**ACTTACAGAACCCATAACGGAGAT-3′ (antisense). *Xho*I sites flanking the coding sequences are shown in bold face. After confirming the sequence, the insert was excised by *Xho*I digestion and recloned into the *Xho*I sites of pcDNA3-Myc-His C (Invitrogen) and pEGFP-N3 (Clontech) to generate expression vectors for DLAD with C-terminal Myc and His tags (pDLAD-Myc-His) and DLAD with a C-terminal GFP fusion protein (pDLAD-GFP), respectively. An expression vector for Myc-His tagged DNase II, pDNase II-Myc-His, was generated by the same procedure. The primers used to amplify a cDNA fragment of human DNase II were 5′-**CTCGAG**-CCACCATGATCCCGCTGCTGCTGGCA-3′ (sense) and 5′-**CT-CGAG**AGACGGTTTCCAGTCTTTCACC-3′ (antisense). In the construction of the DNase II–DLAD chimeric protein expression vector (pII/DLAD), cDNA fragments for the DNase II signal peptide and DLAD without a signal peptide were generated by PCR using the primers 5′-**CTC-GAG**CCACCATGATCCCGCTGCTGCTGGCA-3′ (sense) and 5′-GCAGGTCAGGGCGCCGGC-3′ (antisense), and 5′-AGC-TAGGCGCCCTCTCATGCAGAAATGAA-3′ (sense) and 5′-**CT-CGAG**ACTTACAGAACCCATAACGGAGAT-3′ (antisense), respectively. *Hae*II sites flanking the coding sequences are underlined. The resulting fragments were ligated after *Hae*II digestion and subcloned into pBluescript KS+. After confirming the sequence, the insert was recloned into pcDNA3-Myc-His C and pII/DLAD was generated as described above.

Transfection and purification of recombinant DNases

HeLa S3 cells (5×10^6) grown in RPMI 1640 supplemented with 10% fetal calf serum, were transfected with 25 μg of pDLAD-Myc-His (or pDNase II-Myc-His) using a FuGene 6 transfection reagent (Boehringer). Cells were harvested 48 h after transfection and homogenized in 2 ml of ice-cold buffer A [10 mM Tris–HCl (pH 7.8), 3 mM $MgCl_2$, 1 mM 2-mercapto-ethanol, 0.3 mM PMSF] containing 0.1% Nonidet P-40 by10 strokes of a Teflon-glass homogenizer. The homogenate was centrifuged at 10 000 *g* for 10 min and the supernatant was collected as the cell extract. His-tagged recombinant protein was purified from the cell extract with a Ni-NTA spin column (Qiagen) according to the manufacturer's protocol. Purified DLAD (or DNase II), eluted in 300 μl of elution buffer [50 mM sodium phosphate (pH 8.0) containing 250 mM imidazole and 300 mM NaCl], was dialyzed against 20 mM Mes–NaOH (pH 5.6) containing 1 mM 2-mercaptoethanol and used in the characterization of enzymatic properties.

Western blot analysis

HeLa S3 cells (2×10^5) were transfected individually with 1 μg of pDLAD, pII/DLAD, or pDNase II as described above. The cells were cultured for 48 h and the culture medium and the cells were collected separately. The affinity-purified Myc-His-tagged proteins were subjected to 10% SDS–PAGE and transferred onto Immobilon-P membranes (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% BSA for 1 h and probed with anti-Myc antibody (Novagen). After washing with TBST, the antibody retained on the membrane was detected using alkaline phosphatase-conjugated anti-mouse IgG (Promega) and a Proto Blot western detection kit (Promega). An image of the blot was scanned with a CCD camera (Atto) and the optical densities of the bands recognized by the anti-Myc antibody were quantified by densitometry (NIH image 1.60).

Microscopic analysis of the DLAD-GFP fusion protein

HeLa S3 cells (2×10^5), grown on a coverslip, were transfected with 1 μg of pDLAD-GFP as described above. Cells were cultured for 48 h and fixed with 1% glutaraldehyde in PBS without Ca^{2+}/Mg^{2+}, PBS(–), at room temperature for 10 min. The coverslip was washed with PBS(–), and the cells were stained with 1 mM Hoechst 33258 in PBS(–). The GFP and DNA images were observed by fluorescence microscopy (Olympus).

