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The conformational modification of serpins transforms Leukocyte Elastase Inhibitor into an endonuclease involved in apoptosis.

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Abstract:

The best characterized biochemical feature of apoptosis is degradation of genomic DNA into oligonucleosomes. The endonuclease responsible for DNA degradation in caspase-dependent apoptosis is Caspase Activated DNase (CAD). In caspase-independent apoptosis, different endonucleases may be activated according to the cell line and the original insult. Among the known effectors of caspase-independent cell death, L-DNase II (LEI derived DNase II) has been previously characterized by our laboratory. We have thus shown that this endonuclease derives from a serpin super-family member LEI (Leukocyte Elastase Inhibitor) by post-translational modification [Torriglia et al, (1998) Mol. Cell. Biol 18, 3612-3619]. In this work we assessed the molecular mechanism involved in the change of the enzymatic activity of this molecule from an anti-protease to an endonuclease. We report that the cleavage of LEI by elastase at its reactive center loop abolishes its anti-protease activity and leads to a conformational modification that exposes an endonuclease active site and a Nuclear Localization Signal (NLS). This represents a novel molecular mechanism for a complete functional conversion induced by the conformational change of a serpin. We also show that this molecular transformation affects cellular fate and that both endonuclease activity and nuclear translocation of L-DNase II, are needed to induce cell death.

Introduction

Apoptosis is a form of cell death characterized by specific morphological features. At early stages of the process, DNA is cleaved into large fragments (50-200 kb) that will be later on degraded into oligonucleosomes (180 bp). Degradation of genomic DNA into oligonucleosomes is one of the most studied features of apoptosis. In many cases, apoptosis is triggered by the activation of specific proteases, called caspases. Upon activation of caspases, DNA degradation is triggered by the activation of CAD (Caspase Activated DNase). Nevertheless, in systems where caspases are not activated, other proteases, like serine proteases or cathepsins, and different endonucleases are activated also leading to a cell death, morphologically indistinguishable from caspase-dependent cell death (19). Indeed, many enzymes have been proposed as responsible for DNA degradation, such as DNase I (29), DNase II (3), DNase γ (35) and NUC18/cyclophilin A (24). However, none of them appeared to fulfill completely the criteria for an apoptotic DNase. Besides, recent studies of apoptotic degradation *in vivo* and *in vitro* indicate that two independent systems are involved in DNA degradation during programmed cell death (26): a cell-autonomous system that functions in dying cells (involving the enzymes mentioned above) and another system taking place after dying cells are engulfed by phagocytes.

In mammalian cells, caspase-independent apoptotic DNA degradation has been associated with two mitochondrial proteins: endonuclease G (20) and Apoptosis Inducing Factor (AIF) (37). These proteins are translocated to the nucleus upon release from the mitochondria. They may be involved in a pathway alternative to CAD/ICAD leading to genomic DNA fragmentation in a caspase-independent manner.

Another effector of caspase-independent apoptosis-like cell death recognized nowadays is LEI/L-DNase II (LEI derived DNase II) (34), a protein characterized in our laboratory (41). The activation of this DNase II (acid, cation-independent DNase) was first discovered in lens during its terminal differentiation (39), which is an apoptosis-related cellular process (12). The activation of this enzyme is also seen in other physiological models, such as neural apoptosis during retinal development (38), as well as in different cell lines (5, 9).

As most enzymes involved in apoptosis, L-DNase II is synthesized as a precursor. This precursor is LEI (Leukocyte Elastase Inhibitor), a protein of the serpin superfamily, which has a protease inhibitor activity and a cytoplasmic localization. After cleavage by elastase

(41) or Apoptotic Protease 24 (1) (but not caspases (40)), LEI loses its anti-protease activity and is transformed into L-DNase II, a nuclear protein with an endonuclease activity. Proteins of the serpin superfamily share conformational features present in all crystal structures of serpins and serpin-protease complexes that have been reported to date (36, 42). The key feature for all serpins is the reactive center loop (RCL). The relevant amino acids are tethered between A and C β -sheets which exists in an exposed conformation. The specificity of the serpin is determined by the amino acid sequence encompassed by the RCL. With few exceptions, serpins inhibit serine proteases by an irreversible suicide-substrate inhibitory mechanism. Protease attacks on the P1 residue of the RCL, leading to a covalent ester linkage between P1 residue of the RCL and the active site of the serine. This will cause cleavage of the P1-P1' peptide bond of the serpin. Thus, the RCL inserts into the A β -sheet, thereby imparting enhanced stability upon the complex, disrupting protease structure and rendering it inactive. This mechanism, called the "Stressed to Relaxed Transition", is associated with a change in the apparent molecular weight of the serpin. The preferable conformation of serpin is its relaxed form, more stable from a thermodynamic point of view. Here, we investigate the molecular basis of the change in LEI activity from a protease inhibitor to an endonuclease, as well as the nuclear translocation linked to this transformation. We show that the conformational modification typical of serpins is responsible for this transformation and that both, endonuclease activity and nuclear translocation, are key events for the induction of cell death (2).

Materials and Methods

Anti-protease and endonuclease activities. Site directed mutagenesis was performed on a wild type-LEI-pET 23d(+) construction using the Quick-Change mutagenesis kit from Stratagene. An alanine residue at P10 position of the hinge region of the reactive loop of LEI (AP10T mutant) or histidine 368 (H368A mutant) were changed into threonine and alanine, respectively.

Recombinant proteins were produced in BL21pLysS *E. coli* strain and purified using His-select cartridges (Sigma).

Increasing concentrations of wild type or mutant LEI (17.5 to 280 μ g/ml) were incubated with elastase (0,1 μ g/ml) in the presence of 2 mM of pNaMAAPV, a synthetic substrate. The resulting colored reaction was measured at 405nm.

DNase activity was measured after overnight cleavage of LEI with equimolar quantities of elastase at 37°C (this step was omitted with 358Stop LEI). 841 ng of wild type, AP10T, H368A or 358stop LEI were incubated with 2,5 µg of plasmid DNA in a time dependent manner at 37°C in 20 mM Tris-EDTA pH 5,5. Untreated wild type LEI was incubated with DNA as a negative control.

Nuclear translocation. BHK cells were grown as monolayer at a density of 45 000 cells/cm² in Dulbecco's modified Eagle's medium (D-MEM, Life Technology) supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin (all from Life Technology) at 37°C in a humidified atmosphere containing 5% CO₂.

Porcine LEI cDNA was subcloned into the XhoI/Sall restriction sites of pDsRed2-C1 plasmid (Clontech) and mutated by site directed mutagenesis, as before.

