

Sequential degradation of proteins from the nuclear envelope during apoptosis

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SUMMARY

We have produced new antibodies specific for the integral pore membrane protein POM121. Using these antibodies we show that during apoptosis POM121 becomes proteolytically degraded in a caspase-dependent manner. The POM121 antibodies and antibodies specific for other proteins of the nuclear envelope were used in a comparative study of nuclear apoptosis in staurosporine-treated buffalo rat liver cells. Nuclei from these cells were classified in three different stages of apoptotic progression: stage I, moderately condensed chromatin surrounded by a smooth nuclear periphery; stage II, compact patches of condensed chromatin collapsing against a smooth nuclear periphery; stage III, round compact chromatin bodies surrounded by grape-shaped nuclear periphery. We have performed double labeling immunofluorescence microscopy of individual apoptotic cells and quantitative immunoblotting analysis of total proteins from apoptotic cell cultures. The results showed that degradation of nuclear envelope

marker proteins occurred in a specific order. POM121 degradation occurred surprisingly early and was initiated before nucleosomal DNA degradation could be detected using TUNEL assay and completed before clustering of the nuclear pores. POM121 was eliminated significantly more rapid compared with NUP153 (a peripheral protein located in the nucleoplasmic basket of the nuclear pore complex) and lamin B (a component of the nuclear lamina). Disappearance of NUP153 and lamin B was coincident with onset of DNA fragmentation and clustering of nuclear pores. By contrast, the peripheral NPC protein p62 was degraded much later. The results suggest that degradation of POM121 may be an important early step in propagation of nuclear apoptosis.

Key words: Apoptosis, Nuclear lamina, Nuclear envelope, Nuclear membrane, Nuclear pore complex

INTRODUCTION

The nuclear envelope (NE) separates the nucleus from the cytoplasm (Gerace and Burke, 1988; Stoffler et al., 1999). The NE is composed of an inner and an outer nuclear membrane, which fuse together at the nuclear pores. The nuclear pores harbor multi-protein assemblies termed nuclear pore complexes (NPCs), which are responsible for macromolecular transport between the nucleus and the cytoplasm. The vertebrate NPC has an estimated molecular mass of ~125 MDa and is thought to consist of about a hundred different proteins in multiple copies arranged in an octagonal symmetry around a central channel. Some NPC proteins, termed nucleoporins (Nups), have been localized to different parts of the NPC. RanBP2/Nup358 has been shown to be a part of the cytoplasmic fibers (Yokoyama et al., 1995) and Nup153 is located in the nuclear basket (Sukegawa and Blobel, 1993). P62 (Davis and Blobel, 1986) is believed to be located close to the central channel. Gp210 and POM121 are two integral membrane proteins referred to as pore membrane proteins and are believed to participate in nuclear pore biogenesis (Hallberg et al., 1993; Wozniak et al., 1989). Underlying the inner nuclear membrane and associated with the peripheral chromatin is the nuclear lamina composed of intermediate filament proteins called lamins (Stuurman et al., 1998).

Apoptosis, or programmed cell death, is a highly ordered genetically conserved process of elimination of cells from the body in order to maintain tissue homeostasis (Hengartner, 2000; Wyllie et al., 1980). Regardless of the various means used to initiate apoptosis, it progresses according to a general program involving a set of cysteine-proteases termed caspases (Cohen, 1997; Nicholson, 1999). The activation of the caspase cascade will finally lead to cleavage of a selected group of substrates (Earnshaw et al., 1999; Stroh and Schulze-Osthoff, 1998) that dismantle essential cell components, resulting in the morphological and biochemical changes that characterize apoptotic cell death (i.e. cytoskeletal rearrangements and cell membrane disruption). Nuclear apoptosis is manifested as chromatin condensation, nucleosomal fragmentation, perturbation of the nuclear envelope and clustering of nuclear pores (Gruenbaum et al., 2000; Lazebnik et al., 1993; Robertson et al., 2000). Proteins of the nuclear pores (NUP153), inner nuclear membrane (LBR and Lap2) and the nuclear lamina (lamin B) are targets of specific executioner caspases (Buendia et al., 1999; Duband-Goulet et al., 1998; Gotzmann et al., 2000).

The pore membrane protein POM121 displays a bitopic topology with a short lumenally exposed N-terminal tail, a single transmembrane domain and a large C-terminal portion facing the pore complex (Hallberg et al., 1993; Soderqvist and

Hallberg, 1994). Pore membrane proteins are believed to anchor the NPC to the pore membrane and might thus represent strategic targets for executioner caspases involved in early nuclear apoptosis. In a previous study, using a stably transfected cell line expressing the POM121-GFP fusion protein, we showed that the GFP fluorescence disappeared from the nuclear periphery in apoptotic, but not necrotic cells (Imreh et al., 1998). Decreased GFP fluorescence was detectable at an early stage, before massive DNA fragmentation became evident using the TUNEL assay. Here we answer some of the questions raised in the previous study concerning the cause of the loss of GFP fluorescence and how this is related to apoptotic disassembly of the NPC and the nuclear envelope. Using a new antibody that is specific for POM121, we have investigated the apoptotic degradation of POM121 in relation to the degradation of other proteins from the nuclear envelope and to other hallmarks of nuclear apoptosis. A sequential model for nuclear apoptotic progression is discussed.

MATERIALS AND METHODS

Antibodies

Polyclonal rabbit antibodies were produced against an overexpressed fragment of rat POM121. A cDNA fragment corresponding to amino acid 660-800 of POM121, was cloned into the bacterial expression vector pET-28a(+). The fusion protein containing an N- and C-terminal (His)₆ tag was overexpressed in *E. coli* BL21(DE3) cells and purified according to manufacturer's manual, Novagen (Madison, WI) using a (His)₆ affinity resin, TALON (Clontech Laboratories Inc., Palo Alto, CA). Mouse monoclonal mAb414 antibodies, recognizing a subfamily of nucleoporins (mainly p62 and NUP153) were purchased from BAbCO (Nordic BioSite AB, Täby, Sweden). Anti-GFP monoclonal antibodies were purchased from Clontech Laboratories. Rabbit polyclonal antibodies towards lamin B were a kind gift from Howard Worman (Columbia University, New York, NY). Anti-RanBP2 affinity purified guinea pig polyclonal antibodies were a kind gift from Volker Cordes (CMB, Karolinska Institute, Solna, Sweden). Rabbit anti-peptide antibodies (Rb-68) against gp210 (Greber et al., 1990) was a kind gift from Larry Gerace (Scripps Institute, La Jolla, CA). As secondary antibodies for immunofluorescence we used fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse and anti-rabbit IgG, tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-mouse and anti-rabbit IgG, indocarbocyanine (Cy3)-conjugated donkey anti-guinea pig IgG (all from Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and Extravidin-conjugated FITC (Sigma, Stockholm, Sweden).

