

L-DNase II, a Molecule That Links Proteases and Endonucleases in Apoptosis, Derives from the Ubiquitous Serpin Leukocyte Elastase Inhibitor

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The most widely recognized biochemical change associated with the majority of apoptotic systems is the degradation of genomic DNA. Among the enzymes that may participate in this cleavage, the acidic cation-independent DNase II is a likely candidate since it is activated in many apoptotic cells. To better understand its role, we purified and sequenced a DNase II extracted from porcine spleen. Protein sequencing of random peptides demonstrated that this enzyme is derived from a ubiquitous serpin, the leukocyte elastase inhibitor (LEI), by an acidic-dependent posttranslational modification or by digestion with elastase. We call this novel enzyme L-DNase II. In vitro experiments with purified recombinant LEI show that the native form has no effect on purified nuclei whereas its posttranslationally activated form induces pycnosis and DNA degradation. Antibodies directed against L-DNase II showed, in different cell lines, an increased expression and a nuclear translocation of this enzyme during apoptosis. Since the appearance of the endonuclease activity results in a loss of the anti-protease properties of LEI, the transformation from LEI to L-DNase II may act as a switch of protease and nuclease pathways, each of which is activated during apoptosis.

Apoptosis, or programmed cell death, is a mechanism of cell clearance in many physiological processes such as embryogenesis, metamorphosis, and tumor regression (2). Although the signals inducing apoptosis are very different, nuclear condensation, membrane blebbing, and formation of apoptotic bodies are morphological features common to all apoptotic cells. By far the most widely recognized biochemical change is the degradation of genomic DNA. The identity of the enzymes responsible for this cleavage is the subject of considerable debate. Several endonucleases have been proposed to be responsible for DNA fragmentation. Ca^{2+} - plus Mg^{2+} -dependent endonucleases in thymocytes, such as NUC 18/cyclophylin A (16), DNase I (21), DNase γ (29), and a new 97-kDa DNase (18), are examples. Mg^{2+} -dependent, Ca^{2+} -independent endonucleases have been implicated in human myeloid cell line apoptosis (7, 8). Barry and Eastman (1) implicated DNase II, a cation-independent acidic endonuclease, as the enzyme that degrades DNA in apoptosis associated with intracellular acidification. We have shown in our laboratory the involvement of DNase II in nuclear degradation in terminally differentiating lens fiber cells (32).

To date, our knowledge of the molecular structure of DNase II is very limited. The enzymatic properties of DNase II from different tissues and animals were found to be very similar, but its physical and chemical properties showed high variability. For instance, the molecular mass of mammalian DNase II ranges between 26 and 45 kDa. The reasons for this variability remain unknown (15).

To better understand the biology of DNase II, the knowledge of its protein sequence seemed to be a mandatory step. In

this study, we showed that this ubiquitous L-DNase II arises from leukocyte elastase inhibitor (LEI) by a posttranslational modification that involves a shift in the molecular weight of LEI. This shift in the apparent molecular weight of LEI is followed by a loss of its elastase-inhibiting activity and the appearance of DNase II activity. We also investigated, using cultured cells or purified nuclei, its involvement in apoptosis.

MATERIALS AND METHODS

Protein sequence. DNase II (200 μg) was purified by polyacrylamide gel electrophoresis (PAGE) from a commercial preparation (Worthington) and transferred to a polyvinylidene difluoride membrane (Millipore). The protein was visualized by staining in 0.001% amido black diluted in 40% methanol–10% acetic acid. The 27-kDa protein was cut out and digested with trypsin or lysine endopeptidase. The resulting peptides were separated by high-pressure liquid chromatography on a DEAE- C_{18} column. The N-terminal peptide sequence and the sequences of selected peptides were determined by Edman degradation (Laboratoire des Biotechnologies, Institut Pasteur, Paris, France).

LEI cloning and nucleotide sequence. Total RNA from porcine spleen was purified with TriINSTAPUR (Eurogentech) and retrotranscribed from a poly(dT) primer by reverse transcriptase from Moloney murine leukemia virus (Bethesda Research Laboratories). The cDNA was amplified by PCR with horse primers. We therefore obtained a porcine sequence that was used to design specific porcine primers. The PCR products obtained with these primers and an anchoring poly(dT) primer (dT₁₇-AGC TAC AGC TGA GCT CAG) were cloned in pGEM.T (Promega). Four partial clones were obtained and sequenced with the universal reverse and forward IRD-41-labeled primers. Sequencing reactions were performed with Thermo Sequenase cycle-sequencing kit (Amersham) and analyzed in a Licor automatic sequencer.

Construction of the recombinant vectors and expression of porcine LEI. The complete coding sequence was reconstituted from two partial clones in pGEM by using the unique Eco0109I site. Isolation of plasmid DNA, conditions for digestion with restriction enzymes, and agarose gel electrophoresis were as described previously (27). Expression of the recombinant protein with the SP6 transcription promoter of pGEM vector was carried out in the presence of [³⁵S]methionine in an in vitro transcription-translation system with rabbit reticulocyte lysate as indicated by the manufacturer (25).

A prokaryote expression system was constructed by inserting the cDNA of porcine LEI into *Nco*I and *Xho*I restriction sites of pET 23d(+) (Novagen). *Escherichia coli* BL21 was electroporated and grown in Luria-Bertani medium. The synthesis of LEI was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 10 mM. The resulting protein bears a poly-histidine tag at its N-terminal end, which allows purification with His-Bind resin

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in the presence of 6 M urea, as specified by the manufacturer (Novagen Inc.) (17).

Southern Blot analysis. A 5- μ g portion of porcine total genomic DNA was digested with *Pst*I, *Eco*RI, and *Hind*III overnight at 37°C. The fragments obtained were separated on a 1% agarose-Tris-acetate-EDTA (TAE) gel and transferred by capillary blotting onto a Hybond N⁺ Amersham membrane. The membrane was prehybridized and hybridized in 6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-5 \times Denhardt's solution-0.5% sodium dodecyl sulfate (SDS) for several hours. The probe used was a 550-bp DNA fragment (from nucleotides [nt] 409 to 850 of the porcine DNA coding sequence), labeled by [³²P]dCTP random priming as specified by the manufacturer (Amersham) and added to a final concentration of 10⁶ cpm/ml. Filters were washed at low stringency and exposed for autoradiography at -80°C, using Kodak X-Omat S film, between two intensifying screens.

Northern blot analysis. Total RNA was electrophoresed in a 1% agarose gel in a formaldehyde running buffer and transferred to a Hybond N⁺ membrane (Amersham). Transfer and hybridization were carried out as described previously (27). The blots were then washed twice for 15 min in 1 \times SSC-0.1% SDS at room temperature and twice for 15 min in 0.25 \times SSC-0.1% SDS at 50°C. The membranes were exposed for 1 week as above. For the probe, 25 ng of LEI complete coding sequence was prepared as above.