Assay of DNase activity

Twenty microliters of reaction mixture [50 mM MES–NaOH (pH 5.2), 1 mM 2-mercaptoethanol, 1 U enzyme and 500 ng *Eco*RI-digested linear pBluescript II KS+] was prepared on ice and incubated at 45°C for 20 min. The enzyme reaction was terminated by phenol/$CHCl_3$ extraction and 5 μl aliquots of the mixtures were analyzed by 1% agarose gel electrophoresis. The gels were stained with ethidium bromide and scanned with a CCD camera (Atto) under UV transillumination, the optical densities of the intact substrate (2961 bp) were quantified by densitometry (NIH image 1.60). DNase activities were determined by reduction of the band intensities corresponding to the intact substrate. In this study, one unit of DLAD or DNase II activity is defined as the amount required to decrease the band intensity corresponding to 200 ng of intact substrate in the assay. Alternatively, DNase activities were assayed using supercoiled plasmid as a substrate to show their endonuclease activities (8; Fig. 6). In these cases, 2 U enzyme was used in the reactions to obtain clear results.

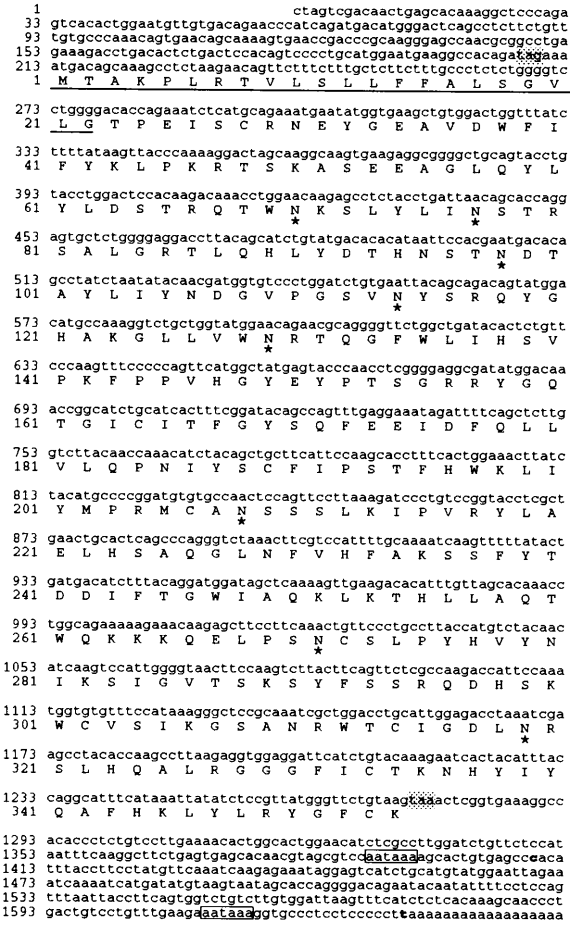

Figure 1. Nucleotide and the predicted amino acid sequences of mouse DLAD. The complete sequences of mouse DLAD cDNA and its translation product are illustrated. The predicted amino acid sequence is shown from the first ATG codon in the open reading frame. In-frame stop codons are shaded. Nucleotide and amino acid numbers are shown at the left. Putative polyadenylation signals are outlined and the polyadenylation sites (1409 and 1634) are indicated in bold letters. Potential *N*-linked glycosylation sites are marked by asterisks. A putative signal sequence is underlined. The GenBank accession number of mouse DLAD is AI128888.

Figure 2. Sequence comparison of DLAD-related proteins. Alignment of the mouse DLAD sequence with the sequences of mouse DNase II and fowlpox virus FP-CEL1. Identical and conserved residues are marked by asterisks and dots, respectively. Dashes indicate gaps introduced for better alignment. The GenBank accession numbers of mouse DNase II cDNA and fowlpox virus FP-CEL1 genome are AB013359 and AB013361, respectively.

