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LBR, a chromatin and lamin binding protein from the inner nuclear membrane, is proteolyzed at late stages of apoptosis

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SUMMARY

Chromatin condensation and apposition to the nuclear envelope is an important feature of the execution phase of apoptosis. During this process, lamin proteins that are located between the inner nuclear membrane and heterochromatin are proteolyzed by the apoptosis-specific protease caspase 6. We have investigated the fate of nuclear membranes during apoptosis by studying the lamin B receptor (LBR), a transmembrane protein of the inner nuclear membrane. LBR interacts through its nucleoplasmic amino-terminal domain with both heterochromatin and B-type lamins, and is phosphorylated throughout the cell cycle, but on different sites in interphase and mitosis. We report here that: (i) the amino-terminal domain of LBR is specifically cleaved during apoptosis to generate an ~20 kDa soluble fragment; (ii) the cleavage of LBR is a late event of apoptosis and occurs subsequent to lamin B cleavage; (iii) the phosphorylation of LBR during apoptosis is similar to that occurring in interphase. As the association of condensed chromatin with the inner nuclear membrane persists until the late stages of apoptosis, we suggest that the chromatin binding protein LBR plays a major role in maintaining this association.

Key words: Apoptosis, Nuclear envelope, LBR, Lamin

INTRODUCTION

Apoptosis or programmed cell death is an active process in cell auto-destruction which is essential for tissue homeostasis (Wyllie et al., 1980). Diverse death signals activate different pathways that converge toward a conserved execution machinery composed of specific apoptotic proteases (Goldstein, 1997). The hierarchical activation of these proteases (CED-3/caspases) provokes the cleavage of key nuclear and cytoplamic proteins as well as the activation of DNases, leading to the nuclear and cytoplasmic lesions characteristic of apoptotic cell death (Orth et al., 1996b; Martins and Earnshaw, 1997). Cell-free systems based on the cytosol of apoptotic or normally growing cells reproduce in vitro several of the changes observed in apoptotic cells (Lazebnik et al., 1993; Newmeyer et al., 1994; Liu et al., 1996).

Apoptotic nuclear modifications are characterized by a progressive condensation of chromatin at the periphery of the nucleus (Wyllie et al., 1980). Condensed chromatin masses further bleb off the nuclear surface and ultimately disperse into the cytoplasm. In parallel with these morphological changes, chromatin is cleaved into nucleosomal and polynucleosomal fragments due to the activation of a specific nuclease (Enari et al., 1998; Sakahira et al., 1998). The nuclear proteins which are substrates for caspases act at different levels, including DNA repair, DNA processing, RNA splicing and RNA maturation (Kaufmann, 1989; Kaufmann et al., 1993; Earnshaw, 1995; Casciola-Rosen, 1995, 1996). Structural proteins of the nuclear matrix such as lamins, NuMa protein, and topoisomerase II, are also key substrates for caspases (Kaufmann, 1989; Hsu and Yeh, 1996; Oberhammer et al., 1994; Weaver et al., 1996; Gueth-Hallonet et al., 1997). Contrasting with the early cleavage of lamins (Neamati et al., 1995; Weaver et al., 1996), the nuclear envelope persists until the late stages of apoptosis in vivo and in vitro (Wyllie et al., 1980; Lazebnik et al., 1993; Rao et al., 1996). In this study, we investigated the fate of nuclear membranes during apoptosis at the molecular level, using the lamin B receptor (LBR) as a membrane marker.

LBR is an integral protein of the inner nuclear membrane which has been characterized in chickens and humans (Worman et al., 1990; Ye and Worman, 1994) and shown to play a major role in nuclear envelope chromatin interactions both during interphase and mitosis. LBR has a nucleoplasmic amino-terminal domain of ~200 amino acids followed by a hydrophobic, carboxyl-terminal domain with eight putative transmembrane segments. The amino-terminal domain of LBR contains three domains, each of them interacting with a nuclear component, B-type lamins, DNA, and HP1 chromatin proteins, respectively (Soullam and Worman, 1993; Ye and Worman, 1994, 1996; Ye et al., 1997; see also Fig. 1). At the onset of mitosis, LBR is phosphorylated by cyclin B/p34^{cdc2} protein kinase (Courvalin et al., 1992) and the nuclear envelope disassembles (Chaudhary and Courvalin, 1993; Buendia and Courvalin, 1997). After metaphase, LBR containing membranes are targeted back to decondensing chromosomes and the nuclear envelope reassembles (Collas et al., 1996: Buendia and Courvalin, 1997; Ellenberg et al., 1997). Here we

show that, during apoptosis, LBR is: (i) cleaved within its amino-terminal domain at a late stage of the process; (ii) phosphorylated as in interphase. Therefore LBR may play a role in the maintenance of chromatin-nuclear membranes interactions until the late stages of apoptosis.

MATERIALS AND METHODS

Cell culture and drug treatments

Chicken DU249 hepatoma cells (Langlois et al., 1974) were grown in monolayer in RPMI 1640 culture medium containing 10% FBS. Apoptosis was induced according to two different protocols. The first protocol was a modification of that of Lazebnik et al. (1993). Cells were blocked in S phase with aphidicolin (1 μg/ml medium) for 15 hours, then released from the block for 4 hours or more as indicated in the text. Alternatively, cells were incubated from 4 to 7 hours with actinomycin D at a concentration of 7.5 μg/ml to block transcriptional activity. Both treatments provoked the detachment of a fraction of the cell population (floating cells). Further shaking of the flasks detached an additional cell population that was combined with the floating cells to provide the apoptotic cell population. Attached cells (preapoptotic) were harvested by trypsinization followed by washing in serum containing culture medium. In some experiments floating cells were collected without shake off, as indicated in the text. Necrosis was induced by addition of 10% ethanol for 4 hours in culture medium and detached cells were recovered by shake off (Re et al., 1994). Cell viability was checked by Trypan blue exclusion.

Kinetics of LBR proteolysis in vivo were performed on floating cells collected without shake off after a 4 hour incubation in drug-free medium following aphidicolin treatment. Cells transferred to a new culture flask were further incubated for 30, 60 and 120 minutes at 37°C before analysis.

Antibodies and drugs

Mouse monoclonal antibody R7 (Bailer et al., 1991), directed against chicken LBR, was provided by Dr E. Nigg (Université de Genève, Switzerland). Human anti-LBR antibodies (Courvalin et al., 1990) and rabbit polyclonal antibodies directed against a peptide of human B-type lamins have been previously described (Chaudhary and Courvalin, 1993). Aphidicolin and actinomycin D obtained from Sigma (St Louis, MO) were solubilized in DMSO at a concentration of 2 mg/ml and 10 mg/ml, respectively, and kept frozen at –20°C until use. They were then diluted as indicated in the text.

Determination of apoptosis

Apoptosis was assessed by analysis of DNA degradation and changes in nuclear structures. Samples of 3×10^6 cells were washed with PBS, then treated as described by Pittman et al. (1994) except for lysis which was performed by an overnight incubation in the presence of 0.5% SDS at 55°C. The DNA pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and samples corresponding to 1.5×10^6 cells were resolved on a 1.8% agarose gel.