Cell culture and apoptosis

Neuroblastoma SH-SY5Y cells and PC12 (adrenal pheochromocytoma) cells, were transfected with POM121-GFP and BHK (Syrian hamster kidney) cells with POM121-(GFP)₃. Stably transfected cells were selected for G418 resistant clones, essentially as described (Imreh et al., 1998). Stably transfected neuroblastoma SH-SY5Y cell line (GI97-4) was cultivated in Eagle's minimal essential medium (MEM), supplemented with Earl's salts, 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1% nonessential amino acids and 0.25 mg/ml G418. Stably transfected BHK cell line was cultivated in in Dulbecco's minimal essential medium, (DMEM), supplemented with 10% heat-inactivated FCS and 0.6 mg/ml G418. Stably transfected PC12 cell line was cultivated in RPMI 1640, supplemented with 2 mM L-glutamine, 5% FCS, 10% heat-inactivated horse serum and 400 µg/ml G418. Buffalo rat liver

cells (BRL) were cultivated DMEM, supplemented with 5% heat-inactivated (FCS) and 50 µg/ml gentamycin. All cell lines were cultivated at 37°C in a humidified atmosphere containing 5% CO₂. All cell culture media and supplements were obtained from GibcoBRL (Life Technologies, Täby, Sweden).

Apoptosis was induced by treating cells with staurosporine, 1 µM for BRL and PC12 cells, and 0.5 µM for GI97-4 cells (Sigma) or by UV irradiation (10-200 J/m²), using an UV crosslinker, Stratallinker 2400 (Stratagene, La Jolla, CA). For inhibition of caspases, the pan-caspase inhibitor, Z-Asp-CH₂-DCB (carbo-benzyloxy-Asp-CH₂OC(O)-2,6-dichlorobenzene), was used (Calbiochem, La Jolla, CA). Monolayers of cells were incubated with 50 µM of the inhibitor, either 1 hour prior to staurosporine addition or the inhibitor was added 1 hour after staurosporine addition.

Fluorescence and confocal laser scanning microscopy (CLSM)

Cells were grown on 13 mm glass coverslips, carefully washed once with PBS, fixed on ice in 3.7% formaldehyde in PBS for 20 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. The cells were blocked in PBS containing 0.1% Tween-20 and 5% dry milk (Semper, Stockholm, Sweden). The samples were then incubated for 60 minutes with primary antibodies in blocking buffer followed by two consecutive 2 minute washes in blocking buffer and a 60 minute incubation with secondary antibodies in blocking buffer. After additional washes in PBS with 0.1% Tween-20 the coverslips were mounted in a solution of 1 mg/ml *p*-phenylenediamine (Sigma) in 90% glycerol, pH 8.3. For visualization of DNA, Hoechst 33258 or BoBo-3-iodide (Molecular Probes, Eugene, OR) was used. For double immunolabeling using antibodies specific for POM121 and gp210, which were both raised in rabbits, the following procedure was followed. The cells were fixed in a 1:1 mixture of ethanol and acetone for 20 minutes, permeabilized and the monolayer cells were first incubated for 60 minutes with rabbit anti-gp210 antibodies followed by four consecutive 2 minute washes and incubation with TRITC-conjugated anti rabbit IgG. After washing, the labeled cells were incubated for 60 minutes with biotinylated anti-POM121 antibodies followed by incubation with FITC-conjugated Extravidin (Sigma).

Images were acquired using a conventional Zeiss Axiovert fluorescence microscope (Zeiss, Stockholm, Sweden). For high resolution confocal images a Leica TCS-SP confocal microscope was used (Leica, Heidelberg, Germany). Images were processed using Adobe PhotoShop 5.5 software (Adobe Systems Inc., CA).

Western blot and quantification

Monolayer of Buffalo Rat Liver (BRL) cells, stably transfected neuroblastoma SH-SY5Y (GI97-4) or PC12 cells, were scraped off in the media, using a rubber policeman. The cells were pelleted by centrifugation (1200 g), resuspended in sample buffer, boiled for 5 minutes and sonicated. Amounts equal to 2×10⁵ cells were loaded on 8% SDS polyacrylamide gels (Laemmli, 1970). For western blotting, SDS-PAGE separated proteins from each experiment were transferred onto a nitrocellulose filter, which were blotted against the primary antibodies, anti-POM121, mAb414 (NUP153 and p62) and anti-lamin B, followed by HRP-coupled secondary antibodies and ECL detection (Amersham Pharmacia Biotech, Uppsala, Sweden). Western blots were analyzed using the Luminescent Image Analyzer, LAS1000plus system and quantified using the ImageGauge3.1 software (Fuji Photo Film Co. Ltd., Tokyo, Japan). The amount of protein in each band was quantified and expressed relative to the control (0 hours). Data were obtained from four different experiments. The apoptotic degradation of the proteins from the nuclear envelope was assumed to follow first order kinetics ($N/N_0 = e^{-kt}$). In N/N_0 versus time was plotted and a regression line with intercept zero was fitted. Values of t_{50} (the time-point where 50% of the protein had been degraded) were calculated from the regression coefficients. All t_{50} values for the proteins were subjected to linear regression analysis, using a linear model (S-PLUS

2000 software MathSoft Inc., Cambridge, MA) and the variation between experiments as the error term. To meet the model assumption of equal variance in all groups, the t_{50} values were reciprocally transformed prior to analysis. Differences in t_{50} values between proteins were evaluated using Tukey multicomparison analysis.

RESULTS

A novel antibody specific for POM121

We have raised rabbit antibodies against a recombinant fragment (amino acid 660-800) of rat POM121. In immunofluorescence microscopy of Buffalo rat liver (BRL) cells the antiserum gave rise to a distinct punctate staining of the nuclear surface in grazing sections and a strong rim staining in equatorial sections (Fig. 1A). Background staining was very low or absent (not shown), suggesting that the antibodies specifically labeled the nuclear pores. In western blotting of SDS-PAGE separated total proteins of BRL cells, the antibodies specifically reacted with a single antigen (Fig. 1B). Purified rat liver nuclear envelope membranes extracted with 7 M urea, which is enriched in integral proteins of the nuclear membrane, but devoid of peripheral proteins (Hallberg et al., 1993) was loaded in a parallel lane as a reference. The results suggest that the antibodies were specific for POM121, which migrates with an apparent mobility of 145×10^3 . The small difference in mobility of the bands in the two fractions may be due to differences in post-translational modifications between cultured cells and intact rat liver.

