Interaction of Leukocyte Elastase Inhibitor/L-DNase II with BCL-2 and BAX

Imène Jaadane a, Atf Nagbou a,1, Francine Behar-Cohen a,b, Alicia Torriglia a,*

a INSERM UMRS 1138, Centre de Recherches des Cordeliers, Université Paris Descartes, Université Pierre et Marie Curie, 15 Rue de l’Ecole de Médecine, 75006 Paris, France
1 Hôpital Ophtalmique Jules-Gonin, Avenue de France 15, Case Postale 133, 1000 Lausanne 7, Switzerland

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Leukocyte Elastase Inhibitor (LEI, also called serpin B1) is a protein involved in apoptosis among other physiological processes. We have previously shown that upon cleavage by its cognate protease, LEI is transformed into L-DNase II, a protein with a pro-apoptotic activity. The caspase independent apoptotic pathway, in which L-DNase II is the final effector, interacts with other pro-apoptotic molecules like Poly-ADP-Ribose polymerase (PARP) or Apoptosis Inducing Factor (AIF). The screening of LEI/L-DNase II interactions showed a possible interaction with several members of the BCL-2 family of proteins which are known to have a central role in the regulation of caspase dependent cell death. In this study, we investigated the regulation of LEI/L-DNase II pathway by two members of this family of proteins: BAX and BCL-2, which have opposite effects on cell survival. We show that, in both BHK and HeLa cells, LEI/L-DNase II can interact with BCL-2 and BAX in apoptotic and non-apoptotic conditions. These proteins which are usually thought to be anti-apoptotic and pro-apoptotic respectively, both inhibit the L-DNase II pro-apoptotic activity. These results give further insight in the regulation of caspase independent pathways and highlight the involvement of the intracellular environment of a given protein in the determinism of its function. They also add a link between caspase-dependent and independent pathways of apoptosis.

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1. Introduction

Programmed cell death is a process allowing the elimination of harmful cells from tissues. During aging, an unbalance of this process may cause a dysfunction in cell homeostasis which is the basis of many pathological states, like neurodegenerative diseases or cancer. Among the different pathways of cell death [1], apoptosis is the best defined. It is characterized by typical morphological changes, such as chromatin condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies [2]. The morphological state of apoptosis can be achieved by the activation of caspases or non-caspase proteases, in the so-called caspase independent apoptosis [3].

Concerning caspases, there are two major pathways of caspase dependent apoptosis: an extrinsic (via the death receptors located to the plasma membrane) and the intrinsic (mitochondria-associated) pathways. The mitochondrial pathway is highly regulated by the BCL-2 (B-cell lymphoma 2) family members [4,5]. The BCL-2 family of proteins is composed of 3 groups of proteins: anti- and pro-apoptotic members control the permeabilization of the mitochondrial outer membrane and the BH3-only proteins detect the apoptotic signal and activate pro-apoptotic proteins like BAX (BCL-2-associated X protein) or BAK (BCL-2 antagonist/killer-1). This activation allows BAX homo-oligomerization and pore formation in the mitochondrial membrane through which pro-apoptotic factors will be released [6,7]. The process of pore formation is tightly controlled by the anti-apoptotic members of the BCL-2 family. They antagonize pro-apoptotic members by a complex interplay of protein–protein interactions [5].

The BCL-2 proteins have been extensively analysed in the framework of mitochondrial membrane permeabilization, irrelevantly of caspase activation [8–10]. However, mitochondria are not systematically involved in cell death [11–15]. This is the case of the LEI/L-DNase II pathway, which depends on the activation of serine and lysosomal proteases [16–22]. LEI (Leukocyte Elastase Inhibitor) is a cytosolic serine

**Abbreviations:** AIF, Apoptosis Inducing Factor; BAK, BCL-2 antagonist/killer-1; BAX, BCL-2 associated X protein; BCL-2, B cell lymphoma 2; BHK, Baby hamster kidney cells; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; HeLa, Henrietta Lacks cells; HMA, Hexamethylene Amiloride; L-DNase II, LEI-derived DNase II; LEI, Leukocyte Elastase Inhibitor; PARP, PolyADP ribose polymerase

⁎ Corresponding author at: INSERM UMRS 1138, Centre de Recherches des Cordeliers, 15 Rue de l’Ecole de Médecine, 75006 Paris, France. Tel.: +33 144278173; fax: +33 144278163.

E-mail address: alicia.torriglia@inserm.fr (A. Torriglia).