Reverse transcription-PCR (RT-PCR) and nested PCR. mRNA was purified from 150 μ g of porcine spleen total RNA with poly(dT)-containing magnetic beads (Dynal). Retrotranscription was performed as above. mRNA was eliminated by incubation for 1 h at 65°C in 0.2 M NaOH, and free nucleotides were eliminated on a spin column (Pharmacia Biotech). Poly(dG) was added at the 5' end of the cDNA with a polynucleotide terminal transferase (Boehringer Mannheim). The first PCR step was performed with primers oligo(dT) and 354; nested PCR was performed with primers 374 plus 812, 374 plus 877, and 374 plus 1044. The primer sequences are as follows: 354, 5'-AAA CCT ACG GGG CTG AAC TG-3'; 374, 5'-AGC GTG GAC TTC CTG CGG-3'; 812, 5'-ACC TCG GGC AGT GGA CAT TG-3'; 877, 5'-AAA GAG ATC CTG CAC GCC CA-3'; 1044, 5'-CAT TGA AAT TTT CCT CTG GCA-3'.

Posttranslational modification of the recombinant protein. The recombinant protein produced by the reticulocyte lysate was incubated at different pHs overnight. The different pHs were obtained with the following reagents: pH 1.0, 50 mM H₂SO₄; pH 2.0, 50 mM H₂SO₄ (adjusted with NaOH); pH 4.0, 50 mM acetic acid; pH 6.0, 50 mM H₃PO₄. Alternatively, the mixture was diluted in phosphate-buffered saline (PBS) and digested overnight with 1 μ g of bovine elastase (Sigma). A 3- μ l volume of each sample was mixed with 30 μ l of Laemmli sample buffer and loaded on a 12% polyacrylamide gel. The gel was subsequently fixed, treated with En³Hance enhancer (NEN Bioproducts), and exposed for autoradiography as above.

Porcine spleen crude extract. A 2-g portion of porcine spleen was homogenized in 20 ml of 25 mM Tris-1 mM EDTA (pH 7.4) containing 1 M NaCl and centrifuged for 15 min at 13,000 \times g, and the supernatant was dialyzed overnight against the same buffer without NaCl. The protein concentration, as determined by the bicinchoninic acid method (Pierce), was 4.5 mg/ml.

DNase II activity assays. A 50- μ l volume of the pH 2-treated protein (obtained from reticulocyte lysate or from *E. coli*) was incubated in a final volume of 600 μ l containing 10 mM Tris and 10 mM EDTA (pH 5.5) with 25 μ g of genomic DNA. Aliquots (100 μ l) were ethanol precipitated at different incubation times, resuspended, and loaded on a 1% agarose gel. DNase activity on plasmid DNA was assayed under the same conditions with 4.5 μ g of pGEM and 5 μ l of the acid-treated protein.

Anti-elastase activity. The anti-elastase activities of the 42-kDa protein and of its posttranslational product were evaluated by the capacity of these polypeptides to bind to elastase (5). A 6- μ l volume of the posttranslational reaction mixture was incubated in PBS containing 414 ng of elastase. The reaction was allowed to proceed for 5 min at 20°C and then stopped by the addition of twofold-concentrated Laemmli sample buffer without S-S reducing agent. The samples were then loaded on a 12% polyacrylamide gel and subjected to autoradiography.

Western blot analysis. Polyclonal antibody against DNase II and immunoblot analyses were performed as described previously (32).

Effect of LEI and pH 2-treated LEI on purified nuclei. Baby hamster kidney (BHK) cells were grown as a monolayer in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% fetal calf serum, 4 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml (all from GIBCO-BRL) at 37°C in a humidified atmosphere containing 5% CO₂. The cells were detached with trypsin, rinsed in PBS, and extracted by incubation in 1.5 mM MgCl₂ for 15 min at 4°C. They were then subjected to five strokes in a Dounce homogenizer and washed by centrifugation three times in 1.5 mM MgCl₂. The obtained pellet containing nuclei was stored in 10 mM Tris (pH 7.4)-200 mM sucrose-60 mM NaCl at a concentration of 10⁸ nuclei/ml. To test the effects of LEI, 10⁶ nuclei were incubated for different periods with 280 ng of *E. coli*-produced LEI, treated overnight at pH 2.0 or untreated. Negative control experiments were done with PBS. The reaction mixture was adjusted to DNase II ionic conditions by adding 2 μ l of a 10-fold appropriate buffer (see above). After incubation of these mixtures at 37°C for 0, 2, or 6 h, nuclei were prepared for morphological or DNA analysis.

To study DNA degradation, the reaction was stopped by the addition of 20 μ l of 10 mM Tris (pH 7.4)-100 mM NaCl-25 mM EDTA-1% Sarkosyl-5 μ l of

proteinase K (10 μ g/ μ l). The mixture was incubated overnight at 37°C, 30 μ g of DNase-free RNase was added, and the mixture was incubated for an additional 1 h. Samples were then loaded on a 1% agarose gel as above.

To analyze nuclear morphology, nuclei were washed in PBS at the end of the incubation time, stained with 4',6-diamidino-2-phenylindole (DAPI), spread on a microscope mounting plate, covered with a coverslip, and analyzed under a Leitz Aristoplan microscope.

The same experiments were performed with 10 μ l of LEI produced in reticulocyte lysate treated at pH 2.0 or left untreated. Control experiments with PBS and reticulocyte lysate crude extracts treated at pH 2 were also performed. Nuclear incubation, DNA analysis, and nuclear morphology investigation were done as described above.

Induction of apoptosis in cultured cells. Chinese hamster ovary (CHO) cells and COS-7 cells were grown as a monolayer in Dulbecco's modified Eagle's medium Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4 mM glutamine, 100 U of penicillin per ml, and 0.1 mg of streptomycin per ml at 37°C in a humidified atmosphere containing 5% CO₂. The cells were seeded on coverslips (5,000 cells/cm²), maintained in culture for 2 days, and then treated for 1 h with 1 nM Thapsigargin (COS-7 cells) or incubated in the same medium for 6 days (CHO cells).