BHK cells were transfected with jetPEI (PolyPlus Laboratories) according to the manufacturer protocol. The pCDNA3-1/NTGFP topo construction contained wild type LEI. Apoptosis was induced with 40µM 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) and pictures taken 16h after induction. All the apoptotic cells present on the well (24 wells plate) were morphologically identified. Cells displaying red and green (colocalized) fluorescence in the nucleus were considered as expressing LEI constructions displaying no impairment in nuclear translocation. A dissociation of fluorescence with an increase of green fluorescence in the nucleus was considered as impaired for nuclear translocation.

Cell survival. Porcine LEI cDNA was subcloned into the BamHI/XhoI restriction sites of pZeoSV2 plasmid (Invitrogen). BHK cells were seeded at a density of 20 000 cells/cm² in 24-well plates. 2h before transfection the culture medium was replaced by fresh medium without FCS. The transfection medium contained 0.4µg of pZeo-LEI and 3µl of Lipofectin reagent (Life Technologies, Inc.) per well. The transfection process occurred at 37°C for 4h and was stopped with DMEM containing 20% FCS.

Apoptosis was induced with 40µM 5-(N,N-Hexamethylene)-Amiloride (HMA). Survival was measured by the MTT method. At the end of cell treatment, culture medium was removed and 250µl of MTT (1 mg/ml) were added to each well. The plate was kept in a CO₂ incubator for 1h. Cells were then lysed by the addition of 250µl of isopropanol. The degree of MTT reduction in each sample was subsequently assessed by measuring absorption at 570 nm versus 630 nm using a microplate reader (BioRad).

Pull down. Recombinant proteins (wild type LEI, K225A LEI mutant and calmodulin) were

produced in BL21pLysS *E. coli* strain and loaded into His-select cartridges (Sigma) according to the manufacturer protocol. HeLa cells were grown at confluence for three days in 75cm² flasks before lysis in RIPA buffer (50mM Tris, pH 7.2, 150mM NaCl, 1% Triton X-100, 1% DOC, 1% SDS, 2mM PMSF, 1µg/ml leupeptine, 5µg/ml pepstatine, 5µg/ml aprotinin) and loaded onto the column. Bound material was quantified with Bradford assay (Bio Rad), 25µg of each one was resolved by SDS-PAGE and immunoblotted for importin α (monoclonal clone IM-75, Sigma). Calmodulin was used as a negative control and crude extract of HeLa cells (25µg) as a positive control.

Protein Explorer. Proteins were analyzed using the Protein Explorer site (http://www.umass.edu/microbio/chime/pe_beta/pe/protexpl/frntdoor.htm). Introducing the Protein Data Base (PDB) code of each protein, the Molecular Electrostatic Potential was calculated with the Advanced Explorer tools.

RESULTS

Conformational change of LEI

In previous studies we have shown that a limited cleavage transforms LEI into L-DNase II (41). However, the mechanism leading to the production of L-DNase II was not fully established. To assess these mechanisms, we first tested whether the transition from LEI to L-DNase II involves proteolytic cleavage of the N- or C-terminal regions. Previous published data show that LEI (42kDa) is preferentially cleaved by elastase, at amino acid 358, in the C-terminal region of the protein (**Fig. 1**) leading to a protein with an apparent molecular weight of 35kDa (32). Even though this cleavage induces LEI transformation, it does not lead to the release of the C-terminal peptide. Indeed, the Edman's degradation indicated the presence of this peptide in L-DNase II (41). Moreover, trypsin digestion of p42 and p35 followed by mass spectrometry of the obtained peptides showed no significant difference between digests (data not shown), other than the changes expected from a cleavage described earlier (32). This suggested that, in spite of their different apparent molecular weights, the peptide content was comparable in these proteins. Besides, we introduced a stop codon at nucleotide 1074 corresponding to residue 358, which is the cleavage point of elastase in LEI (32). This produced an inactive endonuclease, although its ability to bind DNA was preserved (**Fig. 2b, 358 stop mutant**). Indeed, a shift effect but not a plasmid degradation was observed when the 358stop mutant was incubated with DNA at different times (compare with LEI wild type before and

after cleavage by elastase on **Fig. 2b**). This result suggested that the DNA binding site was in the peptide upstream of residue 358. It also might suggest that the C-terminal peptide supported the endonuclease active site.

We then asked whether the change in LEI activity might be associated with splicing of the protein (43). This post-translational modification has already been described in prokaryotes and lower eukaryotes (6) and concerns proteins which are often related to DNA metabolism (11). This possibility was not assessed further since no consensus site for protein splicing was found in LEI.

We finally studied a possible conformational change as a source of the enzymatic switch in LEI activity. Concerning this possibility, it is worth noting that LEI, as all serpins, is a metastable protein. Its reactive center loop (RCL) (P5-P15 for LEI) is flexible and can assume different conformations (**Supplementary Fig 1**). We have previously shown that cleavage of LEI (42kDa) by elastase results in the appearance of a 35kDa band in denaturing gels. This is a mandatory condition for the molecule to display an endonuclease activity. Treatment of wild type LEI with an inactivated elastase (heat treated or inhibited with PMSF) (13) did not cause its cleavage and transformation into L-DNase II (data not shown). This indicated that only active elastase can induce endonuclease activity.

In order to further investigate this hypothesis we introduced a single base mutation in the hinge region of LEI (**Fig. 1**). This mutation resulted in the replacement of a small non charged alanine into a bigger polar threonine (AP10T). This transforms LEI interaction with its substrate from a tight binding to a competitive inhibition. This mutant lost its anti-protease activity (**Fig. 2a, AP10T compared with WT**). Nevertheless, after overnight treatment with elastase the endonuclease activity was conserved (**Fig. 2b, AP10T**). It is worth noting that incubation of this mutant with elastase overnight has been done in order to keep identical experimental condition with the wild type molecule. In fact the same activity is obtained with shorter incubations (2-3 hours).

Taken together, these experiments show that cleavage of the RCL of LEI is involved in the appearance of the endonuclease activity. They also suggest that endonuclease and anti-protease activities are not physically linked in the molecule.

Therefore, from the three hypotheses rose before, the conformational modification induced by the cleavage of the RCL appears the most likely to explain the change in LEI activity.

Endonuclease activity of L-DNase II

Three possibilities may then explain the emergence of the endonuclease activity generated by the insertion of the RCL into the A β -sheet: first, the RCL establishes new interactions in the A β -sheet; second, it creates a new interaction elsewhere in the molecule; or, third, it uncovers a preexisting active site hidden underneath.