Modifications of the nuclear structure were documented by fluorescence microscopy examination of DAPI (0.1 μg/ml) stained cells after fixation in methanol for 10 minutes at –20°C. Nuclei with a bright staining localized either at the periphery of the nucleus or within globular bodies were considered as apoptotic.

Indirect immunofluorescence

Apoptotic cells were pelleted on polylysine-treated coverslips as previously described (Buendia et al., 1990), while untreated cells were grown on glass coverslips. Coverslips were rinsed in PBS, fixed as described above, then analyzed by double immunofluorescence as previously described (Buendia and Courvalin, 1997). Primary antibodies were a mixture of mAb R7 directed against LBR and rabbit polyclonal anti-lamin B antibodies diluted at 1:1,000 and 1:150, respectively. Secondary antibodies were a mixture of Texas Red goat anti-mouse antibody (1:120 dilution) and FITC goat anti-rabbit antibody (1:50 dilution). DNA was stained with DAPI as described above.

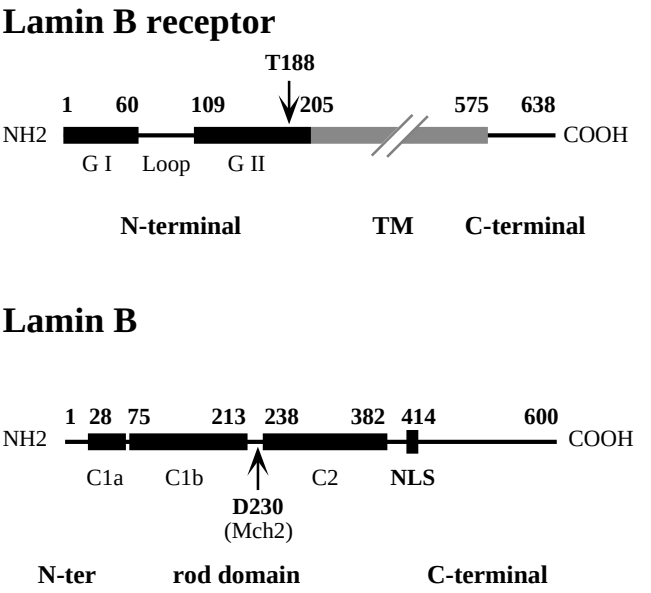

Fig. 1. Schematic representation of the secondary structure of LBR and B-type lamins. For LBR, the amino-terminal domain (N-terminal), the transmembrane domains (TM) and the carboxyl-terminal domain (C-terminal) are shown. Bold lines refer to two globular domains of the N and that have been described previously (Ye et al., 1997). Numbers refer to the amino acids that mark the limits of the different domains. T188 refers to the threonine residue that is phosphorylated in mitosis (Courvalin et al., 1992). For B-type lamins, the three coils of the rod domain (bold line), the amino-terminal (N-ter) and the carboxyl-terminal domains (C-terminal), are shown. NLS refers to the nuclear localization signal and D230 to the aspartic residue upstream of the cleavage site recognized by Mch2/caspase 6 (Rao et al., 1996).

Cell fractionation in the presence of non-ionic detergents

Apoptotic DU249 cells (5×10^6) were washed in PBS, then collected by centrifugation at 400 ***g*** for 5 minutes. The pellet was resuspended in 1 ml of medium containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, digitonin (40 μg/ml), proteases inhibitors (1 mM PMSF, 100 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin, 10 μg/ml antipain), and phosphatase inhibitors (20 mM β-glycerophosphate, 5 mM NaFl and 5 mM Na pyrophosphate). After a 15 minute incubation at 4°C, permeabilized cells were collected by centrifugation as described above. The supernatant containing cytosolic proteins was precipitated with 10% TCA, washed in 90% ethanol, and finally resuspended in Laemmli sample buffer (Laemmli, 1970), while the pellet was directly solubilized in the same medium. Untreated monolayers of DU249 cells were treated for 3 minutes by trypsin-EDTA. After detachment, cells were washed successively in serum containing culture medium, then PBS, before permeabilization by digitonin and further processing as described above. All SDS solubilized fractions were made 100 mM DTT, then heated for 15 minutes at 70°C. DNA was sheared by repeated passages through 21G then 26G needles before loading of the samples on polyacrylamide gels.

Triton extraction was performed at a concentration of 10^6 cells per

ml in a medium containing 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, and the proteases inhibitors indicated above, either in the presence or absence of NaCl (150 mM). After a 20 minute incubation at 4°C, cells were centrifuged at 18,000 ***g*** for 20 minutes, and supernatant and pellet were further processed as described above.

Immunoblotting and immunoprecipitation

Apoptotic and control cells were washed in PBS, pelleted by centrifugation, then resuspended in lysis medium containing 50 mM Tris-HCl, pH 8, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% BSA and a mixture of phosphatases and proteases inhibitors as described above. Lysates were sonicated, then centrifuged at 18,000 ***g*** for 15 minutes. Proteins of the supernatants were precipitated with 10% TCA before solubilization in SDS sample buffer. Proteins were resolved by SDS-PAGE on 10, 12 or 15% polyacrylamide gels according to the method of Laemmli (1970). Immunoblotting experiments were performed as previously described (Buendia and Courvalin, 1997). Nitrocellulose sheets were probed with mAb R7 (1:3,500 dilution), or with human anti-LBR antibodies (1:1,000 dilution), or with polyclonal rabbit anti-lamin B serum (1:1,000 dilution), then incubated with the appropriate secondary antibodies coupled to horseradish peroxidase and finally revealed with the ECL technique, following the instructions of the manufacturer (Amersham).

For immunoprecipitation experiments, untreated and aphidicolin-treated cells were incubated for one hour in phosphate-free medium, then for an additional hour in the presence of $^{32}P_i$ (2.5 mCi/ml). After $^{32}P_i$ incorporation, apoptotic cells were recovered by shake off, while attached cells were collected by scraping. Cells were collected by centrifugation then processed for immunoprecipitation and thin layer chromatography analysis of immunoprecipitated material as previously described (Favreau et al., 1996).

In vitro proteolysis of LBR in apoptotic cell extract

Cell extracts were prepared from aphidicolin-treated cells collected by shake off 4.5 hours after drug removal. Cells (80% apoptotic) were sedimented at 800 ***g*** for 10 minutes, washed in cold PBS, then resuspended in 20 volumes of extraction medium containing 50 mM Pipes, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 1 μg/ml cytochalasin D and a mixture of proteases inhibitors (1 mM PMSF, 5 μg/ml of leupeptin, pepstatin, antipain and chymostatin). Cells were collected by centrifugation, resuspended in one volume of the same medium, then pelleted at 800 ***g*** for 10 minutes in a 1 ml volume Dounce glass homogenizer. Cells disrupted by 5 cycles of freezing and thawing combined with grinding with a pestle, were cleared by centrifugation at 10,000 ***g*** for 15 minutes. The supernatant (S10) brought to 2 mM ATP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase was incubated at 37°C for 0, 30, or 60 minutes. Reactions were stopped by addition of SDS sample buffer and samples were resolved by SDS-PAGE on 12% polyacrylamide gels, followed by immunoblotting analysis.