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde for 15 min, washed in PBS, and permeabilized with 0.3% Triton X-100 in PBS for 30 min. After being washed with PBS, the cells were saturated for 30 min at room temperature with PBS containing 1% skim milk and then incubated for 1 h at room temperature with anti-DNase II (1/100) in PBS containing 0.1% skim milk (32). This incubation was followed by five washes for 5 min per wash in PBS-0.1% skim milk. The antibody was localized with tetramethylrhodamine-5-isothiocyanate (TRITC) goat anti-rabbit immunoglobulin G (1/500 dilution) in PBS-0.1% skim milk (1 h room temperature). The cells were then washed five times for 5 min in PBS. During the last wash in PBS, nuclei were stained with the fluorescent nuclear stain DAPI and then mounted with 50% glycerol in PBS. The cells were evaluated under a Leitz Aristoplan microscope equipped with an epi-illuminator HBO and filters for rhodamine and DAPI fluorescence. They were photographed with Ilford HP5 film (400 ASA). Control experiments with anti-DNase II preimmune serum and PBS-0.1% skim milk instead of anti-DNase II were also performed.

RESULTS

Nucleotide sequence of DNase II. DNase II obtained from porcine spleen (Worthington) was purified by PAGE and digested by lysine-endopeptidase and trypsin. The resulting peptides were separated by high-pressure liquid chromatography and sequenced by Edman degradation. The N-terminal sequence was also determined. We obtained the sequence of seven peptides, representing 78 amino acids and accounting for about one-third of the 27 kDa estimated for DNase II. An identity search in data bank showed that the sequence of these peptides all corresponded to the LEI (31), an antiprotease of the serpin superfamily. The LEI coding sequence is known for humans and horses but unknown for pigs (9, 26). By using PCR primers from the horse (Fig. 1), we amplified pig spleen cDNA and obtained the complete nucleotide coding sequence of porcine LEI. At the nucleotide level, the open reading frame showed 81% homology between pigs and horses. The mRNA from porcine spleen showed three putative polyadenylation signals (Fig. 1). At the protein level, our deduced sequence showed a difference of two amino acid substitutions with respect to the previously published protein (31).

Since DNase II and LEI had different biological activities and molecular weights, the existence of different genes carrying related sequences was verified by genomic Southern blot analysis (Fig. 2A). Total pig genomic DNA was digested with three restriction enzymes, transferred, and probed with a 550-bp DNA fragment from the middle region of the LEI coding sequence (from nt 409 to 850). After washing at low stringency, only one band was seen in each digestion.

DNase II displayed the N- and C-terminal regions, of LEI but they had different molecular masses: 27 and 42 kDa, respectively. An alternative splicing of LEI mRNA might therefore be responsible for the synthesis of DNase II. To verify this hypothesis, we studied the mRNA from different porcine tissues (lens, neural retina, retinal pigmented epithelium, brain,

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1/1                               31/11                               61/21
ATGGAGCAGCTGAGCGCGGCAACACCCGCTTCGCCTTGGACCTGTCCGCGCCCTGAATGAGAGCAATCCTGCTGGGAACATCTTCATC
M E Q L S A A N T R F A L D L F R A L N E S N P A G N I F I
91/31                               121/41                               151/51
TCCCCCTTCAGCATTTCGTCAGCACTGGCCATGATCCTTCTGGGACCAGAGGCAACCGGAGGCCAGATGTCCAAGGCTCTCCATTTC
S P F S I S S A L A M I L L G T R G N T E A Q M S K A L H F
181/61                               211/71                               241/81
GACACTGTCAAGGACATTCATTCAGGATTCAGAGCTCGAATGCGGATATCAACAAGTGTGGAGCCTCCTATATCTGAAACTCGCTAAT
D T V K D I H S R F Q S L N A D I N K C G A S Y I L K L A N
271/91                               301/101                               331/111
AGGTTGTTGGCGAAAAACCTATCACTTCTCCCGAAATCTTAGCTTCAACTCAGAAAACCTACGGGGCTGAECTGGCCAGCGTGGAC
R L F G E K T Y H F L P E F L A S T Q K I Y G A E L A S V D
361/121                               391/131                               421/141
TTCCTGCGGGCCTCGGAAGAGGCCGGAAGCGATAAACGAGTGGTCAAAGAGCAGACGGAAGGGAATAATCCAGAGCTGCTGGCATCA
F L R A S E E A R K A I N E W V K E Q T E G K I P E L L A S
451/151                               481/161                               511/171
GGTGTGGTTGATAGCGCACTAAACTGGTGGTGAATGCCATCTATTTCAAAGGCAGCTGGCAGGAGAAGTTCATGACAGAGGCCAGC
G V V D S A T K L V L V N A I Y F K G S W Q E K F M T E A T
541/181                               571/191                               601/201
AAGGATGCCCGTTCAGGCTGAATAAGAAAGACGCAAAACGGTGAATGATGATCAGAAGAGAAATCCCATTCGGCTACATCAAG
K D A P F R L N K K D S K T V K M M Y Q K K K F P F G Y I K
631/211                               661/221                               691/231
GAGCTCAAGTGCCTGGTCTGGAATTGCCCTATCAGGGCAAGGACCTCAGCATGGTCTCCTGCTGCCGACAGCATCGAGGACGAGTCC
E L K C R V L E L P Y Q G K D L S M V I L L P D S I E D E S
721/241                               751/251                               781/261
ACGGGTCTGAGGAAGATTGAGCAACACCTGACCTGGAAAAGCTGCGTGAGTGGACCAAACCTGACAACTGGAGCTCCTGGAGGTCAAT
T G L R K I E Q H L T L E K L R E W T K P D N L E L L E V N
811/271                               841/281                               871/291
GTCCACCTGCCAGGTTGAGGCTGGAGGAGACTACGACCTCAACGCCCGCTGGCCCGCCTGGGCTGCAGGATCTTTGGCAGCAGG
V H L P R F R L E E S Y D L N A P L A R L G V Q D L F G S R
901/301                               931/311                               961/321
GCTGACCTGACTGGCATGTGCGAAGCCCGCAGCTTTCATATCCAAGTGGTCCACAAGTCTTTCTGGAAGTGAACGAGGAGGGCAGC
A D L T G M S E A R D L F I S K V V H K S F V E V N E E G T
991/331                               1051/351
GAGGGCGCCGCTGCCACCGCGGGCATCGCTGTCTTCCGCTGCTGATGCCAGAGGAGATTTCATCGCTGACCACCGTTCATTTTCTTC
E A A A A T A G I A V F A * * M L M P E E D F I A D H P F I F F
1081/361                               1111/371
ATCCGGCATAATCCCTCCTCAACATCCTCTTCTCGGGCCGCTCTCTTCCCGTAGAGGCGAGATGCAAGGTGACGCTTAGGGCTCCCC
I R H N P S S N I L F L G R L S S P STOP
1171
CGACCTTTTCATGGCGACCTTCTCATCTCGGAGATGCAAGGTGACGCTTAGGGCTCCCGGACCTTTTCATGGCGACCTTCTCATCTG
TCTTTACCAATACCCTCCTCCTCTCCAAAAACAATCTTTAACCTCCTTTGTAAGCTCAGATCTGTTGGCTGTTACACCCGTTAACT
TGGCATGGGACCTGTTCTTCTTTTCCGTAAGTGAAAAAACAATCCAGCATCAGGTTAGAGAAAGGAAGAAATCATCAGATGTGTAGA
TTCTTGACCATGTAGCGACCTGAGCAGTTCCTCTCTTTTCTGAGAGGCAAGTGAACATGCTCACACGGTCACTGTCTTACAGACAG
ACTTCTGGAGCCCTCAGCGAGACCCAGATTGTTGGAGGGAGGCTTAAAGCGGATATTCTATTGACATACAGGAGCAGCCCAATGAA
GGGCCCCGGCTGTGCGGACAGACTGCCTTGGCAACGTCCTCCGACCGCTGCGCTGACAGGCCCGGGTCCCATTTACGCCATATGGGTGT
CACACTCTCGAAGGGCCGCTGGCGATAACCTCACTCCCTTTTCTGCTCTCCTTGGTCTTCTGCTTGGGTTTGGTGGGGTACAGG
GAATCAAGCAGCAGGTGTCTGTATATCATGAAAGGTGCCAAGGAGTGACAAAAAGCAGAGCATCACAGGAGAGACTGTTAAGCAAGAG
GTGGAGCAGAGACCCGTCGCGAATGGCTTCTCCGAGATTCCGATCAAGGCTCTTACAGCTGGCATCTGGTACAGATTTATGGCAG
ATGACATGGCTTGTGCAATGCTGCAGTGGGAGATCGCTGGGGGGGGTCTTTGATTATAGAAACCTGTCAATATGCTCCTTGGAC
ACGGGACCTGCCGCTGCTGATTTTCTGCTCCCAACAAGCTGCTGGGCCAGTTAATAAAGCTACCTAAGGTTCTGCAAAAAAAAAAAAA