Apoptotic degradation of POM121

BRL cells and stably transfected SH-SY5Y neuroblastoma cells (GI97-4) permanently expressing POM121 fused to GFP (POM121-GFP) were treated with staurosporine in order to induce apoptosis. In both cell types staurosporine treatment gave rise to morphological changes including rounding up of nuclei, decreased nuclear diameters and condensation of chromatin, which are significant signs of apoptotic cell death. The GFP fluorescence decreased or completely disappeared from the nuclear periphery in apoptotic GI97-4 cells (Fig. 2A). A similar decrease or complete lack of immunostaining of nuclear pores was observed in apoptotic BRL cells using the anti-POM121 antibodies. Western blot analysis displayed significantly reduced levels of POM121 and POM121-GFP in staurosporine-treated cultures (Fig. 2B), indicating that disappearance of GFP fluorescence and immunostaining for POM121 was due to proteolytic degradation. The absence of fragments recognized by either anti-POM121 or anti-GFP antibodies suggest that the degradation of the POM121 portion and the GFP moiety were completed rapidly and simultaneously. The anti-POM121 antibodies did not recognize the endogenous human POM121 homologue of neuroblastoma cells (not shown). We also examined apoptotic cells of a stable rat PC-12 cell line overexpressing POM121-GFP, which enable parallel detection of endogenous POM121 and the overexpressed fusion protein (Fig. 2C). After staurosporine treatment these cells displayed a synchronous degradation of endogenous POM121 and overexpressed POM121-GFP, indicating no or only minor differences in the kinetics of apoptotic breakdown between endogenous POM121 and the reporter protein. The GFP fluorescence

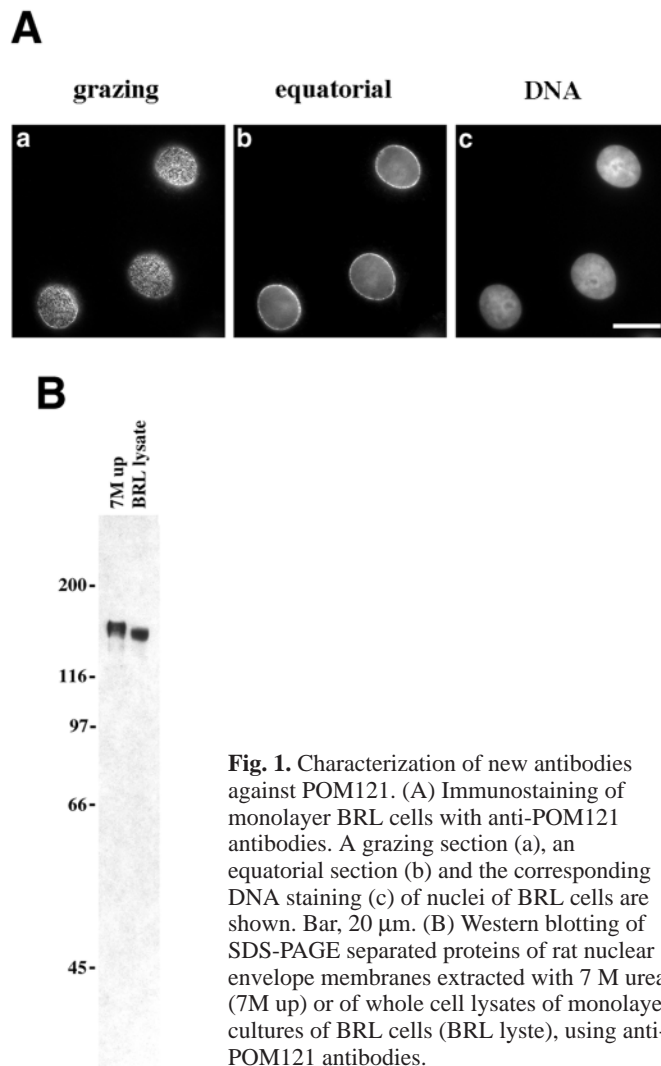


Fig. 1. Characterization of new antibodies against POM121. (A) Immunostaining of monolayer BRL cells with anti-POM121 antibodies. A grazing section (a), an equatorial section (b) and the corresponding DNA staining (c) of nuclei of BRL cells are shown. Bar, 20 μ m. (B) Western blotting of SDS-PAGE separated proteins of rat nuclear envelope membranes extracted with 7 M urea (7M up) or of whole cell lysates of monolayer cultures of BRL cells (BRL lysate), using anti-POM121 antibodies.

serves as an important control to exclude decreased immunostaining due to apoptosis-induced changes in epitope conformation and/or accessibility as suggested by others (Faleiro and Lazebnik, 2000). In another experiment, where apoptosis was induced in BRL cells by UV irradiation, the disappearance of POM121 immunostaining from the nuclear periphery was evident after 24 hours (Fig. 3A). Furthermore, proteolysis of POM121 occurred in a dose-dependent fashion as judged by western blotting (Fig. 3B). Similar results were obtained in BRL cells using actinomycin D (not shown). The results suggest that proteolysis of POM121 is a general phenomenon in apoptotic cell death and not restricted to staurosporine treatment.

Sequence of morphological events in nuclei of apoptotic BRL cells

We attempted to relate the disappearance of POM121 and POM121-GFP to other apoptotic alterations of the nuclear envelope. Staurosporine-treated BRL cells displayed apoptotic morphology (i.e. scanty cytoplasm and rounded up nuclei with small diameters). Based on DNA staining, three subclasses with progressively more apoptotic nuclei could be distinguished (Fig. 4). Stage I nuclei displayed a smooth

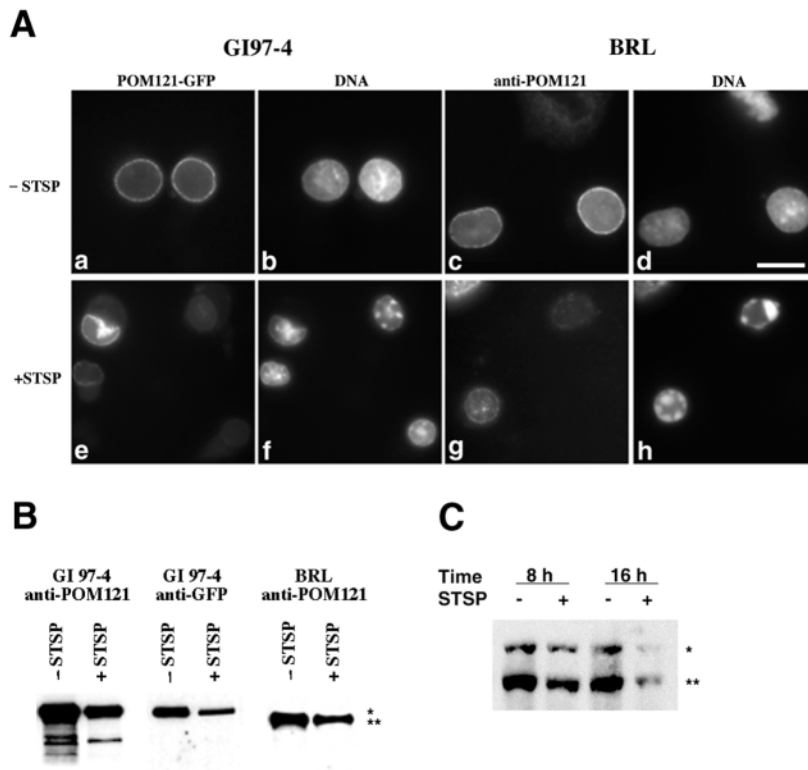
Fig. 2. Proteolysis of overexpressed POM121-GFP and endogenous POM121 during apoptosis.