In order to distinguish between these possibilities, we analyzed the three-dimensional crystal structure of cleaved horse LEI. This was done by using Protein Explorer and structural data from the Protein Data Bank (PDB entry 1HLE) which pointed to the charge distribution of cleaved LEI. The molecule seems quite polarized (top panel of **Fig. 3**) with a higher number of positive charges in the RCL pole. The negative charge of DNA would better interact with this region which is, in addition, exposed after cleavage of the RCL. Moreover, comparison of LEI with two well characterized endonucleases, DNase I and CAD, shows a similar charge distribution (**Fig. 3**). By consequence, the uncovering of a preexisting endonuclease active site should be investigated in priority.

All apoptotic endonucleases show a DH doublet in their active site. Analysis of LEI three-dimensional structure shows the presence of two histidines (H287 and H368) in the more positively charged region. From these histidines, we retained His368 that is conserved in all species, while His287 is absent in human (**Fig. 1**). We therefore introduced a point mutation on His368 and replaced it by an alanine (H368A). We expressed it in bacteria, produced the mutant protein and measured its endonuclease activity (**Fig. 2b, lower panel**). As seen on this figure, no endonuclease activity was detected in this mutant when exposed to DNA. This was not due to a lack of efficient cleavage of the mutant, as it was able to inhibit elastase. Therefore, this mutation did not affect the anti-protease activity of LEI (**Fig. 2a, lower panel**).

Nuclear translocation of L-DNase II is due to its binding to importin α

The change in the enzymatic activity of LEI induced during its transformation into L-DNase II is also followed by a change in its cellular localization (41). We therefore analyzed the LEI structure looking for a Nuclear Localization Signal (NLS). We found a consensus bipartite NLS in positions 212-213 and 224-226 (**Fig. 1**). In order to verify that these lysines are really responsible for the nuclear import of L-DNase II, we systematically

mutated them into alanine. This led to the generation of 31 constructs with 1 to 5 mutated lysines in different combinations. To be able to follow subcellular localization of the protein, we performed the mutation on a DsRed2-LEI chimera. In order to exclude artifactual nuclear translocation due to a change in the nuclear envelope permeability, we made a GFP (Green Fluorescent Protein) wild type LEI construct that we systematically cotransfected with the red chimeras. A mutant was considered as impaired for nuclear translocation when cells expressing both plasmids presented a green nucleus and a yellow or red cytoplasm after induction of apoptosis with 40 μ M 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) (**Fig. 4a**). Right panel of Fig 4a shows a default in nuclear translocation of a DsRed2-LEI NLS mutant (the K225A LEI mutant). Analysis of the cotransfected cells allowed us to classify mutants as having a “normal” or an “altered” nuclear translocation pattern during apoptosis. Relevant results are presented on the table (**Fig. 4b**). In these experiments, some red and/or green aggregates rendered difficult to evaluate the nuclear translocation of a few LEI mutants. There were also mutants that did not fluoresce sufficiently to give a score. Nevertheless, by counting the mutants impaired for nuclear translocation for a given amino acid and using this table as a support, we were able to score, the importance of the different lysines of the NLS (**Fig. 4c**).

Proteins bearing a bipartite NLS are transported to the nucleus by carriers, as importin α (16). In order to verify the ability of the K225A LEI mutant to bind importin α , we pulled down importin α with recombinant LEI. To do this, His-tagged wild type and K225A mutant were produced in bacteria, fixed to a Ni²⁺ column and a protein extract from HeLa cells was added to each column. Extraction was loaded on an acrylamide gel and western blot was revealed with an anti-importin α antibody (**Fig. 4d**). As seen on this figure, when compared with wild type LEI, the K225A mutant has an impairment on its ability to bind importin α . His-tagged calmodulin was used as a negative pull down control. HeLa cells whole extract was used as a positive control for immunoblot.

LEI must be transformed into L-DNase II to mediate apoptosis

The apoptotic effect of L-DNase II probably depends on its endonuclease activity, but also on its nuclear translocation. In order to explore this point, LEI/L-DNase II inactivated for the endonuclease activity (H368A mutant) or presenting an impairment of the nuclear translocation (K225A mutant) were transfected into BHK cells. After induction of apoptosis

with 5-(N,N-Hexamethylene)-Amiloride (HMA, a Na⁺/H⁺ pump inhibitor), cells transfected with wild type LEI show a significant decrease on their survival rate (**Fig. 4e**), in agreement with previously shown data (2). On the contrary, the NLS mutant (K225A) and the endonuclease active site mutant (H368A) showed a higher survival recovery compared to the wild type. It is worth noting that the K225A mutation does not modify significantly endonuclease or anti-protease activities (**Fig. 4f** and data not shown).

DISCUSSION

LEI belongs to the intracellular sub-group of ov-serpins (17). With the exception of ovalbumin, all members of this sub-group contain conserved residues in the hinge region that are essential for their inhibitory activity (**Fig. 1**). Modification of cellular behavior is the main function throughout this sub-group (8): some regulates lysosomal proteases (squamous cell carcinoma antigen), monocyte/granulocyte proteinases (Proteinase Inhibitor-6 (PI-6)), fibrinolysis (plasminogen activator inhibitor 2), bone marrow differentiation (bomapine); others are tumor suppressors (maspin) or are implicated in angiogenesis (10). Several serpins can inhibit apoptosis, as the viral serpin Crm A inhibits Fas or TNF α -induced apoptosis (14). Overexpression of PAI-2 or PI-9 protects cells from TNF α (15) or granzyme B induced apoptosis (7), respectively. All of these data and some of our results suggested that LEI, as a member of this serpin sub-group, could act as a molecular switch between life and death by apoptosis.

In previous studies we have shown that a limited cleavage transforms LEI into L-DNase II (41). Three hypotheses were initially raised to explain the change in LEI activity: 1) the transition may result from proteolytic cleavage of the N- or C-terminal regions; 2) it may be linked to a splicing of the protein (43); 3) the transition may involve a conformational change. From these hypotheses the first was not proven. Many experiments were performed in our laboratory to find the released peptide, without success (not shown). The second hypothesis was also rejected since no consensus site for protein splicing was found in LEI (43). Proteins that undergo post-translational splicing have junction sequences flanked by two cysteines and these are only found in pig LEI (**Fig. 1**). Moreover, peptides between these cysteines were found in both LEI and L-DNase II by

Edman degradation (41) (**Fig. 1**)

The third hypothesis was first investigated by introducing a single base mutation in the hinge region of LEI (**Fig. 1**). Previous studies showed that this replacement slows down the insertion of the RCL into the A β -sheet (30), without changing protease specificity. This LEI mutant loses its anti-protease activity but keep its endonuclease activity after elastase cleavage (**Fig. 2**). This indicates that endonuclease and anti-protease activities are not physically linked in the molecule and suggest that the insertion of the RCL in LEI's A β -sheet is involved in establishment of the endonuclease activity.