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FIG. 1. Nucleotide and deduced protein sequence of porcine LEI. The sequences of internal peptides obtained by Edman degradation are indicated by a dotted underline. The obtained N-terminal sequence of DNase II is indicated by a continuous underline. Grey boxes represent the localization of the nucleotide sequences from horse LEI used to clone porcine LEI. Open boxes represent the difference between our deduced sequence and the already published sequence for porcine LEI. The arrow indicates the *Eco*109I site. **, the P₁-P₁' site. Presumptive polyadenylation sites are indicated by a double underline. The most frequently used site is located at the 3' end.

liver, and spleen) by Northern blot hybridization with a radioactively labeled cDNA probe of LEI (Fig. 2B). In all the tissues, a single band of 2,200 nt was labeled, corresponding to the size expected for LEI transcript polyadenylated at the 3' foremost site. No smaller mRNA was recorded. Since the presence of a rare alternatively spliced transcript would not be detected by this technique, we studied porcine spleen mRNA by RT-PCR. Figure 2C shows the result of a representative experiment: poly(A)⁺ mRNA was retrotranscribed and then amplified with primers 354 and poly(dT). The product of this amplification was amplified a second time with internal primers (primers 374 plus 812, 374 plus 877, and 374 plus 1044). Only one band, corresponding to the mRNA size expected for full-length LEI, was observed. Similar experiments performed

in the 5' region (nt 35 to 392) gave identical results (not shown).

In vitro and in vivo expression of DNase II. These results raised the possibility that DNase II was derived from LEI by a posttranslational modification, leading to a size shift. To verify this hypothesis, the complete coding sequence was reconstructed in a pGEM plasmid. The protein was then expressed in an in vitro transcription-translation system with a TNT rabbit reticulocyte lysate (Promega). The reaction was performed in the presence of [³⁵S]methionine. The major protein band obtained (42 kDa) (Fig. 3A), was not recognized in Western blots but could be immunoprecipitated by an antibody raised against the 27-kDa DNase II (32) (Fig. 3B).

An aliquot of this protein was incubated at 37°C under

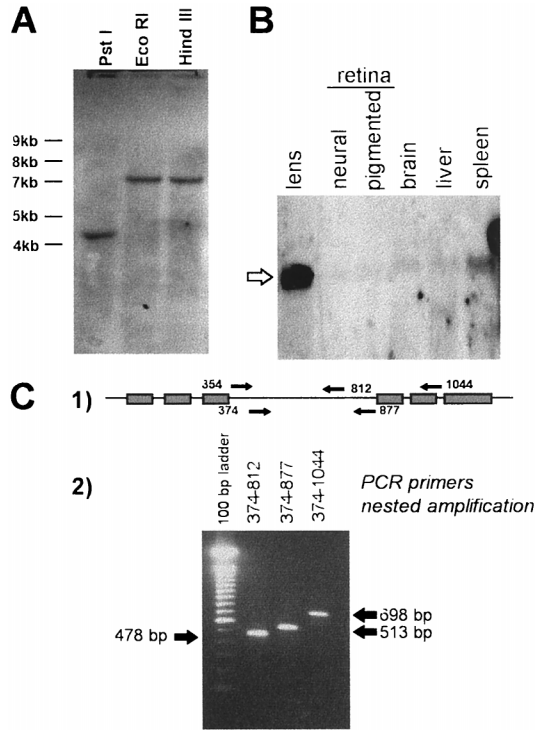


FIG. 2. Southern blot, Northern blot, and nested-PCR analyses. (A) A 25- μ g portion of porcine DNA was digested with *Pst*I, *Eco*RI, and *Hind*III, loaded onto a 1% agarose gel, and transferred to an N^+ membrane. The membrane was hybridized with a 32 P-labeled probe of porcine LEI. A single band is detectable in each lane. (B) Total mRNAs from porcine lens, neural retina, pigmented retina, brain, liver, and spleen were separated on a 1% agarose gel, transferred to a N^+ membrane, and labeled with a 32 P-labeled probe of porcine LEI. The membrane was then subjected to autoradiography. The open arrow indicates the band of 2,200 bases labeled in each tissue. (C) poly(A) $^+$ mRNA from porcine spleen was retrotranscribed and amplified with primers 354 and poly(dT). A second amplification was performed with three pairs of internal primers (labeled 1), where the line represents LEI mRNA and the boxes indicate the positions of the peptides sequenced by Edman degradation of DNase II. The analysis of the second amplifications is also shown (labeled 2).