RESULTS

Induction of apoptosis in DU249 cells treated with aphidicolin or actinomycin D

Chicken DU249 cells growing as a monolayer, were treated for 15 hours with aphidicolin, then grown for 4.5 hours after removal of the drug. Thirty per cent of cells were detached by shaking off, among which 63 to 70% were alive, as judged by Trypan blue exclusion. Fig. 2 shows a comparison of some biochemical and morphological features of the detached cells with that of cells attached to the substratum. Agarose gel

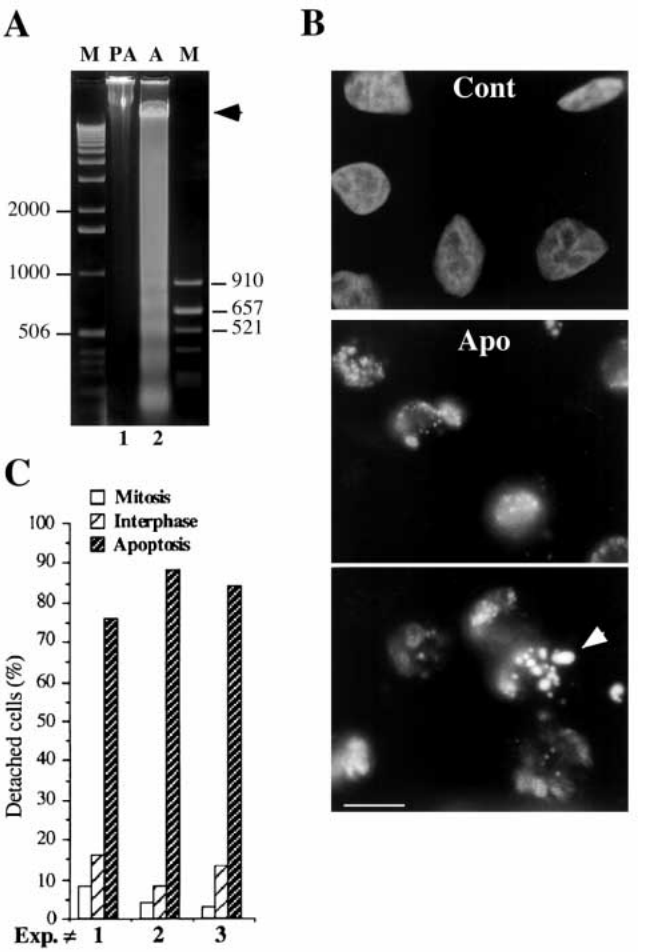

Fig. 2. Aphidicolin treatment induced apoptosis in DU249 cell line. DU249 cells previously treated by aphidicolin for 15 hours were then grown in a drug-free medium for an additional 4.5 hours. (A) Agarose gel electrophoresis of DNA extracted from attached pre-apoptotic cells (lane 1) and detached apoptotic cells (lane 2). Molecular mass markers in lanes M are indicated in base pairs. Note the oligonucleosomal ladder in the apoptotic cells (lane 2). (B) DAPI staining of chromatin in control cells (Cont, upper panel) and apoptotic cells (Apo, lower panels). At an ultimate stage of apoptosis chromatin masses disperse in the cytoplasm (arrowhead). Bar, 10 μm. (C) Percentage of interphasic, mitotic and apoptotic cells among the detached cells in three independent experiments.

electrophoresis of DNA extracted from detached cells (Fig. 2A, lane A) revealed undegraded DNA similar to DNA from attached cells (Fig. 2A, lane PA, arrowhead) and control cells (data not shown), but also DNA fragments the size of oligonucleosomes (Fig. 2A, lane A) as previously described in apoptotic cells (Wyllie, 1980). Changes in the nuclear structure of apoptotic cells were analyzed in parallel by DAPI staining and immunofluorescence microscopy (Fig. 2B). Compared to the DNA of control untreated cells (Fig. 2B, upper panel), the DNA of most detached cells (Fig. 2B, two lower panels) was condensed into dense masses lining the nuclear envelope, or dispersed throughout the volume of the cell (Fig. 2B, arrowhead). The counting of cells in the detached cell population showed that 80% were apoptotic, 16% interphasic-like, and 4% mitotic (Fig. 2C). Due to the high percentage of

apoptotic cells among detached cells, this cellular fraction was refered to as 'apoptotic cells', while cells remaining attached to the flask were referred to as 'preapoptotic'. The latter term was appropriate since with time all cells were committed to apoptosis.

Actinomycin D was used as an alternative for aphidicolin in the induction of apoptosis (Naora et al., 1996; Barge et al., 1997; Shiokawa et al., 1997). A five-hour treatment with actinomycin D was able to induce DNA degradation and chromatin condensation in detached cells (data not shown). However, the percentage of apoptotic cells in this cell population (50%) was lower than obtained with aphidicolin treatment and increased only slightly with longer treatment (60% at 7 hours), at the expense of cell survival as judged by trypan blue exclusion (55%). Therefore aphidicolin was chosen as an apoptosis inducer in most of the following experiments.

Proteolysis of LBR and lamin B_2 during apoptosis

Since two ligands of LBR in the nucleoplasm, lamin B_2 and DNA are cleaved during apoptosis, the putative proteolysis of LBR during apoptosis was investigated. Apoptotic and preapoptotic cells obtained after aphidicolin and actinomycin D treatment were lysed and cellular proteins analyzed by SDS-PAGE, followed by nitrocellulose transfer and detection with antibodies to LBR (Fig. 3A). Two antibodies were used, a monoclonal antibody (R7) specific for chicken LBR (Bailer et al., 1991) and a human polyclonal antibody of autoimmune origin (HAb) which recognizes both human and chicken LBR (Courvalin et al., 1990). With both antibodies a protein with an apparent molecular mass of ~58 kDa, corresponding to intact LBR, was revealed in all samples (Fig. 3A, lanes 1-6, arrow). However, in apoptotic cells, this signal was fainter (Fig. 3A, compare lane 2 to lane 1) and an additional polypeptide with an apparent molecular mass of ~20 kDa was observed when both protocols were used to induce apoptosis and when either antibody was used to reveal LBR (Fig. 3A, lanes 2, 4, and 6, arrowhead). Since the 20 kDa (p20-LBR) immunoreactive component was recognized by both antibodies, it is likely that it corresponds to a proteolytic fragment of LBR. The epitope recognized in LBR by the human serum has been localized in the first 60 amino acids of the human protein (Lin et al., 1996), whose sequence is 72% identical to that of chicken LBR (Ye and Worman, 1994). Therefore, p20-LBR corresponds to the amino-terminal domain of chicken LBR before the first transmembrane domain (calculated M_r 23,790; see Fig. 1). Since the 20 kDa fragment was detected by mAbR7, the epitope recognized by this antibody should also be contained in the amino-terminal part of LBR. As there was no epitope for the antibodies in the carboxyl-terminal domain of LBR, other proteolytic fragment(s) of LBR were not detected. Attempts to raise antibodies against two conserved peptides flanking the hydrophobic domain of LBR were unsuccessful.

To rule out the possibility that the cleavage of LBR could be the result of cell death by a mechanism different from apoptosis, the putative proteolysis of LBR in cells committed to necrosis by a 10% ethanol treatment was investigated. No morphological chromatin modification characteristic for apoptosis (data not shown) nor any proteolysis of LBR were observed (Fig. 3A, compare lanes 7 and 8).