The endonuclease activity may be generated from new interactions in the A β -sheet either locally or in other parts of the molecule or by uncovering a preexisting active site. From these hypotheses the last one was explored, since the cleavage of the RCL uncovers a basic region and transforms LEI in a polarized molecule, a property common to many endonucleases. This region of the molecule that could potentially interact with DNA should then carry the DNase active site. Studies performed in our group did not reveal any consensus sequence for endonuclease activity (22). However, it is worth noting that the active site of endonucleases activated in apoptosis displays a DH doublet. From these two amino acid residues, the histidine moiety is involved in the proton exchange during DNA cleavage (28). Two histidines (H287 and H368) were good candidates. We retained His368 because it is conserved in the species shown on fig 1, as well as in mouse (UniProtKB/TrEMBL entry Q5SV42), rat (UniProtKB/TrEMBL entry Q4GG075) and zebrafish (UniProtKB/TrEMBL entry Q6DG30). This histidine is surrounded by a basic ring that may bind DNA. Mutation of this histidine completely abolishes endonuclease activity and pro-apoptotic properties of L-DNase II, suggesting that this histidine is essential for the endonuclease activity. It is interesting to note that the entire basic region is exposed by the RCL insertion in the A β sheet and this insertion also uncovers a bipartite nuclearization signal. This is not surprising since L-DNase II has to be translocated to the nucleus where its new target is located. This feature is not due to the increase of nucleus permeability in late apoptosis (33), since in apoptosis induced by caspases LEI remains in the cytoplasm (2).

Bipartite NLS are formed by 5 lysines divided in two groups and are thought to interact with importin α . Here we showed that mutation of these lysines impairs nuclear translocation and binding to importin α . However, it should be stressed that the highest

nuclearization impairment corresponds to the lysine on position 225, the central lysine of the major cluster. This result is in agreement literature data concerning the relevance of the second lysine cluster of a bipartite NLS to bind importin α (16).

We have previously shown that under certain apoptotic conditions LEI is transformed into L-DNase II (2). In addition, several authors have demonstrated that the overexpression of a DNase in a cell may induce apoptosis (18, 21, 25, 27, 31). This is also true for L-DNase II (2) (and **Fig. 4**). The apoptotic effect of L-DNase II depends on its endonuclease activity, but also on its nuclear translocation because overexpression of the K225A mutant decreases the pro-apoptotic activity of L-DNase II as much as the endonuclease inactive protein. This effect depends upon the disability of the K225A mutant to be nuclearized, since this mutation does not affect endonuclease activity.

Taken together, these results indicate that the active endonuclease site of L-DNase II is located underneath the RCL. This site is flanked by a positive charged region that contains a bipartite NLS. Transformation of LEI into L-DNase II is then explained by the conformational modification induced by the cleavage of the RCL, which uncovers the endonuclease active site. Besides, this conformational modification also unmask a bipartite NLS that allows the nuclear translocation of L-DNase II by binding to importin α (**Fig. 5a**).

Current data concerning dual activity of LEI/L-DNase II, including those obtained in the present study, are summarized in **Fig. 5b**. They indicate that LEI, as well as other members of the intracellular sub-family of serpin, is highly implicated in cellular fate. Thus, LEI mediates caspase-independent apoptosis in some particular conditions, i.e. when serine proteases having LEI as a target are massively activated. This takes place during metabolic and oxidative stress, when cells are unable to activate caspases, due to terminal differentiation (like in retinal pigmented epithelium cells (9)) or to acquired mutations in cancer cells (4, 5). In this work we show that the change in activity of LEI is mediated by the classical conformational modification mediating anti-protease activity of serpins. This is a novel example of the use of the conformational modification of serpins to confer tasks different from protease inhibition.

The involvement of this serpin in the activation of a cell death program might turn to be of interest in the development of therapeutic strategies against cancer cells, for instance. Malignant transformation is sometimes followed by lost of apoptotic effectors, leading to

chemotherapy resistance. Induction of apoptosis by stimuli leading to the transformation of LEI into L-DNase II may overcome this resistance. The LEI AP10T mutant described in this work could also be used to increase the efficiency of anti-cancer therapy. Indeed, this mutant is not very efficient to inhibit its target protease and releases it quickly. The protease is no longer trapped in a covalent complex as opposed to wild type LEI. As a result, equilibrium will be displaced towards production of L-DNase II, as the released target protease will be able to cleave more LEI molecules. This would trigger apoptosis in cancer cells. Moreover, a point mutation of the RCL might allow changing the protease specificity of the serpin, which may then be adapted to the protease expression of the target cell.

The existence of a protein being able to induce apoptotic-like cell death by a single conformational modification should encourage research on other key molecules involved in cell fate decisions.

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References

1. **Altairac, S., S. C. Wright, Y. Courtois, and A. Torriglia.** 2003. L-DNase II activation by the 24 kDa apoptotic protease (AP24) in TNF α -induced apoptosis. *Cell Death Differ* **10**:1109-11.
2. **Altairac, S., S. Zeggai, P. Perani, Y. Courtois, and A. Torriglia.** 2003. Apoptosis induced by Na⁺/H⁺ antiport inhibition activates the LEI/L-DNase II pathway. *Cell Death Differ* **10**:548-57.
3. **Barry, M. A., and A. Eastman.** 1993. Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch Biochem Biophys* **300**:440-50.
4. **Belmokhtar, C. A., J. Hillion, C. Dudognon, S. Fiorentino, M. Flexor, M. Lanotte, and E. Segal-Bendirdjian.** 2003. Apoptosome-independent pathway for