1 Present Address: Ad Rem Technology, 162, rue du Faubourg Saint-Honoré, 75008 Paris.
protease inhibitor. During apoptosis, LEI is transformed, after cleavage, into an endonuclease, the L-DNase II (LEI-derived DNase II), and translocated to the nucleus where it degrades DNA [23,24].

From the data obtained so far, it seems that L-DNase II activation strongly depends on the apoptotic stimulus; L-DNase II is easily induced by metabolic but not by genotoxic stresses. For instance, the LEI/L-DNase II pathway is activated by HMA (Hexamethylene Amiloride), a Na+ /H+ exchanger inhibitor that induces an intracellular pH acidification. Other stimuli, like etoposide (cytotoxic agent), are not able to induce this transformation [21]. Actually, LEI overexpression experiments confirm its pro-apoptotic effect in HMA-induced apoptosis and show that LEI protects cells from etoposide-induced apoptosis, which is caspase-mediated [25].

Indeed, it is likely that the pro- or anti-apoptotic effects of LEI and its derived species depend both on molecular events leading to the molecular transformation of LEI into L-DNase II and on the interaction of these molecules with other apoptotic pathways, including their regulating molecules. A screening of protein–protein interactions between LEI and cellular proteins involved in apoptosis was performed in our laboratory revealing several candidates to LEI/L-DNase II regulation [26]. Among them, several members of the BCL-2 family showed some affinity for LEI suggesting an interaction between LEI and this family of proteins, yielding the hypothesis that the LEI/L-DNase II pathway may be regulated by BCL-2 and related proteins.

In this study, we investigate the putative interaction between LEI and the BCL-2 family of proteins in order to investigate if this caspase independent pathway can also be modulated by BCL-2 and related proteins. As this family of proteins involves at least 22 members, we concentrated on the two best studied proteins having opposite effects on cell survival: BCL-2 and BAX.

2. Materials and methods

2.1. Cell lines and culture conditions

BHK cells (baby hamster kidney) were grown as monolayer at 37 °C in humidified atmosphere containing 5% CO2. BHK were cultured in DMEM glutamax (Dulbecco’s Modified Eagle’s Medium, Gibco) supplemented with 10% Fetal Calf Serum (FCS) and 0.5% of streptomyocin/penicillin (10,000 IU/mL) (all reagents were from Celebio).

HeLa cells (53 clone) were cultured in DMEM (Dulbecco’s Modified Eagle Medium) with Glutamax™, supplemented with 4.5 g/L (25 mM) of D-Glucose, 0.11 g/L (1 mM) Sodium Pyruvate, 10% FCS and 0.5% penicillin/streptomycin at 37 °C under 5% CO2 and 90% of relative humidity.

2.2. Cell death induction

BHK and HeLa cells were seeded at a density of 20,000 cells/cm², maintained in culture for 2 days and then treated for 18 or 24 h with different inducers of apoptosis. Cells were treated with 5-(N, N-hexamethylene) amiloride (HMA; stock solution: 40 mM in DMSO, Sigma-Aldrich, A9561) at 40 μM for 18 h. As a positive control of caspase dependent apoptosis, cell cultures were incubated for 3 h with the topoisomerase II inhibitor, etoposide (Sigma-Aldrich, E1383), at 100 μM. Thereafter, the drug was removed and cells were maintained in culture with a fresh medium for 24 h. In some experiments, cells were treated with staurosporine at 1 μM for 18 h (Sigma).

2.3. Pull down assay

Recombinant proteins (wild-type LEI, and calmodulin) were produced in Escherichia coli strain BL21/pLysS and loaded into His-select cartridges (Sigma) according to the manufacturer’s protocol. Briefly, the E. coli BL21(DE3)pLysS strain (Promega) was transformed with a pET 23d (+) plasmid (Novagen) coding for pig LEI with a 6xHis tag. Cultures were grown in L-Broth containing 34 μg/mL chloramphenicol and 50 μg/mL ampicillin. The expression of the protein was induced when the optical density at 600 nm was 0.6 by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Boehringer, Mannheim) for 2.5 h. Proteins were then extracted with a binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris–HCl pH 7.9). The protein was then bound to a Ni-NTA column (Sigma).