End-labeling of DNA

The cleaved ends of the DNA digested by DLAD or DNase II were analyzed by 3′ and 5′ end-labeling as described previously with some modifications (27). Supercoiled pBluescript II KS+, digested in the assay described above, was isolated by phenol/chloroform extraction. The 3′ ends were labeled in 50 µl of a reaction mixture composed of 20 U of terminal deoxynucleotidyl transferase (Toyobo), 0.83 mCi/ml of [α-^{32}P]dCTP, 100 mM sodium cacodylate (pH 7.2), 0.2 mM DTT and 1 mM $CoCl_2$. The 5′ ends of the DNA fragments were labeled in 50 µl of reaction mixture composed of 20 U of polynucleotide kinase (Toyobo), 0.83 mCi/ml of [γ-^{32}P]ATP, 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The phosphoryl groups at the ends of the DNA chains were removed by pretreatment with 20 U of calf intestine alkaline phosphatase (Takara) in the presence of 50 mM Tris–HCl (pH 9.0) and 1 mM $MgCl_2$. Unincorporated nucleotides were removed by ethanol precipitation and the labeled DNA was subjected to 1% agarose gel electrophoresis, transferred to nylon membrane, and analyzed with a BAS 1500 image analyzer (Fuji film).

RESULTS

Isolation and characterization of a cDNA for DLAD

In an attempt to identify a novel DNase II-like mammalian DNase, we screened the GenBank expressed-sequence tag (EST) database using the deduced protein sequence of human DNase II (19) as a query. As a result, an EST clone (AI048641) encoding part of a protein with homology to DNase II was identified. On the basis of the nucleotide sequence, a full length cDNA was isolated from mouse liver cDNA by a combination of 3′ and 5′ RACE reactions (Fig. 1). During the 3′ RACE reaction, we identified two cDNA fragments that differ in their poly A sites (Fig. 1, bold faced). Consistent with this observation, two possible poly A signals, AATAAA, were found in the 3′ untranslated region at 14 and 18 nt upstream of the first (nt number 1409) and second (nt number 1634) poly A sites, respectively (Fig 1, boxed). Sequence analysis revealed an open reading frame of 1065 bp encoding 354 amino acids of a novel DNase II-like protein, sharing 37.1% amino acid identity with DNase II, with a calculated molecular mass of 40 767 (Fig. 2). On the basis of its enzymatic properties (see below), we designated this new enzyme as DLAD (DNase II-Like Acid DNase).

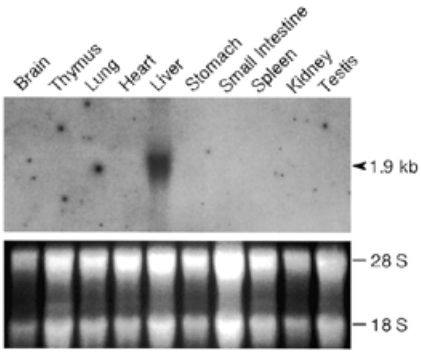

Figure 3. Tissue distribution of DLAD mRNA. DLAD mRNA expression in adult mouse tissues was analyzed by northern blot. The identities of the RNAs are indicated at the top of each lane. The position of the 1.9 kb DLAD mRNA is indicated by the arrowhead. For quantitation of lane loading, the ethidium bromide stained gel is shown at the bottom for comparison. The 28S and 18S RNAs are indicated.

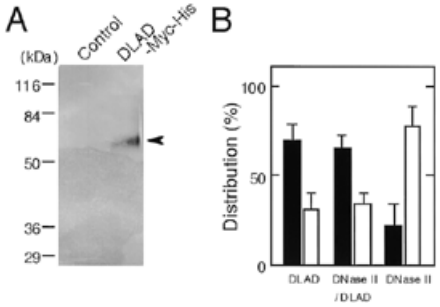

Figure 4. Western blot analyses of recombinant DLAD. (**A**) Cell extracts prepared from pDLAD-Myc-His and empty vector transfected cells were subjected to Ni-NTA column chromatography and the Myc-His-tagged DLAD protein was detected with anti-Myc antibody as described in Materials and Methods. The band representing the DLAD protein is marked by an arrowhead (right lane). No detectable band appeared in the control lane (left lane). (**B**) Quantitation of intra- (closed bar) and extra- (open bar) cellular amounts of DLAD (left), DNase II/DLAD chimera (middle) and DNase II (right) proteins. HeLa S3 cells were individually transfected with pDLAD-Myc-His, pII/DLAD-Myc-His or pDNase II-Myc-His, and the Myc-His-tagged proteins in the extra- and intracellular fractions were purified and detected by western blot as described in Materials and Methods. Band intensities were analyzed by densitometry and the results are summarized. Values are the averages of three independent experiments and are shown with standard deviation.