- apoptosis. Biochemical analysis of APAF-1 defects and biological outcomes. *J Biol Chem* **278**:29571-80.
5. **Belmokhtar, C. A., A. Torriglia, M. F. Counis, Y. Courtois, A. Jacquemin-Sablon, and E. Segal-Bendirdjian.** 2000. Nuclear translocation of a leukocyte elastase Inhibitor/Elastase complex during staurosporine-induced apoptosis: role in the generation of nuclear L-DNase II activity. *Exp Cell Res* **254**:99-109.
 6. **Bezerra, W. M., C. P. Carvalho, A. Moreira Rde, and T. B. Grangeiro.** 2006. Establishment of a heterologous system for the expression of *Canavalia brasiliensis* lectin: a model for the study of protein splicing. *Genet Mol Res* **5**:216-23.
 7. **Bird, C. H., V. R. Sutton, J. Sun, C. E. Hirst, A. Novak, S. Kumar, J. A. Trapani, and P. I. Bird.** 1998. Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Mol Cell Biol* **18**:6387-98.
 8. **Bird, P. I.** 1999. Regulation of pro-apoptotic leucocyte granule serine proteinases by intracellular serpins. *Immunol Cell Biol* **77**:47-57.
 9. **Brossas, J. Y., R. Tanguy, F. Brignole-Baudouin, Y. Courtois, A. Torriglia, and J. Treton.** 2004. L-DNase II associated with active process during ethanol induced cell death in ARPE-19. *Mol Vis* **10**:65-73.
 10. **Cao, Y.** 2001. Endogenous angiogenesis inhibitors and their therapeutic implications. *Int J Biochem Cell Biol* **33**:357-69.
 11. **Cooper, A. A., Y. J. Chen, M. A. Lindorfer, and T. H. Stevens.** 1993. Protein splicing of the yeast TFP1 intervening protein sequence: a model for self-excision. *Embo J* **12**:2575-83.
 12. **Counis, M. F., E. Chaudun, C. Arruti, L. Oliver, M. Sanwal, Y. Courtois, and A. Torriglia.** 1998. Analysis of nuclear degradation during lens cell differentiation. *Cell Death Differ* **5**:251-61.
 13. **El Ouriaghli, F., H. Fujiwara, J. J. Melenhorst, G. Sconocchia, N. Hensel, and A. J. Barrett.** 2003. Neutrophil elastase enzymatically antagonizes the in vitro action of G-CSF: implications for the regulation of granulopoiesis. *Blood* **101**:1752-8.
 14. **Enari, M., H. Hug, and S. Nagata.** 1995. Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature* **375**:78-81.
 15. **Furlong, I. J., R. Ascaso, A. Lopez Rivas, and M. K. Collins.** 1997. Intracellular acidification induces apoptosis by stimulating ICE-like protease activity. *J Cell Sci* **110 (Pt 5)**:653-61.
 16. **Hodel, M. R., A. H. Corbett, and A. E. Hodel.** 2001. Dissection of a nuclear localization signal. *J Biol Chem* **276**:1317-25.
 17. **Irving, J. A., R. N. Pike, A. M. Lesk, and J. C. Whisstock.** 2000. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res* **10**:1845-64.
 18. **Krieser, R. J., and A. Eastman.** 1998. The cloning and expression of human deoxyribonuclease II. A possible role in apoptosis. *J Biol Chem* **273**:30909-14.
 19. **Leist, M., and M. Jaattela.** 2001. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* **2**:589-98.
 20. **Li, L. Y., X. Luo, and X. Wang.** 2001. Endonuclease G is an apoptotic DNase

- when released from mitochondria. *Nature* **412**:95-9.
21. **Los, M., D. Neubuser, J. F. Coy, M. Mozoluk, A. Poustka, and K. Schulze-Osthoff.** 2000. Functional characterization of DNase X, a novel endonuclease expressed in muscle cells. *Biochemistry* **39**:7365-73.
 22. **Martin, E., M. F. Counis, P. Perani, Y. Courtois, and A. Torriglia.** 2002. LEI/L-DNase II : les implications structurales d'un détournement de fonction. *Médecine Sciences* **18**:111-120.
 23. **Martz, E.** 2002. Protein Explorer: easy yet powerful macromolecular visualization. *Trends Biochem Sci* **27**:107-9.
 24. **Montague, J. W., M. L. Gaido, C. Frye, and J. A. Cidlowski.** 1994. A calcium-dependent nuclease from apoptotic rat thymocytes is homologous with cyclophilin. Recombinant cyclophilins A, B, and C have nuclease activity. *J Biol Chem* **269**:18877-80.
 25. **Mukae, N., M. Enari, H. Sakahira, Y. Fukuda, J. Inazawa, H. Toh, and S. Nagata.** 1998. Molecular cloning and characterization of human caspase-activated DNase. *Proc Natl Acad Sci U S A* **95**:9123-8.
 26. **Nagata, S.** 2005. DNA degradation in development and programmed cell death. *Annu Rev Immunol* **23**:853-75.
 27. **Oliveri, M., A. Daga, C. Cantoni, C. Lunardi, R. Millo, and A. Puccetti.** 2001. DNase I mediates internucleosomal DNA degradation in human cells undergoing drug-induced apoptosis. *Eur J Immunol* **31**:743-51.
 28. **Pan, C. Q., J. S. Ulmer, A. Herzka, and R. A. Lazarus.** 1998. Mutational analysis of human DNase I at the DNA binding interface: implications for DNA recognition, catalysis, and metal ion dependence. *Protein Sci* **7**:628-36.
 29. **Peitsch, M. C., B. Polzar, H. Stephan, T. Crompton, H. R. MacDonald, H. G. Mannherz, and J. Tschopp.** 1993. Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *Embo J* **12**:371-7.
 30. **Perani, P., S. Zeggai, A. Torriglia, and Y. Courtois.** 2000. Mutations on the Hinge Region of Leukocyte Elastase Inhibitor Determine the Loss of Inhibitory Function. *Biochemical and Biophysical Research Communications* **274**:841-844.
 31. **Polzar, B., M. C. Peitsch, R. Loos, J. Tschopp, and H. G. Mannherz.** 1993. Overexpression of deoxyribonuclease I (DNase I) transfected into COS-cells: its distribution during apoptotic cell death. *Eur J Cell Biol* **62**:397-405.
 32. **Potempa, J., A. Dubin, W. Watorek, and J. Travis.** 1988. An elastase inhibitor from equine leukocyte cytosol belongs to the serpin superfamily. Further characterization and amino acid sequence of the reactive center. *J Biol Chem* **263**:7364-9.
 33. **Roehrig, S., A. Tabbert, and E. Ferrando-May.** 2003. In vitro measurement of nuclear permeability changes in apoptosis. *Anal Biochem* **318**:244-53.
 34. **Samejima, K., and W. C. Earnshaw.** 2005. Trashing the genome: the role of nucleases during apoptosis. *Nat Rev Mol Cell Biol* **6**:677-88.
 35. **Shiokawa, D., H. Ohyama, T. Yamada, K. Takahashi, and S. Tanuma.** 1994. Identification of an endonuclease responsible for apoptosis in rat thymocytes. *Eur J Biochem* **226**:23-30.