HeLa cells were grown to confluence in 75-cm² flasks before lysis in RIPA buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% sodium dodecyl sulfate, 1 mM phenylmethysulfonyl fluoride, 1 μg/mL leupeptin, 5 μg/mL pepstatin, 5 μg/mL aprozin) and loaded onto the column. After washing, the columns were eluted with 1 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl pH 7.9. In this way the protein bound to the column matrix by the tag together with the interacting proteins were eluted. Samples were dosed and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted for BCL-2, BAX, BID, and BCL-XL. Calmodulin was used as a negative control, and a crude extract of HeLa cells was used as a positive control. Immunoblots were developed by using the following antibodies: BAD: Santa Cruz C7, sc-8044, BCL-XL: Santa Cruz H5, sc-8392, BID: Santa Cruz C20, sc-6538. LEI/L-DNase II: polyclonal anti-rabbit prepared in the laboratory, BCL-2: Santa Cruz, N19, sc-492, BAX: Santa Cruz, N20 sc-493. Experiments were done at least 3 times.

2.4. Co-immunoprecipitation

Co-immunoprecipitation was performed by using the ProFound™ Mammalian Co-Immunoprecipitation Kit (Pierce) in which the antibody was covalently immobilized onto an amine-reactive resin. Before CO-IP, we prepared purified IgG LEI (rabbit) with a DEAE anion-exchange column (Wathman) followed by affinity chromatography on a home-made affinity column. Commercially available anti-BAX and anti-BCL-2 IgGs were also purified by affinity chromatography. 100 μg of proteins from HeLa cell extracts was loaded per experiment. The eluted material was then denatured in Laemmli buffer and analysed by Western blot with LEI or BCL-2, or BAX antibody, using the antibodies described before. The column without IgG was used as a negative control.

2.5. Glycerol gradient

10⁷ BHK or HeLa cells were recovered after 24 h of induction of apoptosis with either etoposide or staurosporine at the concentrations described above. Cells were lysed with 750 μL of lysis buffer (20 mM Hesper, 150 mM NaCl, 0.5 mM DTE). The preparation was sonicated 10 times for 10 s, waiting 15 s between each pulse. 0.2% Triton X-100 was added to each sample and cleared by centrifugation for 10 min at 15,000 g. The supernatant is layered on top of a continuous linear glycerol gradient (40% to 5% glycerol in 20 mM Hesper, 150 mM NaCl, and 0.5 mM DTE, 0.2% Triton X-100). The tubes were centrifuged in a Kontron T-1065 ultracentrifuge at 100,000 g for 18 h. The protein fractions were then recovered by a hole at the bottom of the tube (5 drops each fraction). The different fractions were then analysed by Western blot. The gradient was calibrated using the following proteins: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), and beta-amylose (200 kDa). All proteins were from Sigma (MWGF 200).

2.6. Immunocytochemistry

BHK and HeLa cells were seeded in coverslips placed at the bottom of a 12-multiwell plate at the density of 5 × 10⁴ cells/mL in 2 ml of complete medium. 48 h after seeding they were treated as before. At the end of incubation cells were washed twice with PBS (19 mM Na2HPO4, 1 mM KH2PO4, 140 mM NaCl, 15 mM KCl pH 7.5) containing Ca²⁺ and Mg²⁺, fixed with 4% paraformaldehyde for 15 min (all steps were
performed at room temperature) and then washed twice with PBS. Permeabilization was performed by incubation with 0.3% Triton X-100 for 20 min. The cells were then washed twice with PBS. Non-specific binding sites were blocked by 1 h incubation in a blocking buffer containing 1% non-fat milk in PBS. Cells were then incubated with anti-BAX, anti-BCL-2, or anti-LEI/L-DNase II antibodies at a 1/100 dilution, in 0.1% non-fat milk in PBS for 1 h. For these experiments, we used the same antibodies than before, except for some experiments in which the anti-LEI was from chicken origin, also produced in our laboratory. This was followed by two washes with PBS and incubation for 1 h with a 1/500 dilution of anti-rabbit Alexa Fluor® 488 conjugated antibody, or anti-rabbit Alexa Fluor® 488 conjugated antibody, or anti-chicken Alexa Fluor® 488 conjugated antibody (Invitrogen). Cells were finally washed twice with PBS, incubated for 1 min with 4,6-diamidino-2-phenyl indole dichloride (DAPI) 1/5000 (Sigma-Aldrich) and washed three times with PBS. Coverslips were mounted on microscope slides with fluoromount (SIGMA). Immunoreactivity was visualised using an Olympus microscope BX51 or a Zeiss LSM710 confocal microscope.