DLAD is a highly basic protein with a calculated pI of 9.67 containing eight potential *N*-glycosylation sites (Asn-X-Thr/Ser) and an N-terminal signal peptide. The possible stretch of the signal sequence was predicted to be the first 22 amino acids by von Heijne's method (29). A homology search of the GenBank database revealed that DLAD has apparent homologies with three predicted proteins encoded by putative ORFs in the nematode *Caenorhabditis elegans* genome. DLAD shares 32.1, 25.1 and 19.4% amino acid identities with the proteins C07B5.5, F09G8.2 and K04H4.6, respectively (data not shown). Furthermore, the third ORF of the fowlpox virus (FWPV) genome (30) is found to encode a protein sharing 37.5 and 28.4% identity with DLAD and DNase II, respectively, at the amino acid level (Fig. 2).

Tissue distribution of DLAD mRNA

We performed northern blot analysis to assess the expression of DLAD mRNA in various adult mouse tissues. The blots of total RNAs were hybridized with a ^{32}P-labeled DLAD cDNA fragment under high stringency conditions. As shown in Figure 3, the expression of a single transcript of a 1.9 kb DLAD mRNA was detected only in the liver. This is quite different from the ubiquitous distribution of the DNase II mRNA (19), suggesting a distinct cellular function of DLAD from that of DNase II.

Intra- and extra-distribution of the DLAD protein

As described above, DLAD has a highly hydrophobic domain in the N-terminal region that satisfies the conditions of a signal sequence. That is, DLAD is suggested to be an extracellular protein. To assess this possibility, we expressed DLAD as a C-terminal Myc-His-tagged form in HeLa S3 cells and compared the amounts retained within the cells and secreted into the extracellular medium. Myc-His-tagged DLAD was detected by an anti-Myc antibody as a single band of 58 kDa (Fig. 4A) and the relative amounts of cellular and extracellular DLAD were determined by densitometric analysis. In accordance with previous reports (18,21), exogenously introduced DNase II was secreted efficiently and ~80% of the DNase II protein was found in the extracellular fraction (Fig. 4B). In contrast, ~70% of DLAD was retained within the cells and the rest was released into the culture medium (Fig. 4B). This is not due to the rapid turnover of secreted DLAD, because DLAD is as stable as DNase II in the conditioned medium of HeLa S3 cells (data not shown). The inefficient secretion of DLAD was not improved by replacing its signal sequence with that of DNase II (Fig. 4B, middle column), suggesting that this is not due to a weak secretion signal in the peptide itself but to the existence of some targeting motif(s) for intracellular retention in the mature DLAD protein.

Subcellular localization of the DLAD-GFP protein

To determine the subcellular distribution of DLAD, we constructed an expression vector for DLAD with a C-terminal GFP tag and introduced it into HeLa S3 cells. Fluorescence microscopic analysis detected DLAD-GFP as a granular pattern predominantly in the cytoplasm (Fig. 5A and C), whereas GFP alone gave a diffuse image expanded in both the cytoplasmic and nuclear regions (Fig. 5B and D). These observations indicate the cytoplasmic localization of DLAD and suggest its association and/or targeting to some organelle such as lysosomes or peroxisomes.

Characterization of DLAD activity

On the basis of sequence homology, DLAD is predicted to have an endonuclease activity as in the case of DNase II. Therefore, we examined the DNase activity of DLAD using affinity-purified recombinant DLAD protein. DLAD has an endonuclease activity that catalyzes the DNA degradation of