Proteolysis of lamin B_2 was analyzed in parallel with that of LBR under similar experimental conditions. Lysates of preapoptotic and apoptotic cells obtained as described above were analyzed by probing blots with antibodies directed against the nuclear localization signal (NLS) of the carboxyl-terminal domain of B-type lamins (see Fig. 1). A 66 kDa signal corresponding to native lamin B_2 was revealed in control untreated cells (Fig. 3B, lane 1) and in preapoptotic cells (Fig. 3B, lanes 2 and 4). However, in apoptotic cells this signal was faint compared to a major ~46 kDa component (Fig. 3B, lanes 3 and 5) corresponding to the proteolytic fragment of lamin B_2

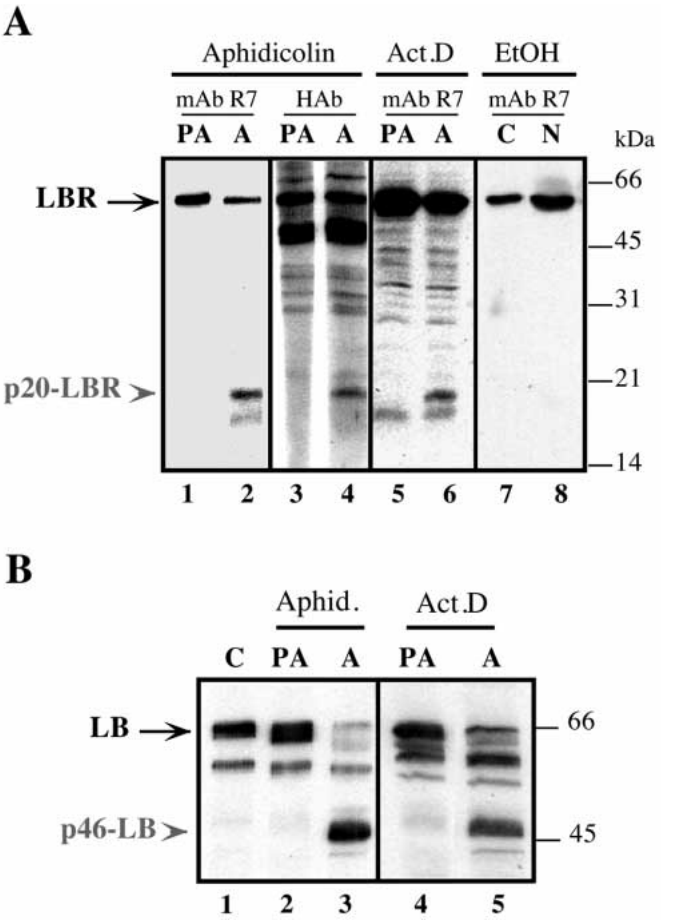

Fig. 3. LBR and lamin B_2 are specifically proteolysed during apoptosis. Control DU249 cells (C), preapoptotic (PA) and apoptotic (A) cells obtained after standard aphidicolin treatment or after a 5 hour actinomycin D (Act. D) incubation, and necrotic cells (N) obtained by ethanol (EtOH) treatment, were lysed in the presence of Triton X-100 and SDS, then revealed by immunoblotting with either anti-LBR (A) or anti-lamin B antibodies (B). (A) Lanes 1, 3, and 5, preapoptotic cells (2×10^5/lane); lanes 2, 4, and 6, apoptotic cells (3×10^5/lane); lane 7, control untreated cells (1×10^5); lane 8, necrotic cells (5×10^5). Immunodetection of LBR was performed using mAb R7 in lanes 1, 2, 5-8 and human autoantibodies in lanes 3 and 4. With both antibodies, a band of ~58 kDa corresponding to the native LBR was detected in all cell lysates (arrow). A ~20 kDa immunoreactive degradation product was detected in apoptotic cells (lanes 2, 4, and 6, arrowhead). Note the absence of proteolysis in necrotic cells. (B) Lane 1, control untreated cells; lanes 2 and 4, preapoptotic cells; lanes 3 and 5, apoptotic cells. Note that a ~46 kDa immunoreactive fragment of lamin B_2 (arrowhead) is only detected in apoptotic cells, in parallel with the disappearance of the 66 kDa signal corresponding to native lamin B_2 (arrow).

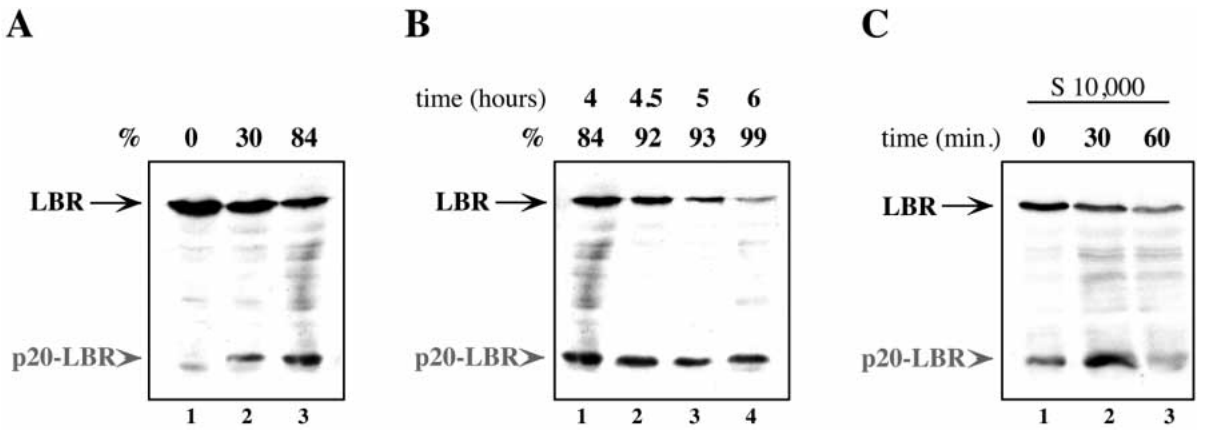

Fig. 4. Time course of LBR proteolysis in vivo and in vitro. (A) apoptotic cells induced by a 15 hours aphidicolin treatment followed by a 4 hour incubation in the absence of the drug were fractionated into three populations, preapoptotic cells (lane 1), loosely attached cells (lane 2) and floating cells (lane 3), then analyzed by immunoblotting with anti-LBR antibodies. In each lane, the percentage of cells of the population with chromatin dispersed in the cytoplasm is indicated (%). (B) The floating cell population collected 4 hours after release of the drug (lane 1) was further incubated at 37°C for an additional 30, 60, or 120 minutes, therefore for a total time of 4.5, 5, and 6 hours after removal of the drug (lanes 2 to 4), before similar blotting analysis. Amount of late apoptotic cells in each population is indicated in %. Note the parallelism between the proteolysis of LBR and the percentage of cells at a late stage of apoptosis. (C) In vitro degradation of LBR in a 10,000 ***g*** supernatant (S 10,000) from an apoptotic cell extract. The extract was incubated at 37°C for 0, 30 or 60 minutes, then processed for immunoblotting as above. Note the time-dependent proteolysis of LBR.