2.7. Stable overexpression of LEI, BCL-2 and BAX

To perform overexpression experiments, BHK cells were transfected with pREP-LEI, pcDNA-BCL-2, or pCPT4-BAX using an AMAXA® cell line nucleofector device. pREP-LEI was a home-constructed vector [24] while BCL-2 and BAX vectors were obtained from Addgene (plasmids n°18003 for BCL-2 and 16587 for BAX). Transfected cells were selected by using hygromycin B (SIGMA) (400 μg/ml) for LEI and BAX, or neomycin (SIGMA) (400 μg/ml) for BCL-2. Cells obtained after transfection were analysed by Western blot to confirm the protein overexpression.

2.8. Flow cytometry

Transfected cells were seeded in 12 wells plates 48 h before treatment with HMA. After treatment, cells were trypsinized and resuspended in regular medium. The cell viability was analysed on a Flow Cytometer with excitation at 488 nm and emission measured at 575 and 640 nm by using the ViaCount software. This assay uses a proprietary mixture of two DNA binding dyes to provide detection of viable, apoptotic and dead cells (Millipore 4000-0040).

2.9. Western blot

Protein concentration was assessed by the BCA method (Pierce) according to the manufacturer’s instructions. After dilution of samples with Laemmli buffer, 25 μg of the extracted proteins was separated by 12% SDS-PAGE, immobilized on nitrocellulose membrane (PROTAN®). Protein bands were visualized by chemiluminescent Substrate (Thermo Scientific) and blotted with: anti-BAX, anti-BCL-2, anti-Actin (Santa Cruz, I19, Sc-1616), anti-Lamin B (Santa Cruz, C20, sc-6216) at 1/500 dilution, in 0.1% non-fat milk in PBS. The secondary antibodies conjugated to HRP (Vector) (Cruz, I19, Sc-1616), anti-Lamin B (Santa Cruz, C20, sc-6216) at 1/500 dilution. The secondary antibodies conjugated to HRP (Vector) were used in a 1/5000 dilution. Finally, SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) was used to reveal the signal.

2.10. Preparation of sub cellular fractions

For detection of BAX, BCL-2 and LEI/L-DNase II redistribution after treatment, both cytosolic and nuclear fractions were isolated. Cells were seeded in a 75 cm² Falcon bottle and treated as before. After treatment, attached and floating cells were collected by scraping and washed 2 times in cold PBS (every time at 37 °C for 5 min at 4 °C). Cells were then resuspended in an ice-cold hypo-osmotic solution of 1.5 mM MgCl₂. After mechanical breakage of cell membranes by using a tight-fit Dounce B potter (10–15 strokes), they were centrifuged and cytoplasmic fractions contained in the supernatants were collected. Pellets were washed 2 times with the MgCl₂ solution and resuspended in 100 μl of the M-PER extraction reagent (Pierce 78503). Finally, protein measurement was performed with bicinchoninic acid (BCA™ Protein Assay Kit (Thermo Scientific) using bovine serum albumin (BSA) as standard. Finally, a Laemmli buffer was added for Western blot analysis. Anti-actin was used as loading reference, unless otherwise stated.

2.11. Mitochondrial fractions

Mitochondrial fractions were prepared by using the Mitochondrial Isolation Kit for tissue from Pierce, according to the manufacturer’s instructions. The fractions were analysed by Western blot as before. Mitochondrial fraction was identified by using an antibody directed against the Voltage dependent active channel (VDAC) (Santa Cruz, N18, sc-8828).

2.12. Statistics

One way analysis of variance (ANOVA) was used unless otherwise stated.

3. Results

3.1. Characterization of the interaction between LEI/L-DNase II and BCL-2, LEI/L-DNase II and BAX

Our laboratory has discovered and characterized a caspase-independent pathway of cell death, the LEI/L-DNase II [24]. In order to get more light on the cellular control of this pathway we investigated the LEI-interacting proteins by using an Affymetrix interaction membrane (not shown). The experiments revealed a possible interaction with proteins of the BCL-2 family. Some of these interactions were verified by pull down. To do this we prepared recombinant His-tagged LEI that was loaded on a Ni column. Afterwards, a whole extract of HeLa cells was charged and LEI, together with its interacting protein, was eluted from the column. Fig. 1A shows the pull down of BAD, BCL-XL and BID, while panels B and C show the same experiment for BAX and BCL-2. These results indicated that several members of the BCL-2 family could interact with LEI. From the different members of this family we choose to study more in detail the most known anti- and pro-apoptotic members, BCL-2 and BAX. Experiments were performed on HeLa cells, cancer cells from human origin and on BHK cells, fibroblast from hamster origin, in order to assess the general character and specificity of interactions. We first verified the interaction of these proteins with LEI/L-DNase II by performing pull down experiments. Fig. 1B shows a Western blot of a HeLa cell extract after elution from a LEI loaded column, developed with an anti-BCL-2 antibody. Ctrl represents the control cell extract. An His-tagged calmodulin was used as a negative control. Fig. 1C shows a representative image of the same type of experiment performed by using anti-BAX to develop the Western blot. These results indicated that the LEI/L-DNase II protein is able to bind BCL-2 and BAX in vitro.