36. **Silverman, G. A., P. I. Bird, R. W. Carrell, F. C. Church, P. B. Coughlin, P. G. Gettins, J. A. Irving, D. A. Lomas, C. J. Luke, R. W. Moyer, P. A. Pemberton, E. Remold-O'Donnell, G. S. Salvesen, J. Travis, and J. C. Whisstock.** 2001. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem* **276**:33293-6.
37. **Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger, and G. Kroemer.** 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**:441-6.
38. **Torriglia, A., E. Chaudun, F. Chany-Fournier, Y. Courtois, and M. F. Counis.** 2001. Involvement of L-DNase II in nuclear degeneration during chick retina development. *Exp Eye Res* **72**:443-53.
39. **Torriglia, A., E. Chaudun, F. Chany-Fournier, J. C. Jeanny, Y. Courtois, and M. F. Counis.** 1995. Involvement of DNase II in nuclear degeneration during lens cell differentiation. *J Biol Chem* **270**:28579-85.
40. **Torriglia, A., C. Negri, E. Chaudun, E. Prospero, Y. Courtois, M. F. Counis, and A. I. Scovassi.** 1999. Differential involvement of DNases in HeLa cell apoptosis induced by etoposide and long term-culture. *Cell Death Differ* **6**:234-44.
41. **Torriglia, A., P. Perani, J. Y. Brossas, E. Chaudun, J. Treton, Y. Courtois, and M. F. Counis.** 1998. L-DNase II, a molecule that links proteases and endonucleases in apoptosis, derives from the ubiquitous serpin leukocyte elastase inhibitor. *Mol Cell Biol* **18**:3612-9.
42. **van Gent, D., P. Sharp, K. Morgan, and N. Kalsheker.** 2003. Serpins: structure, function and molecular evolution. *Int J Biochem Cell Biol* **35**:1536-47.
43. **Wallace, C. J.** 1993. The curious case of protein splicing: mechanistic insights suggested by protein semisynthesis. *Protein Sci* **2**:697-705.

Figure 1 Sequence alignment of LEI protein in horse, human and pig. By convention, amino acids numbering refers to the PDB nomenclature of serpins that aligns all the serpins to α 1-antitrypsin. UniProtKB/Swiss-Prot entries: horse LEI P05619, human LEI P30740 and pig LEI P80229. Hinge region (green) with inhibitory consensus pattern (dark green); P1-P1' elastase cleavage site (blue). Bold, reactive center loop integrated in the main β -sheet of LEI after cleavage; single elastase recognition site is underlined. Cyan, His 287 lost in human LEI and replaced by Ser. The two cysteines of pig LEI are in purple. The sequences obtained by Edman degradation are indicated in yellow (41). Nuclear localization bipartite signal (red); endonuclease active site (orange). Red arrowheads, points of site directed mutagenesis: 1) main nuclear localization signal mutant, 2) hinge region mutant and 3) endonuclease active site mutant.

Figure 2 Anti-protease and endonuclease activities of three different LEI mutants.

Site directed mutagenesis was performed on a wild type-LEI-pET 23d(+) construction using the Quick-Change mutagenesis kit (Stratagene). An alanine residue of the hinge region of the reactive center loop of LEI (AP10T mutant) or histidine 368 of the endonuclease active site (H368A mutant) were changed into threonine and alanine, respectively. Recombinant proteins were produced in BL21pLysS *E. coli* strain, purified using His-select cartridges (Sigma). Increasing concentrations of wild type or mutant LEI (17.5 to 280 μ g/ml) (magenta and blue lines) were incubated with elastase (0,1 μ g/ml) in the presence of 2 mM of a synthetic substrate, pNaMAAPV. The resulting colored reaction was measured at 405nm (**A**). As a control, elastase and its substrate were incubated alone (red). In the AP10T mutant, the anti-protease activity is strongly affected compared to wild type LEI. On the contrary, this activity is not affected in the H368A mutant.

DNase activity (**B**) was measured after overnight cleavage of LEI with equimolar quantities of elastase at 37°C. 841 ng of wild type, AP10T or H368A LEI were incubated with 2,5 μ g of plasmid DNA at different times at 37°C in 20 mM Tris-EDTA pH 5,5. Untreated wild type LEI was incubated with DNA as a negative control. AP10T mutant shows a DNase activity comparable to wild type, while H368A has no endonuclease activity. No endonuclease activity could be detected for the 358stop mutant but DNA was slowed down with incubation time suggesting its binding to this protein.

Figure 3 Comparison of charges of different endonucleases. LEI, DNase I and CAD structures were analyzed using Protein Explorer (23). Perpendicular views of cleaved LEI (PDB number 1HLE) (top), DNase I (PDB number 3DNI) (middle) and Caspase Dependent DNase or CAD (PDB number 1V0D) (bottom) were charge colored (basic regions in blue, acid in red). DNA binding regions of the three molecules are more basic than the opposite side. That area would easily interact with acidic charges of DNA. Aspartate and histidine residues that seem important for endonuclease activity are indicated in yellow. DNA associated with DNase I is indicated in green. CAD seems to be polarized slightly different, maybe because it is the only one that works as a dimmer.

Figure 4 Site directed mutagenesis of the consensus bipartite NLS signal. (a) Impairment of nuclear translocation is shown for K225A NLS LEI mutant. A cotransfection was made with a GFP-LEI plasmid to validate nuclear translocation of wild type LEI. Right panel shows different distribution of wild type and NLS/K225A mutant LEI. Pictures were taken 16h after induction of apoptosis by 40 μ M 5-(N-ethyl-N-isopropyl)-amiloride (EIPA). Nuclei or apoptotic bodies containing nuclear material are indicated by a dotted line. (b) The lysines of L-DNase II putative NLS were changed into alanines and co-transfected with a wild type LEI construct in BHK cells (as described on methods). Evaluation of the constructs nuclearization during apoptosis induced by 40 μ M EIPA is summarized in the table. Numbers on the left correspond to the last number of the mutated lysine. Green squares represent a “wild type” behavior of the mutated protein and red squares, impaired translocation. Black and grey squares represent no florescent or little fluorescent proteins, hatched squares indicate the presence of fluorescent aggregates in cells. (c) The impairment of nuclear translocation of a mutated lysine was scored 1. This allowed giving a score to each mutant represented on the histogram. Lysine 225 is the most important for L-DNase II nuclearization. (d) K225A LEI mutant is impaired in its ability to fix importin α , compared to wild type LEI. Calmodulin was used as a negative pull down control, as it does not bind directly to importin α ; and a brut extract of HeLa cells was used as a positive control of importin α presence and molecular weight. (e) Pro-apoptotic activity of wild type LEI. The rate of survival of BHK cells overexpressing different LEI constructs show an increased cell death for wild type LEI, compared to mutated constructions. Vectors: empty (yellow), LacZ (dark blue), wild type LEI (green), H368A/endonuclease active site LEI

mutant (pink), K225A/NLS LEI mutant (light blue) and non transfected cells (red). Data represents means \pm standards deviations, * Student's test $p < 0,001$ versus H368A and K225A. **(f)** Endonuclease activity of the K225A NLS LEI mutant. Site directed mutagenesis was performed on the middle lysine of the major cluster of lysines of the nuclear localization signal (NLS). K225A LEI mutant was then incubated with elastase overnight at 37°C to obtain the LEI cleaved form. Incubation of this cleaved form with a plasmid DNA for 10 to 30 minutes allowed us to measure the endonuclease activity of the K225A LEI mutant and compare it with wild type (wt) LEI. K225A LEI showed an endonuclease activity comparable to the one displayed by wild type LEI previously treated with elastase. NLS mutant or wild type LEI were not able to cleave DNA if they were not treated previously with elastase. Plasmid DNA showed no autodegradation or cleavage by elastase.