generated in apoptosis (Kaufman, 1989; Lazebnik et al., 1993; Oberhammer et al., 1994). One interpretation for the almost total cleavage of lamin B_2 and the partial cleavage of LBR in the heterogeneous population of apoptotic cells was that lamin B_2 was a substrate for the protease activated at early stages of apoptosis, while LBR was cleaved only at late stages of apoptosis. This interpretation was supported by two sets of experiments. First cells were fractionated after aphidicolin treatment into three populations, a floating cell population, a loosely attached cell population that was further detached by gentle shake off, and the attached cell population (preapoptotic). In each cell population, late apoptotic cells with dense chromatin globules dispersed throughout the cytoplasm (see Fig. 2B, arrowhead) were counted. The percentage of cells of this type was zero in preapoptotic cells, 30% in the loosely attached cell population, and 84% in the floating cell population. A parallel analysis by immunoblotting of native LBR and p20-LBR proteolytic fragment showed that proteolysis of LBR was correlated with the percentage of cells at a late stage of apoptosis (Fig. 4A, compare lane 3 with lanes 1 and 2). In another set of experiments, floating cells obtained 4 hours after removal of aphidicolin were isolated and incubated for additional periods of time. The apoptotic process then progressed until completion since 99% of the cells showed dense masses of chromatin dispersed in the cytoplasm after 2 hours of incubation. Under these conditions, LBR proteolysis also progressed to completion, with an almost complete disappearance of the ~58 kDa signal after 2 hours, i.e. 6 hours after the end of the aphidicolin treatment (Fig. 4B, compare lane 4 to lanes 1-3). The fact that there was no increase in intensity of signal for p20-LBR while LBR signal was disappearing suggests that p20-LBR is also proteolyzed during the process of apoptosis.

Altogether these data show that lamin B and its ligand LBR are sequentially proteolyzed, at early and late stages of apoptosis, respectively.

In vitro degradation of LBR in apoptotic extracts

In vitro LBR proteolysis was studied in a cell free extract obtained from aphidicolin treated DU249 cells lysed by repeated freezing and thawing combined with Dounce homogenization. A 10,000 ***g*** supernatant extract (S10) prepared from the lysate was incubated at 37°C for 30 and 60 minutes, then analyzed by immunoblotting using mAbR7 (Fig. 4C, lanes 1 to 3). The lysate contained both the native and the proteolyzed forms of LBR. Within the first 30 minutes of incubation there was a decrease in the 58 kDa signal for native LBR with a parallel increase in the 20 kDa LBR proteolytic fragment (Fig. 4C, compare lanes 1 and 2). LBR proteolysis progressed for 60 minutes in parallel with the disappearance of p20-LBR, confirming that this peptide is a transient product in the process of LBR proteolysis. When the same assay was performed in the cytoplasmic extract from untreated cells, no proteolysis was observed (data not shown), demonstrating the apoptosis specific origin of the LBR protease.

Cell localization of digested fragments of LBR and lamin B in apoptotic cells

The presence of B-type lamins and LBR at the inner nuclear membrane results from their mutual interactions and also from their interactions with chromatin. As each of these components is cleaved during apoptosis, cell localization of the proteolytic fragments of LBR and lamin B_2 during that process was investigated. Untreated and aphidicolin-treated DU249 cells were extracted with the cholesterol specific detergent, digitonin, under conditions used where plasma membrane but not nuclear membrane were permeabilized (Adam et al., 1990). After permeabilization, cells were fractionated by low speed centrifugation to separate the nuclear fraction from the soluble cytoplasmic fraction. When DNA extracted from both fractions was analyzed by electrophoresis on agarose gel, a high molecular mass DNA signal corresponding to the size of undegraded control DNA (Fig. 5A, lane 4) was still present in

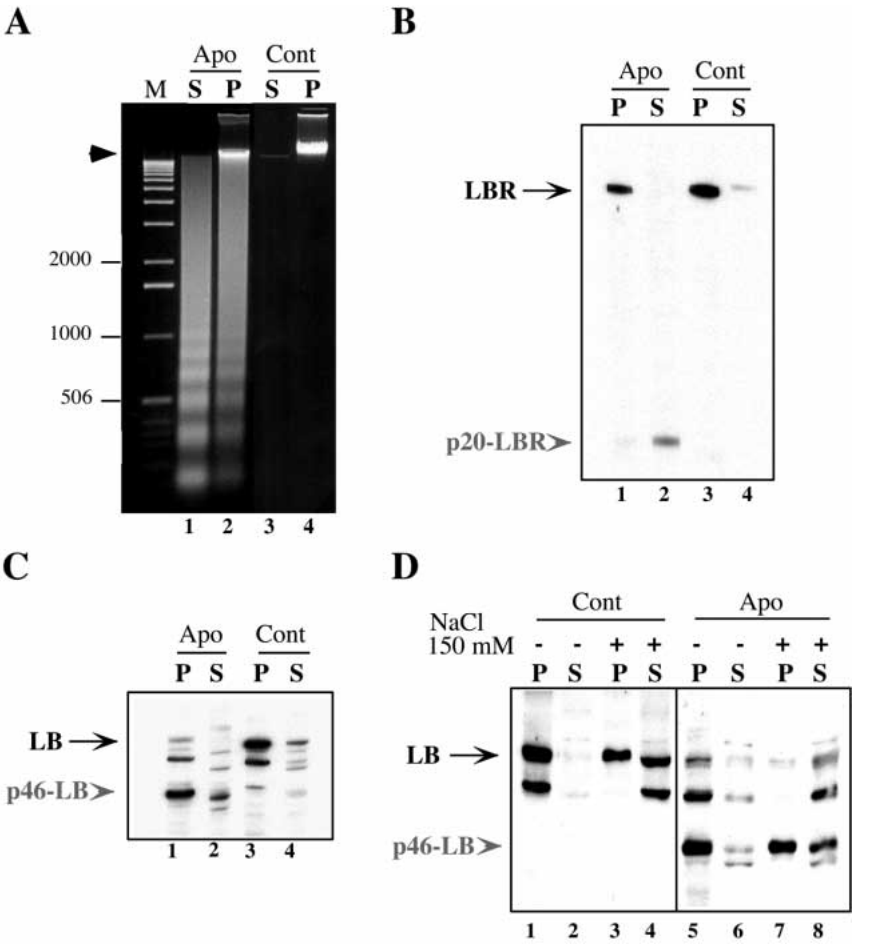

Fig. 5. Non-ionic detergent fractionation of apoptotic cells. (A) Apoptotic cells (Apo) induced by aphidicolin and control cells (Cont) were lysed in the presence of digitonin and soluble (S) and insoluble (P) fractions separated by centrifugation. The various fractions were analyzed for their DNA content by electrophoresis on a 1.8% agarose gel. Undegraded DNA was observed in the insoluble fraction from untreated (lane 4, arrowhead) and treated cells (lane 2, arrowhead), whereas digested DNA from apoptotic cells was present in both the soluble and insoluble fractions (lanes 1 and 2). (B) Proteins present in the cellular fractions described in A were analyzed by immunoblotting for the presence of LBR. The ~20 kDa proteolytic fragment of LBR was present in the soluble fraction from apoptotic cells (lane 2, arrowhead) and virtually absent from the insoluble fraction (lane 1) whereas native LBR was present in the pellet fraction of both apoptotic and control untreated cells (lanes 1 and 3, arrow). (C) Lamin B_2 was revealed in the same cellular fractions by immunoblotting. The ~46 kDa proteolytic fragment specifically generated in apoptotic cells was mainly present in the insoluble fraction of apoptotic cells (lane 1, arrowhead) as the intact lamin B_2 of control cells (lane 3, arrow). (D) Apoptotic cells and control cells were lysed in a medium containing 1% Triton X-100, either in the presence (+) or absence (-) of NaCl (150 mM) and soluble (S) and insoluble (P) fractions were isolated by centrifugation and further revealed by immunoblotting for the presence of lamin B_2. Note that in the absence of salt, lamin B_2 and p46-LB are both present in the insoluble fractions (lanes 1 and 5) and virtually absent from soluble fractions (lanes 2 and 6). In Triton/NaCl, both LB_2 and p46-LB were still found in the insoluble fraction (lanes 3 and 7), as well as in the soluble fraction (lanes 4 and 8).