Pull down experiments offered high concentrations of bite protein, a condition that could force a weak interaction. To confirm the possibility of these interactions at real cellular concentrations co-immunoprecipitations of the proteins of interest were performed. Western blot analysis of these experiments revealed that BCL-2 was co-immunoprecipitated with an anti-LEI antibody and LEI was co-immunoprecipitated with an anti-BCL-2 antibody (Fig. 1D). Non-specific protein interactions were detected in the column without IgG (negative control). In parallel Western blot analysis of co-immunoprecipitaiton experiments using anti-LEI and anti-BAX revealed that BAX was co-immunoprecipitated with an anti-LEI antibody and LEI was co-immunoprecipitated with an anti-BAX antibody (Fig. 1E). Here again, non-specific protein interactions were detected in the column without IgG (negative control). Results
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presented on Fig. 1 were obtained in HeLa cells. The same results, were obtained with BHK cells (not shown). This result suggested that these physical interactions were not cell type specific.

To further verify these interactions and their possible modification during apoptosis, we used a glycerol density gradient loaded with a whole cell extract from cells (Fig. 2). The cells were left untreated or treated with staurosporine (that activates L-DNase II [19]) or etoposide (that activates caspases but not L-DNase II [25]). BHK or HeLa cell extracts were loaded onto the top of a linear glycerol gradient separated by centrifugation. 25 fractions were collected from the bottom of the gradient and analysed by immunoblotting with anti-LEI, BCL-2 and BAX antibodies. As seen on Fig. 2, in extracts from HeLa cells, induced or not for apoptosis, a zone of co-migration of LEI with BCL-2 or BAX was always found. BHK cells showed the same co-migration of molecules (not shown). In control cells, LEI and BCL-2 (but also LEI and BAX) co-sedimented in 6 fractions in the middle of the gradient, suggesting that they belonged to complexes of various sizes. The glycerol gradient using apoptotic cells also revealed that LEI, BCL-2 and BAX co-sedimented. Indeed, although the absolute distribution of the molecules changed with treatment, this change was the most important for LEI. This molecule largely remained over the gradient on etoposide treated cells while it was found only in three fractions in the gradient of staurosporine treated cells. The gradient profile of BCL-2 and BAX was also affected by different apoptotic treatments but in a lesser extent.

These results indicated that LEI/L-DNase II and BCL-2, as well as LEI/L-DNase II and BAX could be associated with each other and probably with other proteins in apoptotic and non-apoptotic conditions. Of note, in control conditions, LEI showed a single band of 42 kDa corresponding to the native molecule. In staurosporine treated cells a band of 35 kDa, corresponding to L-DNase II [24] and spanning only 3 fractions of the gradient was seen. The distribution of the protein dramatically changed again in etoposide treated cells where the 42 kDa species largely spanned the gradient. A very faint band of L-DNase II was seen in heavier fractions.

Concerning BCL-2, the induction of apoptosis also modified its pattern. Staurosporine treated cells showed an almost identical distribution as the control cells but a second band of an apparent molecular weight of 46 kDa, probably a BCL-2 dimer [28], was detected. The distribution pattern of BCL-2 completely changes when cells were treated with etoposide. An important part of BCL-2 moved to the bottom of the gradient and a 20 kDa fragment appeared, probably corresponding to the caspase 3 or 8 generated fragment [29,30].

For BAX, the 18 kDa band, corresponding to its monomer, was found in all gradients. Upon induction of apoptosis with staurosporine or etoposide, there were two additional bands. The band of 42 kDa was already described as the homodimer of BAX that forms during mitochondrial permeabilization in caspase dependent apoptosis [10] as well as a band corresponding to 26 kDa that was also detected.