Figure 5. Molecular structure of LEI and its implication in cellular fate. **(a)** LEI structure was analyzed using Protein Explorer. Native LEI (upper panel) shows the reactive center loop (RCL, green) covering the major cluster of lysines of the bipartite nuclear localization signal (NLS, red). The endonuclease active site is indicated in orange. This three-dimensional structure was obtained from pig LEI sequence. Lower panel shows cleaved horse LEI (PDB number 1HLE) with unmasked NLS (red), after insertion of the RCL in the A β -sheet of the serpin. **(b)** In unstressed cells, LEI is localized in the cytoplasm and has an anti-protease activity, inhibiting preferentially elastase. When the cell receives a stress that will allow transformation of LEI into L-DNase II, the anti-protease activity will be released. L-DNase II will enter the nucleus, cleave DNA and finally lead to apoptosis. Different LEI mutants have been done to understand the LEI/L-DNase II pathway. The NLS mutant (K225A, red) is not able to enter the nucleus. And the endonuclease active site mutant (H368A, green) is able to enter the nucleus but unable to degrade DNA. In both cases apoptosis is reduced. LEI has to be cleaved into L-DNase II and translocated to the nucleus to induce apoptosis.

Supplementary data

Figure 1S: General molecular mechanism of serpins. **(a)** The Stressed to Relaxed Transition: inhibitory serpins are molecules that fold into a native metastable state (here, native antithrombin, PDB number 1E03) and go through a complex conformational change to achieve protease inhibition. Upon docking with a target protease, a flexible exposed region, termed the reactive center loop (RCL) (green), is cleaved on its P1'-P1 site (cleaved antithrombin, PDB number 1ATT). The N-terminal region to the site of proteolysis (termed P2-P15 for antithrombin) inserts then into the center of the large A β -sheet (magenta) of the serpin, forming an additional β -strand and causing a large conformational change throughout the molecule. Loop insertion in the absence of cleavage may also occur. The resulting conformation is called the latent form. The serpin acquires more stability in this way. This latent form is only known for a few serpins *in vivo*, like antithrombin (PDB number 1E04) represented in this figure. Orange lines represent disulfide bridges. **(b)** Tight binding inhibition of a protease by a serpin: the complex formation between a serpin (grey) and a protease (cyan) is mediated by the reactive center loop (RCL) (green) of the serpin (PDB number of the α 1-antitrypsin - trypsin complex 1K9O). When the protease cleaves the serpin on its P1-P1' recognition site, RCL will be inserted and form an additional β -strand in the A β -sheet of the serpin. The protease will then be displaced to the opposite pole of the serpin (PDB number of this cleaved α 1-antitrypsin + trypsin 1EZS). Orange lines represent disulfide bridges.

30 40 50 60 70 80 90
Horse MEQLSTAN IHFAVDLFFA LNESDFIGNI FIEPLSISSA LAMIFLGIRG NITAAQVSKAL YFDIIVE
Human MEQLSSAN IRFALDLFLA LSENFAGNI FISEFSSISA MAMWFLGIRG NITAAQLSKIF HFNIIVE
Pig MEQLSAAAN IRFALDLFFA LNESNEAGNI FISEFSSISA LAMILLGIRG NITAAQMSKAL HFDIVVK
 91 100 110 120 130 140 150 160
Horse DIHSRFQSLN ADINKFGAPY IILKANRLYG ERTYNFLADF LASTQKNYGA ELASVDFQOAP EDARKLINEW
Human EVHSRFQSLN ADINKRGASY IILKANRLYG ERTYNFLPEF LVSTQKIYGA DLASVDFQRRS EDARKINOW
Pig DIHSRFQSLN ADINKRGASY IILKANRLFG ERTYHFLPEF LASTQKIYGA ELASVDFLEFAS EEARKAINEW
 161 170 180 190 200 210 220 230
Horse VKGQTEGKIP ELLVKGWVDNMT KLVLVNRIYF KGNWQEKFMK ZAIRDAPFRL NKKDKITVKM MYQKKKFPYN
Human VKGQTEGKIP ELLASGVDNMT KLVLVNRIYF KGNWQKDFMK ZAITINAPFRL NKKDKRITVKM MYQKKKFAYG
Pig VKEQTEGKIP ELLASGVVDSAT KLVLVNRIYF KGSWQEKEMT ZAINKAPFRL NKKDSKITVKM MYQKKKFPFG
 231 240 250 260 270 280 290
Horse YIEDLKCRLV ELPYQSKELSM IILLPDDIEDESIG LEKIEQLILE KLREWIKPENLY LAEVNWHLFR
Human YIEDLKCRLV ELPYQSELSM VILLPDDIEDESIG LKKEEQILILE KLREWIKPENLD FIEVNVHLFR
Pig YIKELKCRVL ELPYQSKDLSM VILLPDSIEDESIG LRKIEQHLILE KLREWIKPDNLE LLEVNVHLFR
 291 300 310 320 330 340 350 P1-P1
Horse FKLEESYDLI SHLARLGQOD LFNRSKADLSG MSGARDLFVS KIIHKSFVDL NEEGTEAAAA TAGTILMLMLME
Human FKLEESYILN SDLARLGQOD LFNSSKADLSG MSGARDIFIS KIVHKSEVEV NEEGTEAAAA TAGIAIFCMLME
Pig FRLEESYDLN APLARLGQOD LFG.SRADLIG MSLARDLFIS KVVHKSEVEV NEEGTEAAAA TAGIAVFAMLME
 361 370 380 391
Horse EENFNADRRFF IFFIRHNPSA NILFLGRFSSP
Human EENFNADRRFF LFFIRHNSSG SILFLGRFSSP
Pig EEDFNADRRFF IFFIRHNPS NILFLGRLSSP