the nuclear fraction from apoptotic cells (Fig. 5A, lane 2), while DNA fragments of the size of oligonucleosomes were detected in both the nuclear and cytoplasmic fractions of apoptotic cells (Fig. 5A, lanes 1 and 2). Cytosolic DNA likely originated from the cell fraction with disrupted nuclear envelopes. Immunoblotting analysis of proteins from soluble and insoluble fractions showed that, while intact LBR from apoptotic and control cells was only present in the nuclear pellet (Fig. 5B, lanes 1 and 3), p20-LBR from apoptotic cells was found quite exclusively in the supernatant fraction (Fig. 5B, lane 2). On the contrary, the 46 kDa proteolytic fragment of lamin B_2 (p46-LB) was found predominantly in the nuclear fraction (Fig. 5C, lanes 1 and 2) as lamin B_2 from untreated cells (Fig. 5C, lanes 3 and 4). The presence of all p20-LBR in the cytoplasmic fraction is in agreement with its occurence at late stages of apoptosis at the time of NE breakdown. The small fraction of cytoplasmic p46-LB is likely to have the same origin. Concerning the insoluble 46 kDa-LB fraction, we investigated the possibility of this proteolyzed fragment to be organized into a Triton-resistant meshwork as observed for native lamins (Gerace and Blobel, 1980; Stick et al., 1988). As lamin extractability differs among different cell types, lamin B_2 solubilization by Triton in control DU249 cells was first investigated. Fig. 5D (lanes 1-2) shows that lamin B_2 was unextracted by 1% Triton X-100 in the absence of salt, but was partially extracted when the same extraction was performed in the presence of NaCl at a concentration of 150 mM (Fig. 5D, lanes 3 and 4). Fig. 5D (lanes 5-8) shows that p46-LB exhibited the same solubility properties, being largely insoluble in 1% Triton X-100 and partially soluble in Triton/NaCl. These data show that the 46 kDa proteolytic fragment of lamin B_2 is resistant to Triton extraction which is also the case of intact lamin B_2. Therefore they are likely to be organized in a similar network.

Morphological features of the disassembly of lamina and inner nuclear membrane during apoptosis

Since LBR, B-type lamins, and chromatin are cleaved during apoptosis, their relative change of localization was investigated by fluorescence microscopy. Apoptotic cells obtained by shake off after aphidicolin treatment were observed by DAPI staining and double labelling. In Fig. 6 (two left columns), cells from the heterogeneous apoptotic cell population are presented during apoptotic progression, according to phase contrast microscopy and progressive chromatin condensation observed by DAPI staining. At an early stage of apoptosis, when DNA condensation was minimal (Fig. 6F), LBR was distributed in a smooth perinuclear rim (Fig. 6G) as in control nuclei (Fig. 6C).