Taken together these data indicated that LEI/L-DNase II could interact with these two proteins of the BCL-2 family. However, even though this interaction was molecularly possible, the molecules should be able to physically interact in the cell. This is why the cellular localization of the different partners was investigated.
Fig. 3. Immunolocalization of LEI-L-DNase II, BCL-2 and BAX. Panel A: Immunolocalization of LEI and BCL-2 in control and treated HeLa cells with Staurosporine (sts), HMA (HMA) or etoposide (eto). LEI-L-DNase II shows a cytoplasmic labelling in control cells and a nuclear labelling in cells treated with HMA or staurosporine, indicating the activation of the LEI/L-DNase II. In contrast, LEI is cytoplasmic in etoposide treated cells. BCL-2 is located in the same compartments than LEI in all conditions. Scale bars represent 20 μm. Panel B: Fluorescence profiles of control (Ctr) and HMA treated cells of panel A. The fluorescence plot in the axis of control and HMA conditions seen in Panel A was calculated using the Fiji GNU software. Panel C: Immunolocalization of LEI and BAX in control and treated BHK cells, with staurosporine (sts), HMA (HMA) or etoposide (eto). LEI and BAX labellings were cytoplasmic. In contrast, L-DNase II and BAX labelling were nuclear in apoptotic cells treated with HMA or staurosporine. Scale bars represent 20 μm. Panel D: Fluorescence profiles of control and HMA treated cells. The fluorescence plot in the axis of control and HMA conditions seen in Panel C was calculated using the Fiji GNU software. Panel E: Nuclear and cytoplasmic fractions from HeLa cells treated with HMA (HMA) or untreated (control) were analysed by Western blot using anti-LEI/L-DNase II and anti-BAX antibodies. A cytoplasmic localization of LEI and BAX in control cells was seen. In contrast, the nuclear fraction of HMA treated cells showed L-DNase II and also BAX. Actin was used as a control of charge, lamin B as a control of nuclear purification. The histogram seen on the right shows a quantification of nuclear BAX proteins expressed as a ratio to actin. Values are significantly different p < 0.05 according to the t test.
3.2. Cellular localization of LEI/L-DNase II, BCL-2 and BAX

Once the physical affinity between molecules demonstrated, it was necessary to verify if these interactions were possible in the cell by studying the cellular localization of LEI/L-DNase II, BCL-2 and BAX in HeLa and BHK cells. In Fig. 3, immunocytochemistry was used to verify their subcellular localization in normal and apoptotic cells. In normal HeLa cells, LEI and BCL-2 localized at the cytoplasm. In cells treated with HMA or with staurosporine, there was a nuclear translocation of L-DNase II and BCL-2. In contrast, LEI remained cytoplasmic in cells treated with etoposide which was not able to activate the LEI/L-DNase II pathway (Fig. 3, panels A and B). In all circumstances a localization in the same compartment of BCL-2 and LEI was observed.

Concerning BAX, in both control cells and etoposide-treated, LEI and BAX were localized at the cytoplasm. The punctuated staining suggested a partial mitochondrial localization. In contrast, L-DNase II and BAX were translocated to the nucleus in apoptotic cells treated with HMA or staurosporine (Fig. 3, panels C and D).

The nuclear localization of BAX after treatment with HMA seemed important. As this was an unusual situation, we verified this result by analysing nuclear fractions. A cytoplasmic-nuclear fractionation of HEla cells analysed by Western blot confirmed the presence of L-DNase II and BAX in the nuclear fraction of HMA-treated cells that further supported the nuclear translocation of BAX in this apoptotic pathway (Fig. 3E). Note that the WB presented here is not comparable to the results presented in panel 3A. Here the same amount of protein was loaded in each well. As nuclear protein represents 5–10% of the total cellular proteins, the “nuclear” fractions corresponded to a higher number of cells than the cytoplasmic fractions. The same results were obtained with BHK cells (not shown). Taken together, all these results suggested that LEI could interact with BAX and BCL-2 in different conditions, whether the cell was alive or dying.

3.3. The effect of LEI-BCL-2 and LEI-BAX interactions on cell survival

To estimate the functional impact of these interactions on cell survival, overexpression, using stable transfection techniques, of LEI, BCL-2 or BAX was made in BHK and HeLa cells. Not targeted proteins were preferred in order to avoid alterations in their specific activities introduced by the tag. The three proteins (BCL-2, BAX and LEI) were overexpressed separately or in co-transfection, LEI-BCL-2, and LEI-BAX. Protein expression was verified by Western blot (not shown). After establishing permanent clones, cells were treated with HMA for 18 h. These conditions were selected in order to have a rate of apoptosis allowing to easily identify a modification of cell viability.

Cell viability was then measured by flow cytometry.