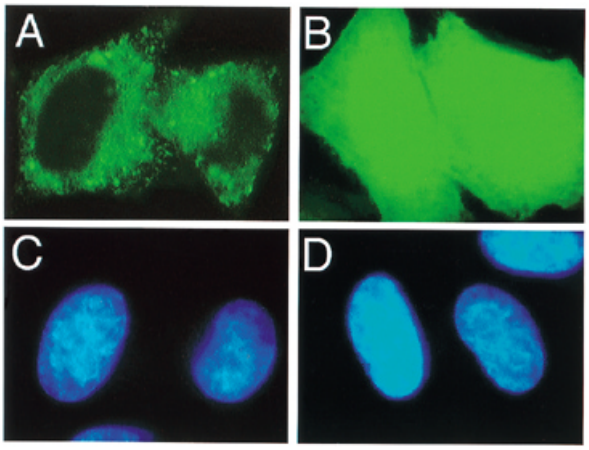

Figure 5. Subcellular localization of DLAD expressed in HeLa S3 cells. Cells were transfected with expression vectors encoding DLAD-GFP (**A** and **C**) or GFP alone (**B** and **D**). At 48 h after transfection, the cells were fixed, stained with Hoechst dye, and the fluorescence of GFP (A and B) and Hoechst (C and D) was monitored by fluorescence microscopy.

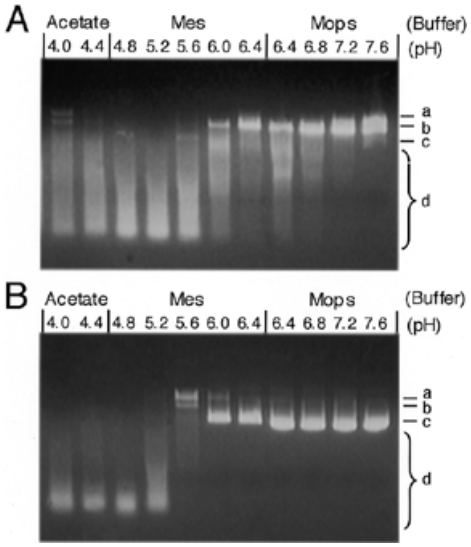

Figure 6. Effect of pH on DLAD activity. The activities of DLAD (**A**) and DNase II (**B**) were assayed using supercoiled plasmid as described under Materials and Methods, except that the following buffers were used: acetate–NaOH (pH 4.0 and 4.4), MES–NaOH (pH 4.8, 5.2, 5.6, 6.0 and 6.4), MOPS–NaOH (pH 6.4, 6.8, 7.2 and 7.6). The forms of plasmid DNA are indicated to the right of the panels; open coil (a), linear (b), supercoil (c) and degraded fragments (d).

supercoiled plasmid. The addition of Mg^{2+}, Ca^{2+} and Mn^{2+}, or divalent cation chelators, EDTA and EGTA, had no effect on DLAD activity (data not shown).

We next analyzed the optimum pH for DLAD activity by performing the assays under various pH conditions. As shown

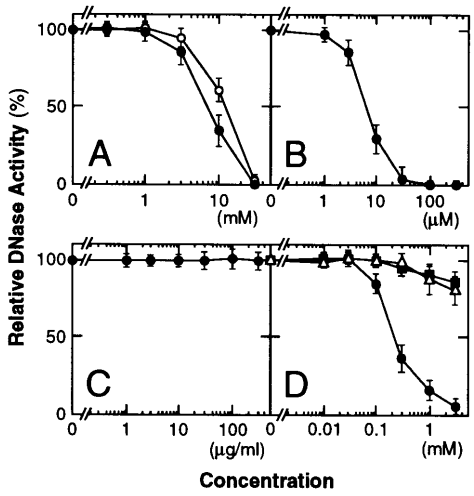

Figure 7. Effects of DNase inhibitors on DLAD. The activity of DLAD was measured using linear plasmid DNA as described under Materials and Methods in the presence of the indicated concentrations of (**A**) $MgSO_4$ (filled circle) or $MgCl_2$ (open circle), (**B**) ATA, (**C**) G-actin, (**D**) $CoCl_2$ (filled square), $NiCl_2$ (open triangle) or $ZnCl_2$ (filled circle). Data represent averages ± standard deviations of results obtained from three independent experiments.

in Figure 6A, DLAD activity was observed over a broad pH range with a maximum at pH 5.2 in MES–NaOH buffer. Under the same assay conditions, however, a high activity of DNase II was observed only at acidic pH below 5.6 (Fig. 6B). Thus, DLAD is shown to be a divalent cation-independent acidic endonuclease.