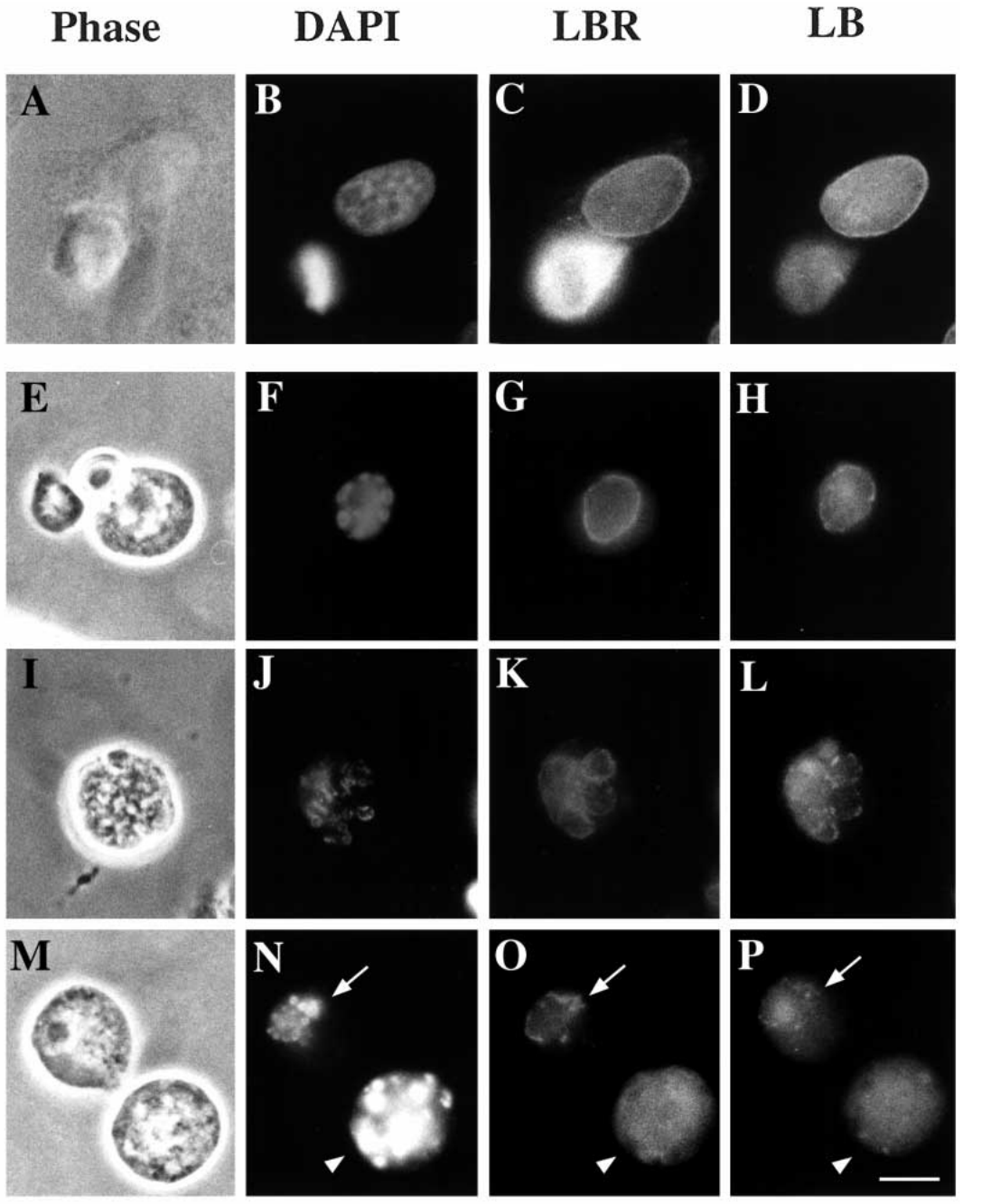

Fig. 6. Disassembly of nuclear lamina and inner nuclear membrane during apoptosis. Control interphasic and mitotic cells (A,B,C,D), and aphidicolin induced apoptotic cells recovered from the culture supernatant as indicated in the legend of Fig. 1 (E to P), were fixed and analyzed either by phase contrast (A,E,I,M), or fluorescence after DAPI staining (B,F,J,N), LBR labelling (C,G,K,O), and LB_2 labelling (D,H,L,P), as indicated in Material and Methods. Note that, at a late stage of chromatin fragmentation (N, arrow), the dispersion of the lamina (P, arrow) precedes that of the inner membrane (O, arrow). Ultimately, chromatin, LBR, and LB_2 disperse in the cytoplasm (N to P, arrowhead). Bar, 10 μm.

In the same apoptotic cell (Fig. 6H), lamin B_2 was also distributed at the periphery of the nucleus, but not as regularly as in control nuclei (Fig. 6D). At later stages of apoptosis, in blebbing nuclei (Fig. 6I-L), chromatin masses (Fig. 6K) were surrounded by a continuous LBR staining (Fig. 6K) but a discontinuous lamin B staining (Fig. 6L). At more advanced stages of apoptosis (Fig. 6N, arrow) LBR labelling became patchy but still surrounded chromatin (Fig. 6O, arrow), while lamin B labelling became dispersed in the cytoplasm (Fig. 6P, arrow). When DNA masses were dispersed in the cytoplasm (Fig. 6N, arrowhead), both LBR (Fig. 6O, arrowhead) and lamin B (Fig. 6P, arrowhead) stainings were diffuse in the cytoplasm.

From these data, obtained with a marker of the inner nuclear membrane and a marker of the nuclear lamina, it was concluded that both structures disassemble late in apoptosis, although dispersion of the membrane may be subsequent to that of the lamina.

LBR phosphorylation during apoptosis

LBR is phosphorylated throughout the cell cycle although on different residues in interphase and mitosis (Courvalin et al., 1992). These modifications may regulate the interactions of LBR with B-type lamins and chromatin during the cell cycle (Chaudhary and Courvalin, 1993; Buendia and Courvalin, 1997; Nikolakaki et al., 1996). Since these interactions may

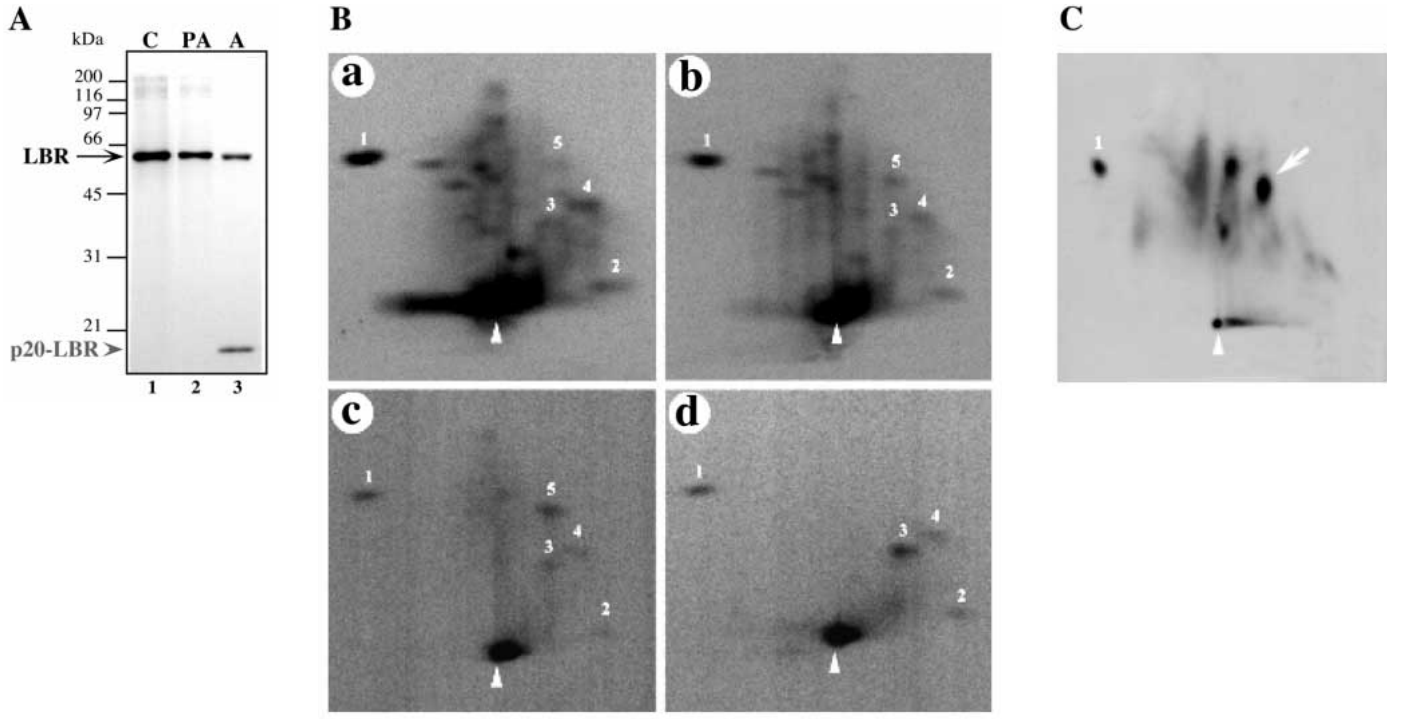

Fig. 7. LBR is not specifically phosphorylated during apoptosis. DU249 cells either control or treated by aphidicolin to induce apoptosis or by nocodazole to induce mitosis were metabolicaly labelled with $^{32}P_i$, lysed, then immunoprecipitated with antibodies directed against LBR. (A) Immunoprecipitates were analyzed by SDS-PAGE on a 10% gel followed by autoradiography. Lane 1, untreated cells; lane 2, preapoptotic cells; lane 3, apoptotic cells. Arrow points to native LBR and arrowhead to the 20 kDa proteolytic fragment of LBR. (B) Phosphorylated LBR and p20-LBR were retrieved from the gel shown in A, then digested with thermolysin. Phosphopeptides were analyzed by bidimensional thin layer chromatography before revelation by autoradiography. (a) LBR from untreated cells; (b) LBR from preapoptotic cells; (c) LBR from apoptotic cells; (d) 20 kDa proteolytic fragment of LBR. The major acidic phosphopeptide detected in all samples is labelled 1, while four other peptides common to the interphasic, preapoptotic and apoptotic intact LBR were numbered 2 to 5. Note that all these phosphopeptides except peptide 5 were detected in p20-LBR. (C) Bidimensional thin layer chromatography of LBR isolated from mitotic cells. Arrow points to the mitosis-specific phosphopeptide. Arrowheads indicate origin.