Fig 1

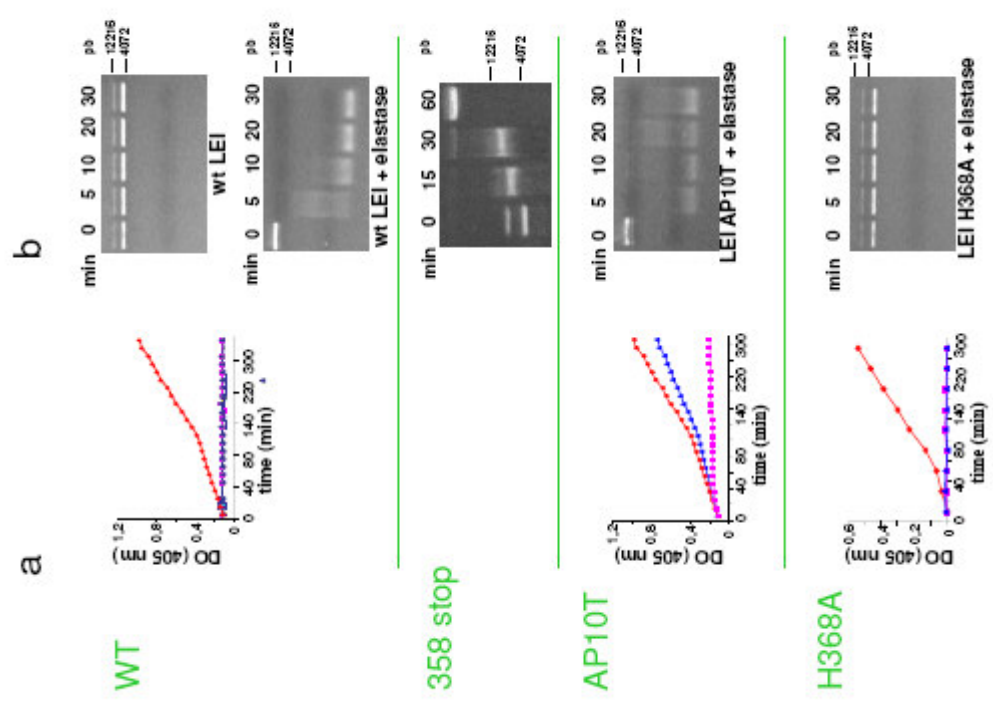


Fig 2

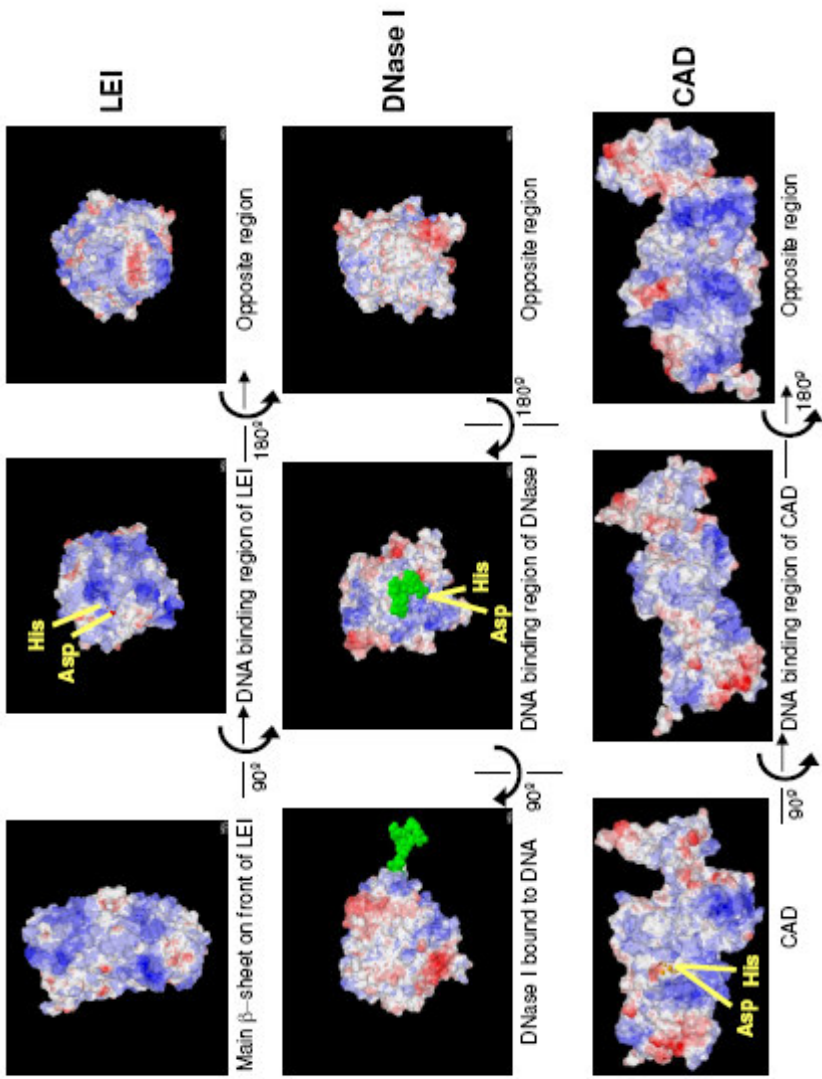


Fig 3

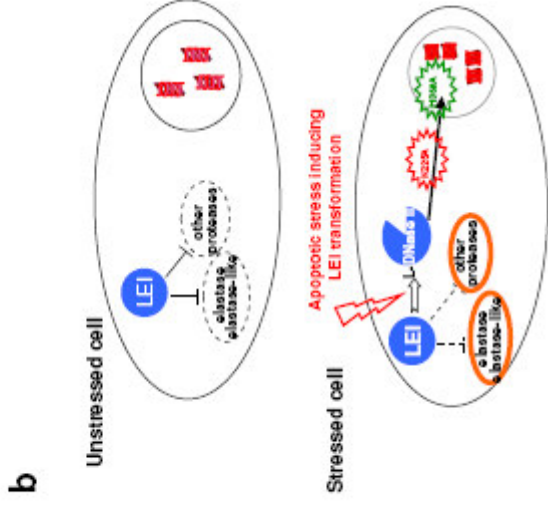
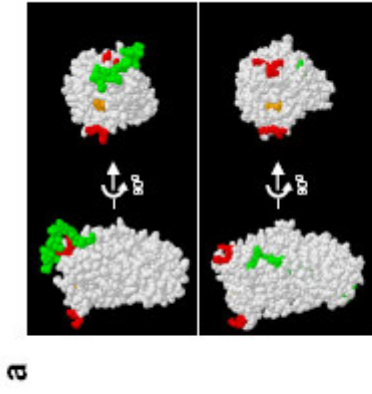


Fig 5