Monolayer cultures of stably transfected neuroblastoma SH-SY5Y cells (GI97-4 cells), stably transfected PC-12 cells or BRL cells, were treated with staurosporine for different times.

(A) Fluorescence micrographs showing equatorial sections of GI97-4 cells (a,b,e,f) and BRL cells (c,d,g,h), treated for 15 hours. Pairs of images of control (–STSP) and treated (+STSP) cells were acquired and processed identically. The GFP fluorescence (a,e) and the immunostaining using anti-POM121 antibodies (c,g) are shown to the left of the corresponding images of DNA staining (b,d,f,h). Bar, 20 μ m.

(B) Western blot analysis of POM121 and POM121-GFP. Treated (+STSP) and control (–STSP) cells were solubilized in sample buffer after 8 hours of treatment and subjected to SDS-PAGE and western blotting using anti-POM121 or anti-GFP antibodies. In apoptotic cells, overexpressed POM121-GFP (★) as well as endogenous POM121 (★★) decreased.

(C) Western blot analysis of overexpressed POM121-GFP (★) and endogenous POM121 (★★) in stably transfected PC12 cells undergoing apoptosis (+STSP). After 8 hours of treatment 56% of the overexpressed and 47% of the endogenous protein remained. After 16 hours the corresponding figures were 14% and 17%, respectively. Data are from one representative experiment.



spherical periphery containing relatively evenly distributed chromatin just starting to condense locally. Stage II nuclei also had a smooth spherical periphery but showed large patches of condensed chromatin distributed in the nuclear periphery. Stage III nuclei displayed a grape shaped periphery, surrounding protrusions of chromatin, distributed in a few spherical apoptotic bodies (0.5 to 3 μ m in diameter). Massive DNA fragmentation detectable using the TUNEL assay was evident in all stage III and in about half of the stage II cells, whereas stage I and control cells were negative (not shown).

We performed a series of double immunostainings of apoptotic BRL cells using antibodies specific for proteins of the nuclear envelope. Images of control and apoptotic cells were acquired and processed identically and compared pairwise. Immunostaining for POM121 was significantly decreased or eliminated in apoptotic nuclei showing retained or only slightly reduced immunofluorescence using monoclonal mAb414 antibodies (Fig. 5a',b'). The converse situation was never observed, suggesting that POM121 disappears from apoptotic nuclei earlier than the nuclear pore proteins recognized by mAb414 antibodies (i.e. p62 and NUP153). RanBP2 appeared to vanish from apoptotic nuclei at approximately the same time as POM121 (Fig. 5d',e'). Since the antibodies against POM121 and lamin B were both obtained from rabbits, we compared overexpressed GFP-tagged POM121 with anti lamin B immunostaining. The fluorescence from POM121-GFP disappeared before the immunostaining for lamin B, in stably transfected BHK cells undergoing apoptosis (Fig. 5g',h'), suggesting that POM121 disappears at an earlier step than lamin B. This is supported by the finding that RanBP2 immunofluorescence, which disappeared from apoptotic nuclei concomitantly with that of POM121 (Fig. 5d',e'), also disappeared at an earlier step than

the lamin B immunofluorescence (Fig. 5p',q'). Other pair-wise combinations showed that both anti-RanBP2 (Fig. 5k') and anti-lamin B immunostaining (Fig. 5n') disappeared before the immunostaining using mAb414 antibodies (Fig. 5j',m'). Anti-

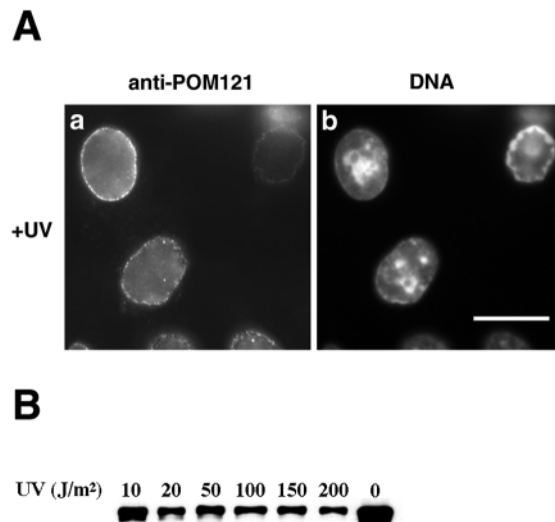
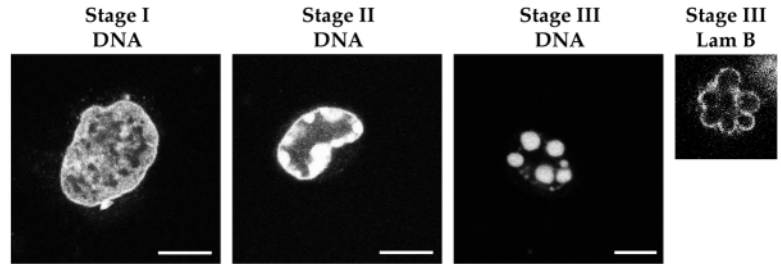


Fig. 3. Proteolytic breakdown of POM121 during apoptosis induced by UV-irradiation. Monolayer cultures of BRL cells were exposed to UV-irradiation and incubated for 24 hours. (A) Fluorescence micrographs of UV-irradiated BRL cells showing three cells in different stages of apoptotic progression. Control cells were similar to those in Fig. 2A panels c,d. Note the almost complete lack of POM121 immunostaining in the cell with condensed chromatin in the nuclear periphery (upper right). Bar, 20 μ m. (B) Western blot of SDS-PAGE separated proteins of whole cell lysates of BRL cells, exposed to different doses of UV-irradiation (as indicated).

Fig. 4. Classification of apoptotic nuclei. BRL cells treated with 1 μ M staurosporine for 15 hours were fixed, permeabilized and stained for DNA using BoBo-3-iodide or for lamin B before analysis by CLSM. The panels show chromatin in 0.3 μ m equatorial sections of apoptotic nuclei classified as stage I-III as indicated. The extremely weak lamin B immunofluorescence signal remaining in a stage III nucleus delineating the grape-shaped nuclear periphery is also shown. Bars, 10 μ m (stage I,II); 5 μ m (stage III).



lamin B immunofluorescence was unaffected in stage I cells, but decreased in intensity in some of the stage II cells. In stage III cells it was very weak or completely absent (Fig. 5h', Fig. 6A). Anti-POM121 or anti-RanBP2 immunofluorescence was never detected in stage III cells and only sporadically in stage II cells, whereas stage I cells displayed normal or weaker immunofluorescence intensity (Fig. 5d',e'). In summary, the immunostaining against RanBP2 and POM121 (or the POM121-GFP fluorescence) disappeared at an earlier step than immunostaining for lamin B, which in turn preceded the disappearance of immunostaining using mAb414 antibodies.