In cells transfected with BCL-2 alone, the rate of cell death was decreased compared with non-transfected control cells (Fig. 4A). This is the classical anti-apoptotic effect of BCL-2, widely described in the literature. In contrast, the overexpression of LEI induces the death of more than 50% of cells which confirms the pro-apoptotic effect of LEI in this condition of apoptosis. In cells co-expressing LEI and BCL-2, BCL-2 protected against cell death induced by LEI and seemed able to counter the pro-apoptotic effect of the L-DNase II.

Concerning BAX overexpression, it induced an important increase in cell death, which confirmed the pro-apoptotic effect of this protein largely described in the literature (Fig. 4B). Surprisingly, in cells with co-expression of LEI and BAX, there was a decrease of cell death induced by HMA suggesting that the pro-apoptotic effects of the molecules were mutually invalidated; e.g. simultaneous overexpression of BAX and LEI apparently cancelled the pro-apoptotic effects of the two proteins, returning to a cell death level similar to the one observed in non-transfected BHK cells. To investigate this protective effect of simultaneous overexpression of LEI and BAX, we studied the subcellular localization of these two proteins in the different transfected cells by immunocytochemistry (Fig. 4C). In all control cells, LEI and BAX localize at the cytoplasm. After treatment with HMA, in non-transfected cells and in cells overexpressing LEI, these two proteins were translocated to the nucleus. In contrast, cells overexpressing BAX and both LEI and BAX, showed a perinuclear localization of LEI and BAX, suggesting that the freely nuclear distribution of LEI was impaired. These results were confirmed by nuclear purification and Western blot (Fig. 4, panel D).

Because the degree of freedom of LEI in the cytoplasm could be involved in the mechanism of control of this pathway by BCL-2, we investigated the distribution of this protein in cytoplasmic and mitochondrial fractions (Fig. 5). In this case, we investigated the mitochondrial compartment because this is where BCL-2 is involved in cell survival. We found that LEI was almost evenly distributed between cytoplasmic and mitochondrial fractions in control cells (Fig. 5A). The induction of apoptosis by a caspase activating agent (etoposide) did not modify this distribution. However, the activation of L-DNase II with staurosporine or HMA clearly mobilized the LEI cytoplasmic pool (Fig. 5A). The expression of BCL-2 changed this distribution (Fig. 5B).

In cells overexpressing BCL-2, an important amount of LEI was retained at the mitochondria after induction of apoptosis and the mobilization of the cytoplasmic pool was less important.

4. Discussion

In this paper we show that LEI/L-DNase II interacts with BCL-2 family members in apoptotic and non-apoptotic conditions. The BCL-2 family of proteins has a central role in the regulation of caspase dependent cell death. Our results indicate that caspase independent cell death mediated by LEI/L-DNase II can also be regulated by this family of proteins. It is interesting to note that the interaction BCL-2/LEI/L-DNase II is protective, confirming the already known anti-apoptotic function of BCL-2 [4,5,8]. However the interaction with the classical pro-apoptotic protein BAX seems not to have the expected effect and turns out to be protective in L-DNase II mediated apoptosis. This highlights the involvement of intracellular environment of the protein to the determinism of its function.

Many studies have been done to understand the molecular mechanisms controlling cell death. The BCL-2 family of proteins is thought to be a key player in the regulation of mitochondrial membrane permeabilization (MMP) and thus a knob in caspase dependent apoptosis [31]. Other than cytochrome C that triggers apoptosisome formation, the MMP will also induce the release of other caspase-independent apoptotic factors like AIF [32,33], HtrA/Omi [34] or endonuclease G. However, MMP is not involved in all apoptotic cell death [3,35–39]. We have characterized the LEI/L-DNase II, a caspase independent pathway activated by serine proteases and cathepsins, and involved in the death of many cellular types, either in vivo or in vitro [23,24]. This pathway uses cytoplasmic effectors and does not require MMP. Though, the interaction of this molecule with proteins regulating MMP does not seem necessary per se. However, we show here that LEI can interact with BCL-2 and BAX in apoptotic and non-apoptotic conditions. The molecular interaction has been demonstrated using several approaches: pull down, co-immunoprecipitation and glycerol gradient, suggesting the existence of a robust interaction between these proteins. Note that the glycerol gradient shows a constant co-fractionation of the molecules independent of the intracellular acidiﬁcation (MMP) and thus a knob in caspase dependent apoptosis [23,24].
seen in vitro are also possible in vivo. It is interesting to note the presence of BAX in the nucleus with HMA treatment, which is unusual. No nuclear localization signal has been described on BAX, suggesting that the nuclear presence of the BCL-2 family members is probably due to their interaction with other proteins bearing this signal [41]. Actually, the nuclear localization of BAX has already been described in the case of a treatment of lung cancer cells by hyperthermia [40,42]. These results added to ours, raise the possibility that BAX may lose its classical pro-apoptotic function when sequestered to the nucleus.