To obtain more information about the properties of DLAD, we next examined the effects of known DNase inhibitors on DLAD activity. High concentrations of $MgCl_2$ inhibit DLAD activity with an IC_{50} of 13 mM (Fig. 7A, open symbol). $MgSO_4$, an inhibitor of DNase II (31), inhibits DLAD more efficiently than $MgCl_2$ (Fig. 7A, closed symbol). The IC_{50} of $MgSO_4$ is estimated to be 7 mM. These results suggest that SO_4^{2-} is a more effective ion on DLAD activity than Mg^{2+}. Aurintricarboxylic acid (ATA), a general inhibitor of nucleases (32), strongly inhibits DLAD (Fig. 7B), whereas G-actin, an inhibitor of DNase I (33) does not (Fig. 7C). The concentration of ATA required for IC_{50} is 6 μM.

In the previous reports, some divalent cation-dependent endonucleases were shown to be suppressed by divalent-metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} (34). Therefore, we tested the effects of some metal ions on DLAD activity. Among the metal ions tested, Zn^{2+} was found to be the most effective inhibitor of DLAD, with an IC_{50} for $ZnCl_2$ of 0.2 mM (Fig. 7D).

Mode of DNA hydrolysis

DNases can be divided into two classes on the basis of their mode of DNA hydrolysis: one includes DNases producing 3′-OH/5′-P cleaving ends and the other includes those that produce 3′-P/5′-OH ends. DNA chains with 3′-OH/5′-P ends,

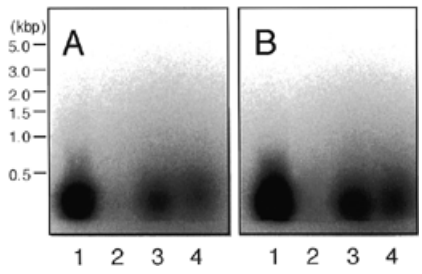

Figure 8. End-labeling of DNA fragments generated by DLAD. Supercoiled plasmid DNA digested by DLAD (**A**) and DNase II (**B**) was subjected to 3′ end- (lanes 1 and 2) or 5′ end- (lanes 3 and 4) labeling with (lanes 1 and 3) or without (lanes 2 and 4) pretreatment with alkaline phosphatase as described in Materials and Methods. Aliquots of the DNAs were separated by 1% agarose gel electrophoresis, transferred to nylon membranes and analyzed by autoradiography.

but not 3′-P/5′-OH, can be appropriate substrates for enzymes, such as DNA ligases and DNA polymerases, involved in DNA replication, repair and recombination. Since determining the mode of DNA hydrolysis of DLAD is important to assess its physiological importance, we performed 3′ and 5′ end-labelings of the DNA cleaved by DLAD. As shown in Figure 8A, the 5′ ends of the DNA fragments were labeled regardless of alkaline phosphatase pretreatment. In contrast, the 3′ ends could be labeled only after the removal of the phosphoryl groups. This labeling pattern is the same as that by DNase II which produces 3′-P/5′-OH ends of DNA (35). These observations clearly show that DLAD catalyzes DNA hydrolysis to produce 3′-P/5′-OH ends.

DISCUSSION

We report here the molecular cloning of a cDNA for DLAD, a novel DNase II-like mammalian endonuclease, and the characterization of its physical and enzymatic properties. The enzymatic properties of DLAD are shown to be quite similar to those of DNase II; both DLAD and DNase II require no cofactors for their catalytic activities, exert their maximum activities under acidic conditions, and produce DNA fragments with 3′-P/5′-OH termini. These similarities are consistent with their partly conserved primary structures, however, our data reveal some important differences between the two enzymes. First, DLAD exerts its DNase activity under neutral to alkaline pH conditions where DNase II is inactive (Fig. 6). Second, as compared with Co^{2+} and Ni^{2+}, Zn^{2+} inhibits DLAD activity strongly (Fig. 7), whereas little difference is observed among these divalent cations in their inhibition efficiencies on DNase II (36). How the difference in the primary structures affects their enzymatic properties is at present unknown, however, these results provide important information to distinguish between the activities of DLAD and DNase II.