Although mAb414 immunostaining was detectable in cells in all stages of apoptotic progression within the timeframe of the experiments, the distribution of the fluorescence was changed (Fig. 5j'). Apoptotic BRL cells were double labeled with mAb414 and lamin B antibodies, and analyzed with confocal laser scanning microscopy (CLSM) (Fig. 6). Stage I nuclei displayed a punctate distribution of mAb414 staining along a smooth spherical periphery, similar to the staining pattern in control cells (not shown). In stage II nuclei, mAb414 staining distributed in an interrupted pattern along the nuclear rim, which remained continuous and spherical, as visualized by anti-lamin B immunofluorescence. The mAb414 staining, although interrupted at some points, distributed in the spherical periphery (Fig. 6A) directly adjacent to the underlying chromatin (Fig. 6B). In stage III nuclei, mAb414 staining accumulated in a number of intense spots or elongated thread like structures, in certain local areas of the grape-shaped nuclear periphery (Fig. 6A) especially in areas between DNA protrusions (Fig. 6B,C). The localization to areas devoid of chromatin in-between or outside apoptotic bodies, suggests that it reflects clustering of NPCs documented previously in electron microscopy micrographs of apoptotic cells and tissue (Falcieri et al., 1994). Clustering of mAb414 staining appeared in some stage II and all stage III apoptotic cells but not in stage I cells and did not appear to depend on complete disappearance of lamin B, although lamin B immunostaining gradually decreased during stage II and III progression.

Sequential degradation of proteins of the nuclear envelope in apoptotic BRL cells

In order to get a quantitative measure of the progression of apoptotic degradation of the nuclear envelope proteins in treated cell cultures we performed a parallel western blotting analysis (Fig. 7A). Degradation of POM121, NUP153 and lamin B were rapid and almost completed after 20 hours. With a 95% confidence interval, statistical analysis (Tukey multicomparison) showed that the rate of degradation of the proteins were significantly different from each other, with the exception of NUP153 and lamin B, which could not be

separated. Comparison of average t_{50} values (the time-point where 50% of the protein had been degraded) showed that POM121 was degraded at a significantly higher rate (t_{50} =3.9 hours) than lamin B (t_{50} =6.6 hours) and NUP153 (t_{50} =6.7 hours), whereas p62 was degraded very slowly (t_{50} =36 hours) (Fig. 7B).

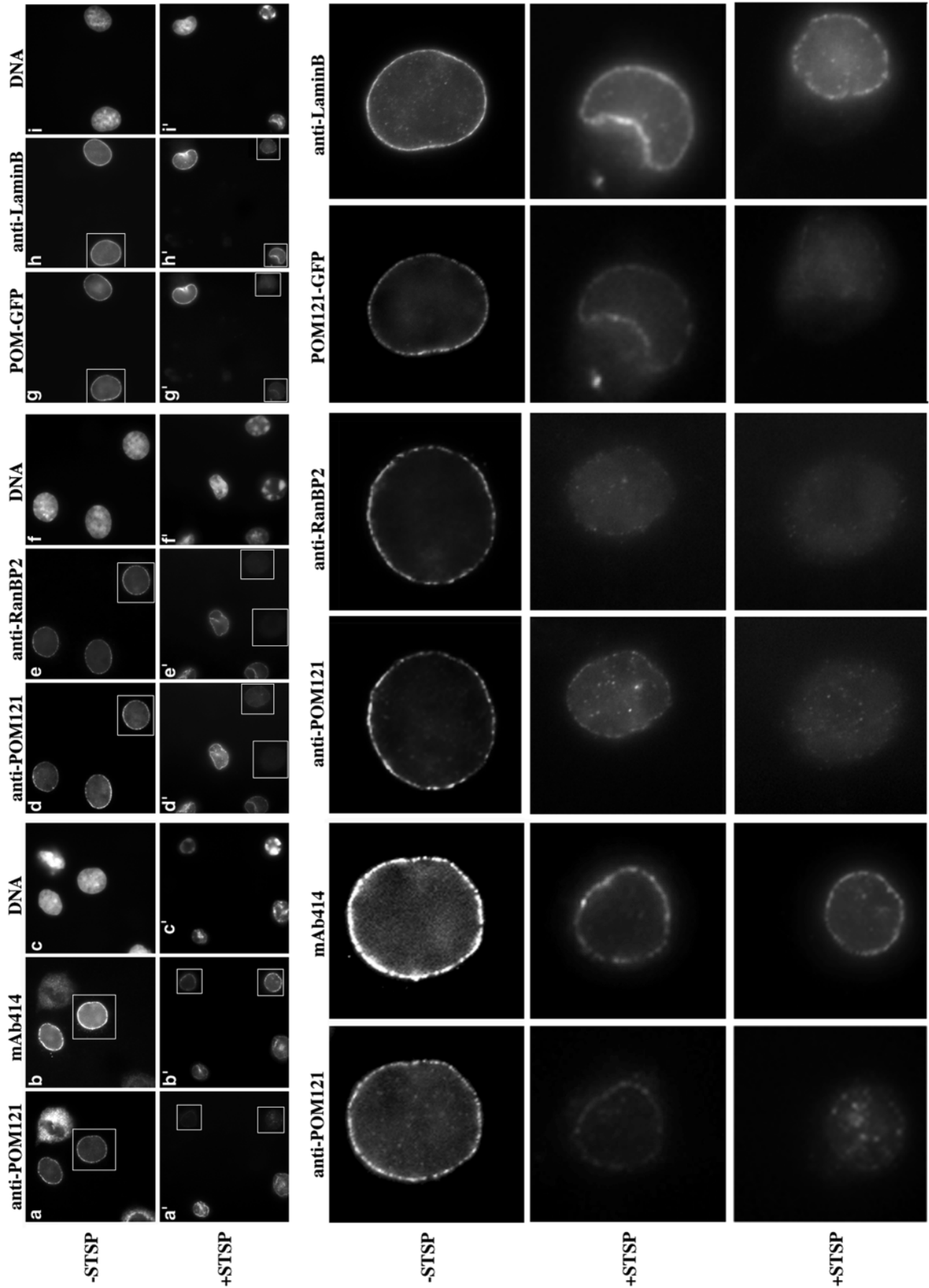
The pan-caspase inhibitor Z-Asp-CH₂-DCB (Posmantur et al., 1997) completely abolished apoptotic degradation of the nuclear envelope proteins when added 1 hour prior to staurosporine and to a large extent when added 1 hour after staurosporine (Fig. 7C). Cell populations treated with both staurosporine and caspase inhibitor showed an apoptotic morphology with rounded up nuclei. However, no stage III apoptotic cells were observed and very few cells showed signs of DNA fragmentation using TUNEL assay (not shown). The data suggest that, during apoptosis, nuclear envelope proteins are degraded by caspases and that POM121 is degraded at an earlier step than NUP153 and lamin B, and that p62 is degraded at an even later step.