DNase II-activating conditions (10 mM Tris, 10 mM EDTA [pH 5.5]), with either a plasmid or genomic DNA. No degradation of the plasmid or the genomic DNA was recorded even after overnight incubation (data not shown).

Since the DNase II purification method of Bernardi et al. (3) included the exposure of spleen extracts to an acidic pH, we incubated the 35 S-labeled protein at 37°C overnight at pH 1, 2, 4, and 6 (Fig. 4A, lanes - spleen). A band shift from 42 to 35 kDa was observed mainly at pH 2 but also at pH 4. Although we obtained the band shift with strong-acid treatment in vitro, some soluble factors might permit this maturation under physiological pH conditions in the cell. Indeed, the incubation of labeled LEI in the presence of a crude spleen extract enhanced the production of the 35-kDa band and made the maturation possible at pH 6 (Fig. 4A, +spleen).

Since the 42-kDa protein did not have endonuclease activity, we verified whether the appearance of the 35-kDa band was related to a DNase II activity. An aliquot of LEI incubated at pH 2 overnight was tested for DNase II activity on plasmid (Fig. 4B) and genomic (Fig. 4C) DNA. Both plasmid and genomic DNA were digested. Control samples containing untreated LEI, as well as the reticulocyte lysate extract treated at pH 2.0 (Fig. 4B and C), showed no DNA-cleaving activity. In addition, polyclonal anti-DNase II was able to inhibit this en-

zymatic activity (Fig. 4B, +Anti-DNase II). In the presence of the antiserum, a complex was formed with DNA, leading to a slower migration of plasmid DNA in the gel. The same results were obtained with the recombinant protein produced and purified from *E. coli* (Fig. 4D). No activity was found in bacterial extracts not expressing LEI treated at pH 2. Therefore, we had identified a protein with DNase II activity, derived from LEI. We called this protein LEI-derived DNase II (L-DNase II).

Since the sequences responsible for the elastase inhibitory action (10) were present in L-DNase II, we investigated if the anti-elastase activity remained in the 35-kDa protein (Fig. 5A). The formation of an SDS-resistant complex between the protease and its inhibitor is a mandatory condition for the anti-elastase activity (24). Therefore, we incubated either the 42-kDa labeled LEI or the acid-treated labeled protein with elastase and used PAGE to separate the complexes formed. We observed a shift of the 42-kDa band (corresponding to the native LEI protein) to about 66 kDa, the expected size for the LEI-elastase complex (42 kDa for LEI and 24 kDa for elastase). In contrast, no shift of the 35-kDa band was observed.

These results indicated that the acid treatment of LEI induced a band shift from 42 to 35 kDa and that this determined the appearance of DNase II activity and the loss of anti-elastase activity.

Because the acid treatment is unphysiological, we submitted the 42-kDa protein to different treatments at neutral pH. Among these, we found that the incubation of LEI with elastase (its preferred substrate) led to a modification similar to the acid treatment, i.e., a shift from 42 to 35 kDa (Fig. 5B). The protein treated with elastase also had DNase II activity (Fig. 5C).

It is worthwhile noting that the major protein appearing after acid and elastase treatment was the 35-kDa protein, while the originally sequenced DNase II was 27 kDa. Since measurement of the DNase activity involved incubation of the 35-kDa protein with DNA, we studied the influence of DNA on p35. Figure 5B shows that this treatment induced the appearance of a band at 27 kDa, which was recognized in Western blots by polyclonal anti-DNase II (Fig. 5D). Note that the p42 and p35 bands, although present at larger amounts, were not recognized by anti-DNase II. Enzyme-linked immunosorbent assays

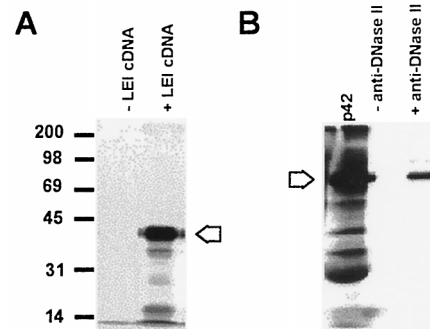


FIG. 3. Expression of porcine LEI in vitro. (A) The cDNA of porcine LEI was inserted in the pGEM vector and expressed with Promega reticulocyte lysate. The reaction was allowed to proceed for 1.5 h at 30°C in the presence or absence of plasmid DNA. A 3- μ l volume of reaction mixture was mixed with the same volume of 2 \times Laemmli sample buffer. The samples were separated on a 12% acrylamide gel and treated for autoradiography. The arrow indicates the 42-kDa main band. (B) A 5- μ l volume of reticulocyte lysate containing [35 S]methionine-labeled LEI was immunoprecipitated in the presence or absence of anti-DNase II and then analyzed by PAGE and autoradiography. The arrow indicates the 42-kDa band.