Subsequently, overexpression of the different molecules allowed us to estimate the functional impact of these interactions on cell survival. Overexpression of BCL-2 has, as expected, an anti-apoptotic effect, indicating that other than regulating MMP, it is also able to regulate mitochondrial independent cell death. We have previously described that L-DNase II can be activated by cathepsin D after lysosomal membrane permeabilization [17,43]. This event can also be regulated by the BCL-2 family of proteins [44]. However, a direct regulation of L-DNase II by BCL-2 cannot be excluded. Actually, the analysis of LEI and
Fig. 5. Intracellular distribution of LEI. Panel A: BHK cells induced in apoptosis by etoposide (eto) staurosporin (sts) or HMA were fractionated into mitochondrial and cytoplasmic fractions. The obtained extracts were developed using anti-LEI. Actin and VDAC were used to evaluate the purity of the fractions. Only the cytoplasmic pool of LEI is mobilized during L-DNase II mediated apoptosis (sts and HMA). Panel B: the same experiment was performed in cells overexpressing BCL-2 (BCL2). Less mobilization of the cytoplasmic pool of LEI was seen, as quantified in the histogram (compare transfected and not transfected cells (NT)). Statistics were performed using the one way ANOVA test, followed by the Newman–Keuls analysis. Only the significance of the difference of interest is indicated (p < 0.005). PR stands for Ponceau Red.

In conclusion, we show here that the BCL-2 family of proteins is able to regulate at least, two apoptotic caspase independent pathways: AIF and LEI/L-DNase II. Moreover, in cases where L-DNase II and AIF are both activated, having a common inhibitor seems reasonable. However, this is not the case for BAX. BAX was shown to be an essential factor in the mitochondria via its ability to condense DNA [46,47]. LEI/L-DNase II has also a dual function. LEI is pro-apoptotic with HMA and staurosporin for instance, due to its transformation into L-DNase II [50] but it has a protective effect when cells are challenged with etoposide [25]. In fact, our results reinforce the idea that all pro-apoptotic proteins have a vital function in the cell [51].

We have previously shown that L-DNase II can interact with the mitochondrial protein AIF. This interaction enhances the pro-apoptotic activity of both proteins [26]. It has been shown that BCL-2 is also able to inhibit the AIF pathway [9,52] suggesting that BCL-2 could also be able to regulate at least, two apoptotic caspase independent pathways: AIF and LEI/L-DNase II. Moreover, in cases where L-DNase II and AIF are both activated, having a common inhibitor seems reasonable. However, this is not the case for BAX. BAX was shown to be an essential factor in the pro-apoptotic activity of AIF [53,54], but seems to be an inhibitor of L-DNase II [9,52].

Concerning BAX, overexpression experiments in which LEI and BAX are overexpressed showed a protection against L-DNase II induced apoptosis in our paradigm. This result is highly unexpected, as this protein has been widely described as pro-apoptotic [46,47]. Here again, this is essentially in a caspase dependent cell death context, where BAX will form pores releasing pro-apoptotic factors. As we previously pointed out, in our system, BAX is translocated to the nucleus, a possible explanation of its lack of pro-apoptotic activity. Actually, the cellular distribution of BAX is regulated by its interaction with different proteins, some of the BCL-2 family, like BCL-XL [48], but others not belonging to this family of proteins, like Humanin [49] or p53 [41]. Our results suggest that BAX can be either pro or anti-apoptotic according to the context of cell death and depending on which pathways are activated. This dual functionality has already been found among different proteins involved in apoptosis. For example, AIF is an anti-apoptotic protein in the mitochondria via its oxido-reductase activity and it is pro-apoptotic in the nucleus via its ability to condense DNA [46,47]. LEI/L-DNase II also has a dual function. LEI is pro-apoptotic with HMA and staurosporin for instance, due to its transformation into L-DNase II [50] but it has a protective effect when cells are challenged with etoposide [25]. In fact, our results reinforce the idea that all pro-apoptotic proteins have a vital function in the cell [51].

Fig. 6. Molecular structure of LEI and L-DNase II and their putative regulation domain. The structure of LEI in its native conformation (accession no. NP_109591.1) or in its cleaved conformation (PDB ID: 1HLE) was represented using the RasMol software. According to the method of analysis, two regions of putative interaction were identified 281-311 (green) and 231-254 (red).