Cleavage of POM121 does not cause release of gp210

The early degradation of POM121 raises questions concerning the fate of gp210, another vertebrate pore membrane protein, since these two proteins have been proposed to interact (Hallberg et al., 1993). We used double immunolabeling fluorescence microscopy for simultaneous analysis of POM121 and gp210 in apoptotic cells. We found that the punctate immunostaining pattern for gp210 along the nuclear rim remained in apoptotic cells lacking POM121 immunofluorescence (Fig. 8). The results indicate that degradation of POM121 precedes that of gp210 and that POM121 cleavage does not cause release of gp210, which would have given rise to an even immunostaining of the continuous ER/nuclear membrane system.

DISCUSSION

Different antibodies against proteins of the nuclear envelope were used in a detailed characterization of nuclear apoptosis in staurosporine-treated BRL cells. Pair-wise comparison of double-labeled individual apoptotic cells by fluorescence microscopy suggested that proteins from the nuclear envelope disappeared in a sequential order. This was confirmed by western blot analysis of nuclear envelope proteins from apoptotic cell cultures. The elimination of proteins from the nuclear envelope in apoptotic BRL cells was compared with other hallmarks of apoptosis (degree of chromatin condensation, DNA degradation and NPC clustering) and



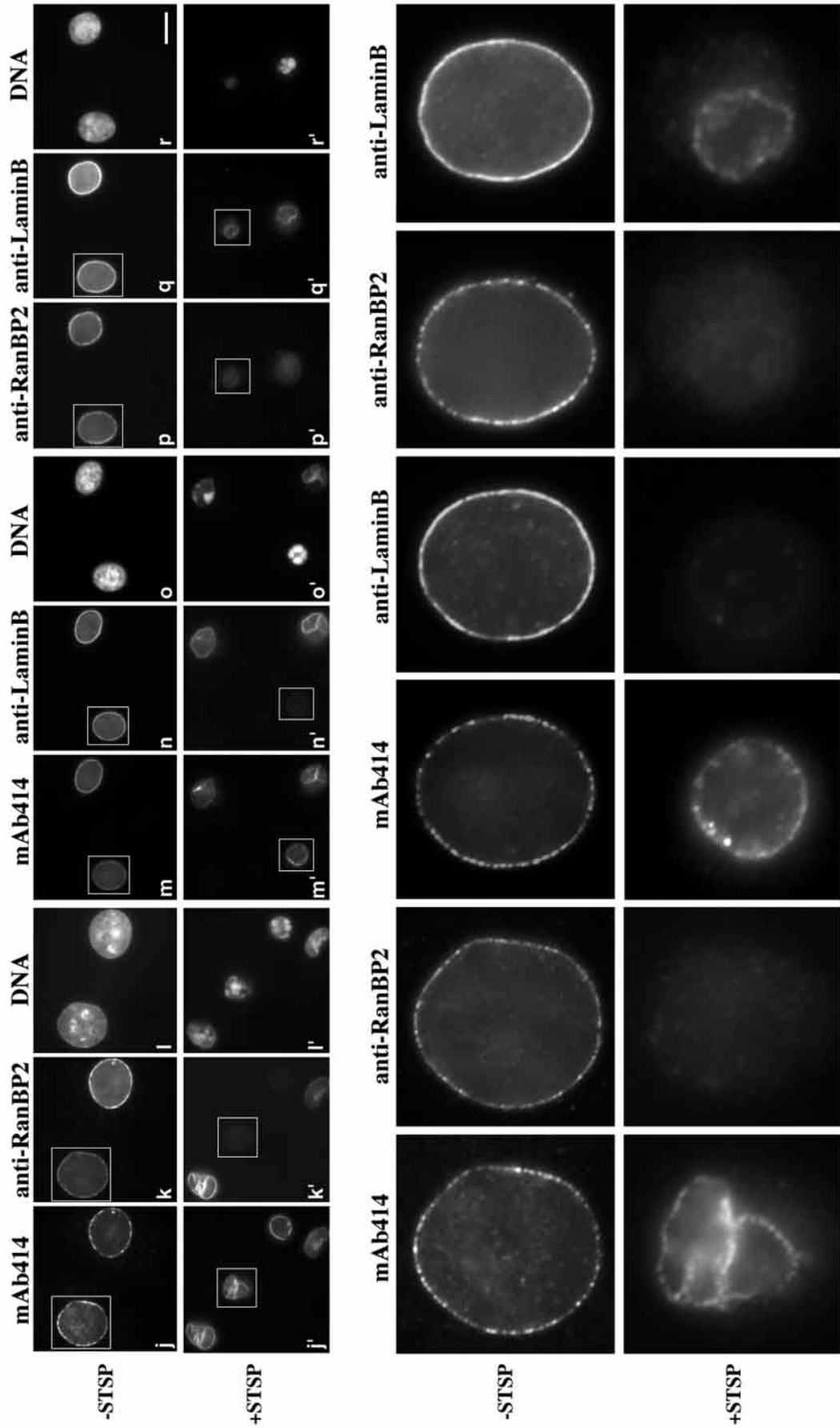


Fig. 5. Subcellular distribution of proteins form the nuclear envelope during apoptosis. Monolayer cell cultures were incubated in the absence, -STSP (a-r) or presence, +STSP (a'-r') of 1 μ M staurosporine. After 15 hours of treatment the cells were fixed and permeabilized and subjected to double immunolabeling using the antibodies indicated. The chromatin was stained using Hoechst 33258. In stably transfected BHK cells, POM121-GFP was visualized by its own fluorescence (g,g'). The smaller panels show fields of cells. The larger panels show enlargements of control or stage II apoptotic nuclei inside the frames. Pairs of images of control and treated cells were acquired and processed identically. Control cells displayed an intense staining around the nuclear rim with all antibodies tested and nuclei with dispersed chromatin and large diameters (a-r). Apoptotic cells (a'-r') showed nuclei with condensed chromatin and a smaller nuclear diameter. The immunolabeling of the periphery of apoptotic nuclei was weaker or absent. However, the immunofluorescence using mAb414 antibodies was retained (b',j',m') in most apoptotic cells, although it frequently displayed a clustered distribution (j'). Bar, 20 μ m.

compiled into a model (Fig. 9). Three different stages of apoptotic progression could be clearly distinguished: stage I, moderately condensed chromatin surrounded by a smooth nuclear periphery; stage II, compact patches of condensed chromatin collapsing against a smooth nuclear periphery; stage III, round compact chromatin bodies surrounded by grape shaped nuclear periphery. Condensation of chromatin was an early event in apoptotic BRL cell nuclei (stage I). Fragmentation of DNA as judged by TUNEL assay, started at a later time point (stage II). Interestingly, disappearance of POM121 and RanBP2 started in stage I and was more or less complete in stage II. Degradation of these two nucleoporins clearly preceded the degradation of NUP153 and lamin B, which started in stage II and proceeded gradually through stage III. By contrast, p62 still remained in stage III, which allowed detection of pore clustering, another hallmark of apoptosis (Buendia et al., 1999; Falcieri et al., 1994). Pore clustering developed gradually, starting clearly after POM121 and RanBP2 had been eliminated. Pore clustering apparently correlated with the disappearance of NUP153 and lamin B, and became more pronounced with alterations of nuclear peripheral shape.