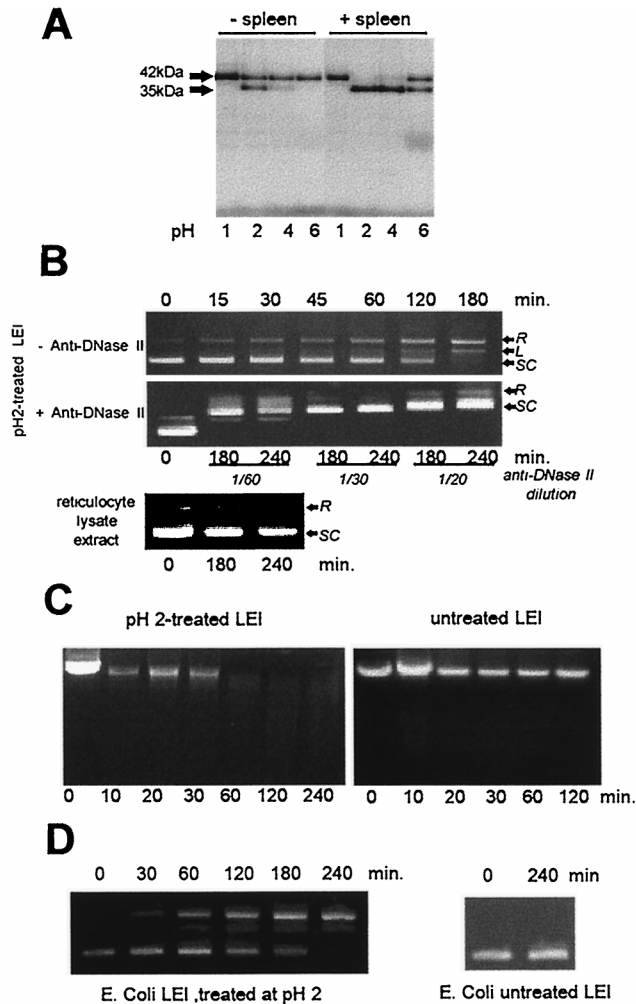


FIG. 4. Posttranslational modification of porcine LEI. (A) Porcine LEI was expressed and [35 S]methionine labeled with the TNT Promega reticulocyte lysate system. Aliquots of 3 μ l were treated at 37°C overnight at different pHs in the presence or absence of crude porcine spleen extract. The reaction was stopped by adding the same volume of 2 \times Laemmli sample buffer. The samples were then separated on a 12% acrylamide gel and subjected to autoradiography. (B) A 4.5- μ g sample of a supercoiled plasmid (pGEM) was incubated in 10 mM Tris–10 mM EDTA (pH 5.5) with 5 μ l of the acid-treated protein; aliquots were ethanol precipitated at different incubation times, resuspended, and loaded on a 1% agarose gel (SC, supercoiled; L, linear; R, relaxed). The same experiment was performed, in the presence of anti-DNase II or with the TNT reticulocyte lysate alone, after treatment at pH 2.0. (C) DNase activity of pH 2-treated or untreated LEI was measured as in panel B, with genomic DNA as the substrate. (D) DNase activity was tested as in panel B, with a pH 2-treated LEI produced by *E. coli* and purified by the His-Bind system (Novagen).

(data not shown) indicated that this antibody had higher affinity for DNase II than for LEI.

Induction of nuclear modification by L-DNase II. To verify if L-DNase II could be responsible for nuclear changes related to apoptosis, we incubated L-DNase II with purified nuclei from normal BHK cells. These experiments were performed with both the protein produced by the reticulocyte lysate system and the recombinant LEI purified from bacteria (Fig. 6). Nuclei (10^6) were incubated for 0, 2, or 6 h at 37°C in the presence of LEI produced in bacteria, LEI treated at pH 2, and PBS. All the samples were loaded onto an agarose gel (Fig. 6A) or DAPI stained for morphological studies (Fig. 6B). As the time of incubation increased, a mild degradation of DNA

was seen in nuclei incubated with either PBS or untreated LEI, but a nucleosomal ladder was seen in LEI acid-activated samples. No major modification in the shape of the nuclei was seen in the presence of PBS or native LEI (Fig. 6B), but a nuclear condensation similar to apoptotic nuclei (4) was observed in the sample incubated with pH 2-treated LEI. The same modifications were seen in nuclei incubated with purified DNase II used as a positive control (data not shown). Similar results were obtained with LEI synthesized by the reticulocyte lysate system (data not shown).

L-DNase II in apoptotic cells. We further investigated the implication of L-DNase II in apoptotic cultured cells by using two cell lines and two different inducers of apoptosis. After induction of apoptosis, the cultured cells were fixed and treated for immunofluorescence with anti-DNase II (fully characterized in reference 32). Representative results with COS and CHO cells are shown in Fig. 7. L-DNase II has a cytoplasmic location in control cells. After induction of apoptosis in COS cells by the Ca^{2+} -ATPase inhibitor Thapsigargin, we observed a strong increase in immunoreactivity in the apoptotic cells (seen by nuclear staining with DAPI). In CHO cells, apoptosis was induced by long-term culture. Although

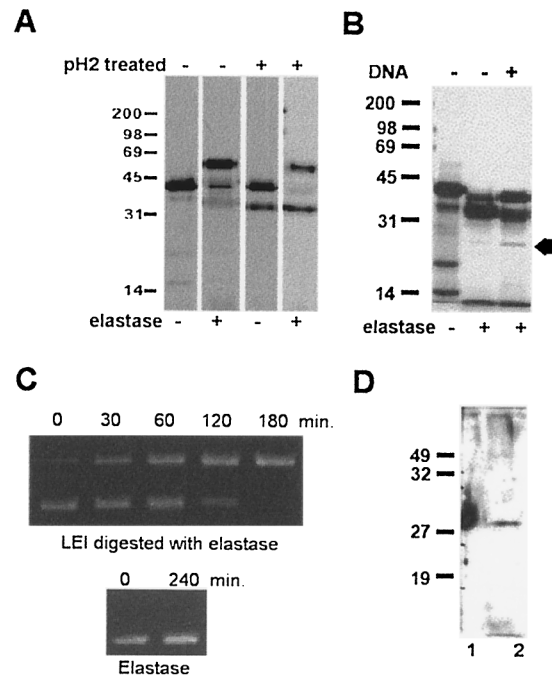


FIG. 5. Posttranslational modification induced by elastase and DNA. (A) pH 2-treated and untreated LEI were incubated in PBS in the presence or absence of 414 ng of elastase. The reaction was allowed to proceed for 5 min at 20°C and then stopped by the addition of 2 \times Laemmli sample buffer without S-S reducing agent. The samples were then loaded on a 12% polyacrylamide gel and subjected to autoradiography. (B) Porcine LEI was expressed and [35 S]methionine labeled with the TNT Promega reticulocyte lysate system. Aliquots of 3 μ l were treated at 37°C overnight with elastase alone or with elastase followed by a second incubation overnight with DNA. The arrow indicates the 27-kDa band. The reaction was stopped by adding the same volume of 2 \times Laemmli sample buffer. The samples were then separated on a 12% acrylamide gel and treated for autoradiography. (C) A 4.5- μ g portion of a supercoiled plasmid (pGEM) was incubated in 10 mM Tris–10 mM EDTA (pH 5.5) with 5 μ l of LEI produced by *E. coli* previously digested overnight with elastase. Aliquots were ethanol precipitated for different incubation times, resuspended, and loaded on a 1% agarose gel. Similar results were obtained with LEI produced by the TNT system (not shown). (D) Porcine LEI treated with elastase and DNA as above (right-hand lane of panel B) was transferred to a membrane and visualized with polyclonal anti-DNase II. Lanes: 1, commercial DNase II; 2, LEI after treatment with elastase and DNA.