also be subjected to modifications during apoptosis, we have examined the phosphorylation of LBR in aphidicolin-treated DU249 cells. Untreated control, preapoptotic and apoptotic DU249 cells were metabolically labelled with $^{32}P_i$ before lysis. Lysates were immunoprecipitated with anti-LBR antibodies and immunoprecipitates analyzed by SDS-PAGE and autoradiography. Fig. 7A shows that native LBR from all cell lysates was phosphorylated (lanes 1-3, arrow), as well as p20-LBR in the apoptotic lysate (Fig. 7A, lane 3, arrowhead). Signals from apoptotic cells were weaker, likely due to the lower amount of radioactive material recovered from apoptotic cells. LBR from all cell lysates and p20-LBR were further submitted to thermolysin digestion and two-dimensional thin layer chromatography. Fig. 7B shows that phosphopeptide maps from native LBR isolated from untreated cells (panel a; see also Courvalin et al., 1992) and preapoptotic cells (panel b) were almost identical, with a major acidic phosphopeptide (labelled 1) and several minor phosphopeptides. Two-dimensional peptide maps of LBR and p20-LBR from apoptotic cells (panels c and d, respectively) also contained phosphopeptide 1 but less minor phosphopeptides, likely because of the lower amount of loaded radioactive material. These minor phosphopeptides (labelled 2 to 4) were also present to a different extent in maps of native LBR (panel a). Fig. 7C shows a phosphopeptide map from LBR isolated from mitotic cells. As previously shown (Courvalin et al., 1992), during mitosis the major interphasic phosphopeptide (labelled 1) decreased in parallel with the appearance of a strongly labelled more basic phosphopeptide (Fig. 7C, arrow). Other phosphopeptides present on the map of mitotic LBR were variable from one experiment to the other. Altogether, the comparison of the phosphopeptides maps of LBR during apoptosis, interphase and mitosis shows that the pattern of phosphorylation of LBR during apoptosis was of the interphasic type. Therefore a specific phosphorylation of LBR is not required for LBR proteolysis during apoptosis.

DISCUSSION

While the disassembly of the nuclear lamina during apoptosis is well documented (Oberhammer et al., 1994; Neamati et al., 1995; Weaver et al., 1996; Rao et al., 1996), the fate of nuclear membranes during that process has not been investigated. Here we present a study of the nuclear membranes disassembly during apoptosis, using the integral protein LBR as a marker of inner nuclear membrane. We show that LBR is cleaved

during the apoptotic process, generating a proteolytic fragment of ~20 kDa that was identified as the amino-terminal nucleoplasmic domain of LBR. Proteolysis of LBR is specific since it was not detected in necrotic cells, where cell death is induced by a mechanism different from apoptosis. The main feature of the proteolysis of LBR in our system is its late occurrence, being completed a few hours after that of B-type lamins. Accordingly, immunofluorescence microscopy shows that LBR was present around chromatin as a smooth continuous rim until late stages of apoptosis. The presence of an intact double membrane around condensed chromatin masses has also been observed by ultrastructural studies of late apoptotic cells and isolated nuclei forced to apoptosis in vitro (Wyllie, 1980; Lazebnik et al., 1993).

Immunolocalization of B-type lamins in parallel with LBR showed that lamins are also present at the periphery of the nuclei until late stages of apoptosis and disperse in the cytoplasm slightly before LBR. The persistance of a lamin labelling at this localization despite the complete conversion of the protein to a 46 kDa proteolytic fragment suggests the persistence of a lamina structure (Weaver et al., 1996). Here we show that the 46 kDa nuclear fragment of lamin B_2 is resistant to Triton extraction as is native lamin B_2 in control nuclei, supporting the above hypothesis. The site of proteolysis in B- and A-type lamins by Mch2/caspase 3 has been mapped to a conserved aspartic residue (Fig. 1) located in a hinge region upstream of coil 2 (Lazebnik et al., 1995; Orth et al., 1996a; Rao et al., 1996; Takahashi et al., 1996). Therefore the 46 kDa proteolytic fragment of lamin B2 contains coil 2. As coil 2 is necessary for the formation of stable B-type lamins homodimers (Ye and Worman, 1995) and higher level lateral interactions that are critical for the integrity of lamin filaments (Heitlinger et al., 1991), its presence in p46-LB may explain the late persistance of a lamina structure in apoptotic cells.

Due to the modular structure of its amino-terminal domain (see Fig. 1), the binding of LBR to chromatin is multivalent. This domain of LBR interacts with chromatin either directly via DNA and chromatin proteins of the HP1 family, or indirectly by association with B-type lamins that also are chromatin binding proteins (Yuan et al., 1991; Glass et al., 1993; Ludérus et al., 1992). The multivalence of the interaction may therefore allow the persistance of chromatin-membrane association during apoptosis even after the processing of one or two of these components. Therefore, we suggest that the protease resistance of LBR until late stages of apoptosis is one of the factors that are responsible for the permanent association of condensed chromatin with nuclear membrane during apoptosis.

LBR is phosphorylated throughout the cell cycle, but on different sites during interphase and mitosis (Courvalin et al., 1992). In the present study, we show that LBR and p20-LBR are phosphorylated during apoptosis with an interphasic pattern. The LBR kinase active in interphase and apoptosis has not been characterized. In vitro phosphorylation of a GST fusion protein containing the amino-terminal domain of LBR with cAMP-dependent kinase, protein kinase C, casein kinase II, or a protein kinase associated with chicken erythrocyte nuclear envelope did not generate the interphasis and apoptosis-specific phosphopeptide (data not shown). Unscheduled activation of cyclin B/p34^{cdc2} protein kinase has been reported during apoptosis in different systems (Shi et al., 1994; Gazitt and Erdos, 1994; Fotedar et al., 1995; Shimizu et al., 1995; Schröter et al., 1996; Furukawa et al., 1996). However, the requirement for this activation during apoptosis is still controversial (Lazebnik et al., 1993; Norbury et al., 1994; Oberhammer et al., 1994; Yoshida et al., 1997). MPM-2 antigens, vimentin, and histones H1 and H3 that are phosphorylated by cyclin B/p34^{cdc2} kinase during mitosis and premature mitosis, are unmodified during apoptosis (Yoshida et al., 1997). Our data show that there is no requirement for mitosis-specific phosphorylation of LBR during aphidicolin induced apoptosis in DU249 cells.

During the ultimate stages of apoptosis, LBR may be proteolyzed as a cause or a consequence of nuclear envelope breakdown, releasing the chromatin membrane association. Although the protease responsible for the cleavage of LBR has not been identified, a protease activity able to generate a LBR proteolytic fragment of the appropriate size has been found in a membranous/particulate fraction (sedimenting at 10,000 ***g***) prepared from apoptotic cells. Preliminary data using protease inhibitors in an in vitro assay (I. Duband-Goulet et al., unpublished data) suggest that proteolysis of LBR is controled directly or indirectly by CPP32/caspase 3, but not by Mch2/caspase 6, as is lamin B_2. The identification of the cleavage site within the amino-terminal domain of LBR should help in the identification of the LBR protease and further in the elucidation of the role plaid by LBR in the irreversible disassembly of the nuclear envelope during apoptosis.

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