Although one must keep in mind that the apoptotic program may differ depending on cell type and the method of initiation, the sequential order of elimination of proteins from the nuclear envelope reported here is consistent with previous publications. For example, it has been shown that degradation of lamin B, Lap2 and NUP153 preceded proteolysis of p62, LBR and gp210 (Buendia et al., 1999). Another study (Gotzmann et al., 2000) showed that degradation of lamin B preceded degradation of emerin. In both of the above reports, lamin B was degraded to

a large extent in cells showing a high degree of chromatin condensation, corresponding to stages II-III in our investigation. Faleiro and Lazebnik reported decreased immunostaining in apoptotic cells due to inaccessibility or modification of the epitope recognized by mAb414 antibodies (Faleiro and Lazebnik, 2000). This was not the case in our study, where data from immunofluorescence and western blotting correlated well (Fig. 5, Fig. 7).

Since NPCs are believed to be connected to the nuclear lamina (Daigle, 2001; Dwyer and Blobel, 1976), it is tempting to speculate that clustering of nuclear pores and loss of membrane integrity is dependent of nuclear lamina disruption. In fact, NPC clustering was one of the most striking features in *Drosophila* embryos lacking B-type lamins (Lenz-Bohme et al., 1997) and mice lacking lamin A displayed nuclei with perturbed discontinuous nuclear envelopes (Sullivan et al., 1999). Apoptotic elimination of lamin B has been reported to

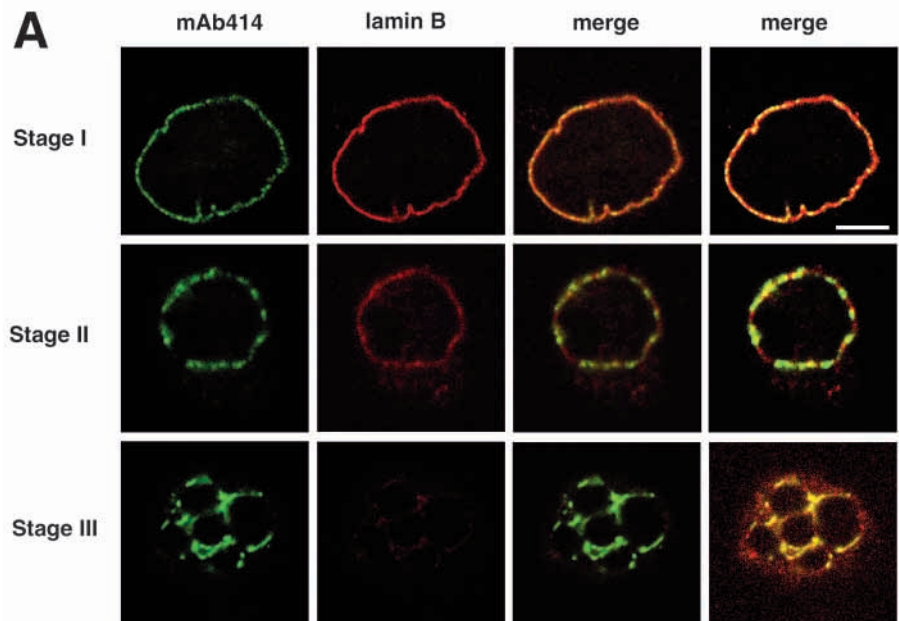
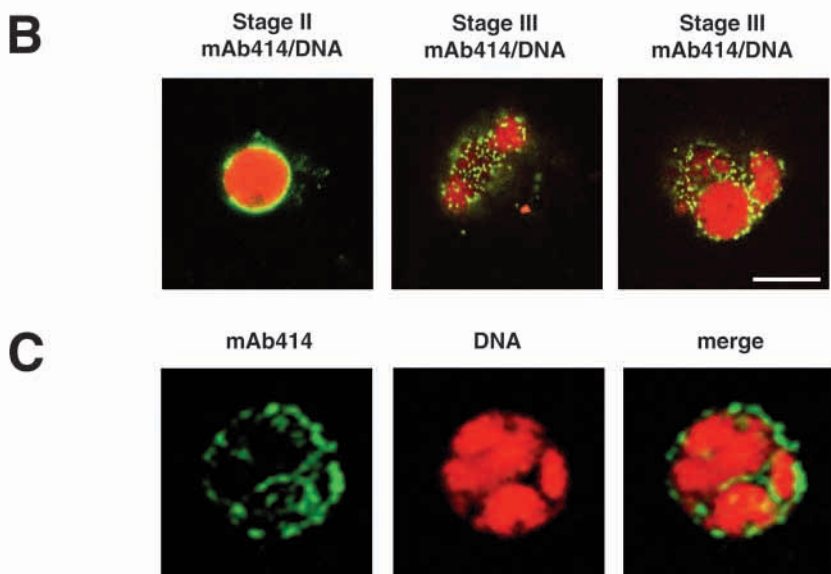


Fig. 6. High resolution confocal images of NPC clustering. BRL cells were incubated with 1 μ M staurosporine for 15 hours and subjected to immunostaining using mAb414 and/or anti-lamin B antibodies. DNA was visualized using BoBo-3-iodide.

(A) Distribution of nuclear pore proteins (green) and lamin B (red) in 0.3 μ m equatorial sections of nuclei in different stages of apoptotic progression. The right panels show enhanced versions of the merged mAb414 and anti-lamin B staining in order to better visualize the weak lamin B staining. Note the interrupted and accumulated pattern of mAb414 immunofluorescence in stage II and III. Bar, 5 μ m. (B) Distribution of nuclear pore proteins (green) and chromatin (red) in (0.3 μ m) sections of stage II and III nuclei, respectively. Bar, 10 μ m. (C) A projection of a stack of optical sections of a nucleus between stage II and III that had been stained with mAb414 antibodies (green) and Bobo-3-iodide (red).



be associated with the appearance of discontinuities in the nuclear envelope of chicken DU249 hepatoma cells (Duband-Goulet et al., 1998).