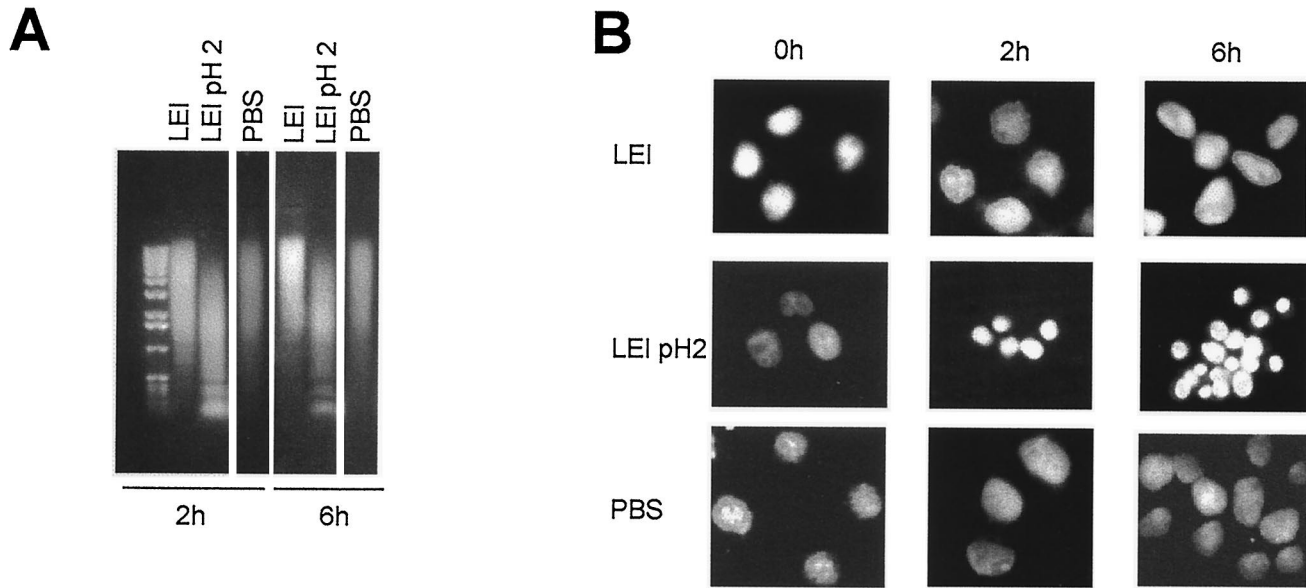


FIG. 6. Induction of apoptotic morphology by posttranslationally modified LEI. Purified nuclei from BHK cells were incubated for 0, 2, or 6 h in the presence of LEI produced by *E. coli* without further treatment (LEI), LEI treated overnight at pH 2.0 (LEI pH 2), or PBS. (A) Neutral agarose gel of BHK purified nuclei after incubation for 2 or 6 h in the presence of the different proteins. (B) The purified nuclei were treated as above, stained with DAPI, and observed with a fluorescence microscope.

the increase of immunoreactivity in this cell line is questionable, a nuclear translocation of L-DNase II is clearly seen in apoptotic cells.

DISCUSSION

L-DNase II is encoded by LEI mRNA. DNase II was studied from an enzymatic point of view in the early 1970s. Later, interest in this nuclease was lost. Recently, its involvement in apoptosis (1, 32) brought it to our attention. To clone the cDNA, we determined the amino acid sequence of seven peptides derived by proteolysis from DNase II. This allowed us to determine the sequence of about one-third of the 27-kDa DNase II. Astonishingly, the sequences obtained belong to porcine LEI, a protein from the serpin superfamily (for this reason, we call the enzyme L-DNase II). The attempt to sequence the N-terminal part of DNase II resulted in a sequence belonging to the C-terminal region of LEI (Fig. 1). This result may have two explanations: (i) this sequence actually represents the N-terminal sequence of L-DNase II (in this case, L-DNase II could result from a novel organization of LEI), or (ii) this result is an artifact. Of these two hypotheses, we favor the second for two reasons. (i) Teschauer et al. (31) have reported that LEI is blocked at its N-terminal end. They show that only the peptide resulting from the natural cleavage of LEI (P_1-P_1') may be directly sequenced by Edman degradation. This sequence corresponds to our N-terminal sequence. (ii) DNase II may arise from gene duplication and/or reorganization of LEI at the chromosomal level (frequently described in the serpin superfamily [6]). Southern blotting of genomic DNA probed with an LEI cDNA shows the presence of one band in all the digestions, suggesting the presence of only one gene coding for this sequence. In addition, the labeled bands do not display very high molecular weights. This makes the possibility of the existence of two genes next to each other very low.

Since L-DNase II is smaller than LEI and displays the N- and C-terminal regions of LEI, we raised the hypothesis of the

existence of alternative splicing of LEI mRNA leading to the production of L-DNase II. The Northern blotting and RT-PCR (which allows the detection of very poorly expressed mRNA) studies demonstrated the presence of only one mRNA with the size expected for the full-length LEI.

L-DNase II is derived from LEI by a posttranslational modification. The results described above suggest that L-DNase II might be derived from LEI by a posttranslational modification. Therefore, we expressed a recombinant LEI by using an in vitro transcription-translation system. We obtained a single protein showing the molecular weight and properties of LEI (i.e., formation of a complex with elastase, a mandatory condition for the elastase inhibitory activity) (24). Since the DNase II purification procedure of Bernardi et al. (3) includes an acidic extraction, we have incubated LEI at different acidic pHs. This treatment produced a 35-kDa protein that is correlated with the appearance of DNase II activity. In addition, this protein has lost its capacity to bind elastase. This indicates that L-DNase II is derived from LEI by a posttranslational modification. It is important to note that soluble factors present in spleen extract facilitate this transition. Indeed, in the presence of this extract, the band shift was observed at pH 6, making this process likely to occur in apoptotic cells, which are subjected to a decrease in intracellular pH. This may explain why cytoplasmic alkalization inhibits apoptosis in some apoptotic models (19, 23, 33).

Three hypotheses can be put forward to explain the nature of the posttranslational modification leading to production of L-DNase II from LEI. (i) LEI belongs to the family of serpin proteins. Several proteins of this family are known to have "relaxed" and "stressed" states which modulate their activity (12, 30) and are correlated with different apparent molecular weights. This may be also the case for LEI. However, the molecular weight shift is too large to be explained by conformational changes alone, and we see this band shift under denaturing conditions (SDS-PAGE). (ii) The transition from LEI to L-DNase II may be the result of a proteolytic cleavage of N- or C-terminal regions. In fact, the elastase releases a