Although DLAD has a molecular structure closely related to that of DNase II, the extra/intracellular ratios of DLAD and DNase II are quite different; DLAD prefers to stay within the cells as compared with DNase II. One possible explanation for the inefficient secretion of DLAD is a weakness of its signal sequence itself. This prediction, however, is disproved by the observation that replacement of the signal sequence with that of DNase II fails to improve the secretion efficiency of DLAD. These results suggest the existence of targeting motif(s) for cytoplasmic compartments within the mature DLAD protein and its physiological roles in some intracellular activities. A motif search using the PSORT II program revealed that DLAD contains no targeting signals for mitochondria or nuclei. Thus, likely targets for the cytoplasmic DLAD may be acidic organella such as lysosomes or peroxisomes. At present, our knowledge about targeting motifs for such acidic compartments is quite limited. Therefore, elucidation of the precise subcellular localization of DLAD and its corresponding targeting motif(s) must await further studies.

On the basis of the molecular structure of the DLAD protein, the existence of an N-terminal signal peptide and eight possible *N*-glycosylation sites, one potential pathway for its biosynthesis is as follows. During *de novo* synthesis, the nascent chain of the DLAD protein is translocated into the endoplasmic reticulum (ER) depending on the signal sequence. After removal of the signal peptide, the mature DLAD is glycosylated and secreted and/or transported to the appropriate cellular compartment via the golgi apparatus. This prediction is supported by the following observations: (i) indicative of glycoproteins, DLAD is detected as a diffused band in SDS gels at a higher apparent molecular mass than that calculated from the amino acid composition of the mature DLAD-Myc-His protein; (ii) the DLAD protein is found both in the culture medium and cytoplasm, and the intracellular granular distribution pattern of the DLAD-GFP fusion protein supports the co-localization of DLAD with some organella (Fig. 5).

A homology search revealed that DNase II- or DLAD-related proteins are widely conserved from invertebrates to mammals. Furthermore, we found several non-mammalian EST clones, including zebrafish (AI330733), *Drosophila melanogaster* (AI402235) and *Schistosoma mansoni* (AA999280), potentially coding a DNase II or related protein. The evolutionary conservation of DNase II-like proteins provides further support for their importance in certain essential biological events conserved beyond the species. A partial amino acid sequence, ATEDHSKW, was previously determined for porcine DNase II, and the fifth, histidine (His), is predicted to be the active site of DNase II catalysis (18). Importantly, this His has been found to be conserved among DNase II and DLAD-related proteins. Although their enzymatic activities have yet to be determined, the striking conservation of the active His residue suggests a DNase II-like DNase activity. It is of note that the viral DNase II-related protein FP-CEL1 has more amino acid homology to DLAD than DNase II. This indicates FP-CEL1 to be a viral orthologue of mammalian DLAD. Although the reason why FWPV carries a DLAD homologue in its genome is unknown, one possible explanation is that the expression of the viral DLAD ensures its successful proliferation by preventing the invasion of another virus that will compete for the replication system of the host cells. This idea is supported by the observation that FP-CEL1 is not essential for the replication or proliferation of FWPV itself and its expression is observed during the late phase of infection (30). The physiological significance of DALD is at present unknown, however, it is important to elucidate the role of DLAD in cellular defence against viral infections.

Recent interest in DNases has focused on the clinical application of DNase molecules as therapy for cystic fibrosis (CF), a lethal disease common in Caucasian populations (37,38). CF is characterized by a defect in the CF transmembrane conductance regulator, and the resulting pulmonary disease, the major cause of morbidity and mortality, by the accumulation of viscous purulent secretions in the airway (39). Human recombinant DNase I has recently been approved as a therapy to improve the lung function of CF patients, because the viscoelastic nature of CF sputum is contributed to by the high concentration of DNA released by leukocytes (37,38). In addition to its action in DNA degradation, DNase I supports the depolymerization of F-actin, which is abundant in CF sputum. However, since the resulting monomeric G-actin inhibits DNase I, the administered DNase I will soon be inactivated by the increasing concentration of G-actin. This problem could be solved in two ways: (i) by generating actin-resistant DNase I by protein engineering (40,41) or (ii) by applying other DNases that are naturally insensitive to G-actin. For the latter possibility, DLAD may be a suitable candidate to substitute for DNase I, because (i) DLAD is not inhibited by G-actin, (ii) DLAD requires no cofactors for its activity, and (iii) DLAD is active under a wide range of pH.

The present study provides important information not only for the understanding of the physiological functions of DNase II family endonucleases but also for the utilization of DLAD in clinical applications such as the prevention of viral infection and as therapy for CF.

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