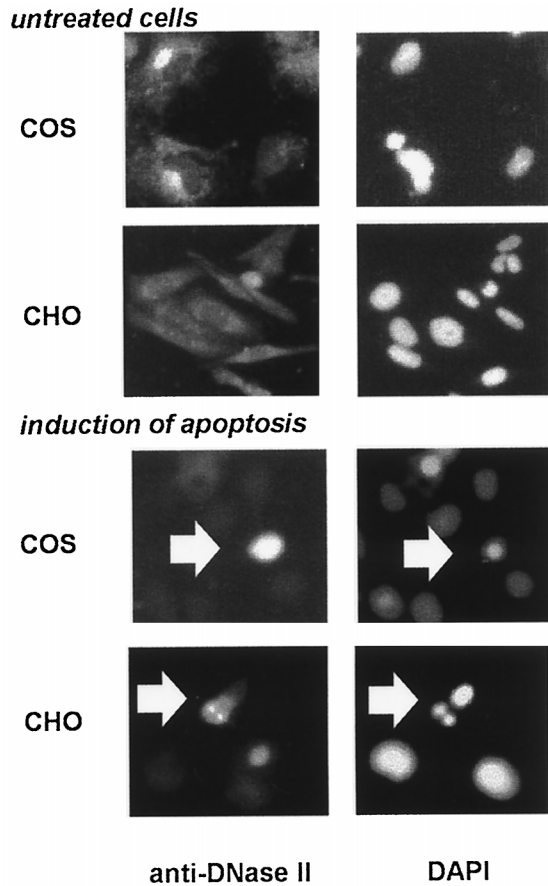


FIG. 7. Involvement of L-DNase II in apoptosis. L-DNase II was located in the cytoplasm of COS and CHO control cells (upper panel, left), compared to the nuclear staining DAPI (right). Apoptosis was induced in COS cells with the Ca^{2+} -ATPase inhibitor, Thapsigargin (1 nM) for 1 h or by long-term culture in CHO cells (lower panel). Apoptotic COS cells show an increased reactivity of L-DNase II; apoptotic CHO cells show a nuclear translocation of the enzyme.

peptide at the N-terminal end of the protein (31) and mimics the exposure of p42 to acidic pH (i.e., a band shift from p42 to p35). This result favors the hypothesis of proteolytic cleavage as the most likely mechanism. Nevertheless, it must be noted that the peptide (4 kDa) liberated from LEI by elastase is found in L-DNase II, and it alone cannot account for the loss in molecular mass. This suggests that the elastase cleavage does not induce the complete transition. We believe that the transition from p42 to p27 takes place in two steps. The first step, induced by acidification (and similar to the elastase cleavage), determines the formation of an intermediate form, p35, that has lost its antiprotease activity. The second step, induced by DNA, completes the modification to p27 L-DNase II. In this model, L-DNase II may be an element of the proteolytic cascade activated in apoptosis (20). (iii) Since we were unable to sequence any polypeptide from the central region of LEI, suggesting the loss of this region in the mature protein, the existence of splicing of the protein might also be hypothesized. Protein splicing is a posttranslational modification in which, in a precursor protein, an internal segment is excised and the external domains are joined by a peptide bond. This process has already been described in prokaryotes and lower eukaryotes and concerns proteins that are often related to DNA metabolism (35).

L-DNase II is involved in apoptosis. DNase II has been partially purified from human gastric mucosa, cervix, and urine (36, 37). In 1985, Liao (14) purified a different enzyme displaying DNase II activity from porcine spleen and established the sequence of its active site. We presume that two or more enzymes with similar activities may exist, as is the case for DNase I (28, 38). The involvement of this DNase in apoptosis was investigated in purified nuclei (a method previously used to identify nucleases involved in nuclear degradation during apoptosis [18]) and in several cell lineages by using different inducers of apoptosis. We found that L-DNase II induces the cleavage of DNA into an oligonucleosomal ladder, a hallmark of apoptosis, and the appearance of nuclear pycnosis, resembling morphological changes seen in apoptosis. In cellular models, an increase of the immunoreactivity for L-DNase II and a nuclear translocation of the enzyme are seen during apoptosis. These facts further support the role of L-DNase II in this process and are in agreement with previous results in differentiating lens cells (32).

L-DNase II activation and the current models of apoptosis. The finding that L-DNase II is derived from LEI is very interesting for the understanding of the molecular mechanisms involved in apoptosis. Two main conclusions can be highlighted. (i) It expands the participation of the serpin superfamily in apoptosis. It has been shown that several human and viral serpins can regulate apoptosis. The best characterized are the cytokine response modifier gene A (*crmA*), which inhibits interleukin- 1β converting enzyme, and the plasminogen activator inhibitor type 2, which inhibits apoptosis induced by tumor necrosis factor (22). (ii) Wright et al. (34) showed the implication of an elastase-like protease activated during apoptosis of U937 cells. Several proteases, such as caspases and other noncaspase proteases, have also been implicated in this phenomenon (39).

We propose that LEI plays a critical role in apoptosis (Fig. 8), acting as a molecular switch between living cells and apoptotic cells. Its double function is to prevent the proteolytic cascade of apoptosis in living cells while, via the transition to

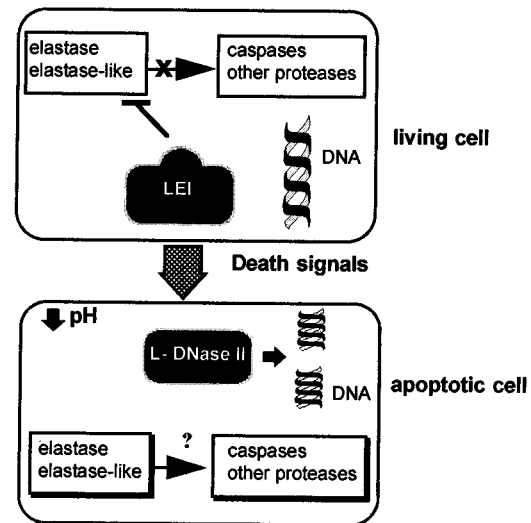


FIG. 8. Hypothetical role of L-DNase II in the apoptotic pathway. In a living cell, LEI is in its native (p42) form. No L-DNase II activity is present in the cell, and protease activities are inhibited by the LEI anti-protease action. During apoptosis, cytoplasm acidification induces the posttranslational modification of LEI, leading to the loss of anti-protease activity and the appearance of L-DNase II. Two degradation pathways are then activated: the endonuclease pathway, by generating L-DNase II, and the protease pathway, by releasing its inhibition.

DNase, releasing this proteolytic inhibition and inducing nuclear degradation in apoptotic cells. We hypothesize that the major control element operating this switch is the intracellular pH. When the intracellular pH decreases in apoptotic cells, the transition from LEI to L-DNase II is made possible. The presence of cofactors (probably acting as other control elements) completes the process. In forms of apoptosis lacking acidification (13), LEI may be a simple substrate for elastase-like activity. This may induce the activation of L-DNase II by an alternative pathway.

These findings establish for the first time a possible link between the activation of endonucleases and proteases involved in apoptosis. In addition, we show here that this link between these systems may not be linear (i.e., activation of proteases leads to activation of endonucleases), as currently believed (4, 11), but that both pathways might be activated at the same time. The discovery of this pathway gives a new insight into apoptosis research and may lead to the development of new tools to regulate cell death.

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