This study presents, for the first time, data that relate the apoptotic elimination of the integral pore membrane protein POM121 to that of other NE proteins and other hallmarks of

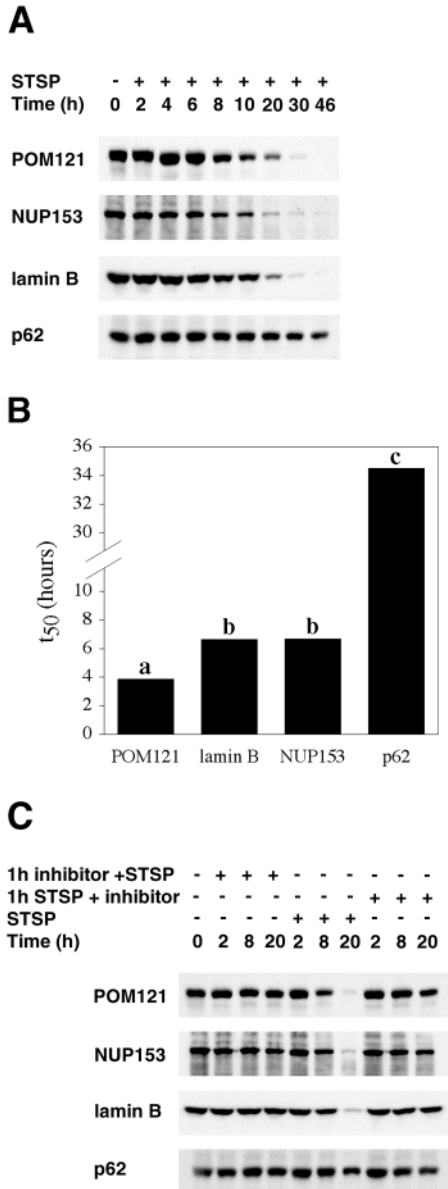


Fig. 7. Sequential proteolysis of proteins from the nuclear envelope in cell cultures undergoing apoptosis. (A) BRL cells were treated with 1 μ M staurosporine for different times. Equivalent amounts of total proteins of control or treated cell cultures were separated by SDS-PAGE and analyzed by western blotting using anti-POM121, mAb414 (NUP153 and p62) or anti-lamin B antibodies. The panel of western blots is representative of one experiment. (B) Mean t₅₀ values of quantified western blots from four separate experiments. Bars denoted with different letters are significantly different (a-c). (C) BRL cells were treated with 1 μ M staurosporine and the caspase inhibitor was added either 1 hour before (1h inhibitor +STSP), or 1 hour after addition of staurosporine (1 h STSP +inhibitor) or cells were incubated with staurosporine alone (STSP). The panel of western blots is representative of one experiment.

apoptosis. Surprisingly, POM121 together with RanBP2 appear to be the earliest NPC proteins to be degraded. At present we have not identified the caspase responsible for POM121 degradation and we have been unable to detect any proteolytic fragment(s). The cytoplasmically exposed C-terminal portion of POM121 contains two tetrapeptides D¹⁴⁶PRD and D⁵²⁸KTD, which could act as potential proteolytic sites for caspases (Cohen, 1997), although this remains to be analyzed experimentally.

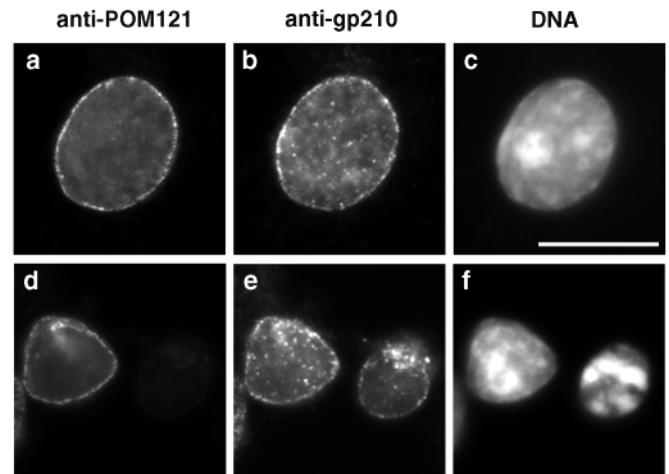


Fig. 8. Distribution of gp210 in apoptotic cells lacking POM121. BRL cells were treated with 1 μ M staurosporine for 15 hours and subjected to double immunolabeling using antibodies specific for POM121 (a,d) and gp210 (b,e), respectively. DNA was visualized using Hoechst 33258 (c,f). Control cells (a-c) displayed a punctate fluorescence pattern along the nuclear rim. The gp210 antibodies also gave rise to a background staining seen as small dots in the nucleoplasm. In staurosporine-treated cells (d-f) both antibodies gave rise to a bright peripheral immunostaining of a type I cell (left), reminiscent of control cells. By contrast, the nuclear periphery of an early type II cell (right) only stained positive for gp210 (e), whereas POM121 was absent (d). Bar, 20 μ m.

Apoptotic disassembly of the nuclear envelope

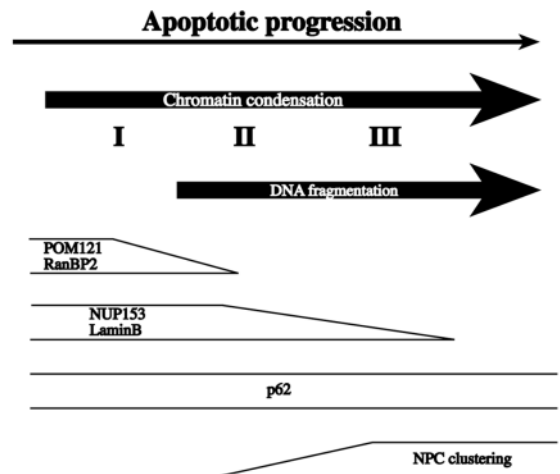


Fig. 9. Sequential steps in nuclear envelope breakdown during apoptosis.

The sequential order of elimination of the NE proteins observed can be interpreted in two different ways. If the NE proteins were simply degraded by a common set of downstream effector caspases one would expect the order of degradation to be limited by accessibility. It appears possible that apoptosis proceeds in a centripetal direction as a gradient of activated caspases starting in the cytoplasm and working its way into the nucleus. In such a scenario, proteins accessible from the cytoplasmic side of the NPC would be degraded first. This is true for both RanBP2, located on the cytoplasmic fibrils (Yokoyama et al., 1995) and POM121, whose C-terminal portion has been shown to be accessible for antibodies in digitonin-treated BRL cells (Soderqvist and Hallberg, 1994). In a centripetal mechanism, activated caspases would first have to enter the nucleus before getting access to NUP153 and lamin B on the nucleoplasmic side explaining their delayed degradation. However, this model does not explain the resistance against degradation shown by p62, gp210 and emerin even in late apoptosis (this study; Buendia et al., 1999; Gotzmann et al., 2000). Furthermore, an ultra-structural study show that clustered pore complexes in late nuclear apoptosis essentially maintain their overall structure and density (Falcieri et al., 1994), indicating that most of the NPC proteins are still intact. A more likely interpretation of the sequential degradation observed in our study is that initially only a selective group of strategic targets are attacked by upstream proteases. This initial attack may pave the way for more substantial destruction in later stages, perhaps facilitated by loss of pore function and increased permeability. The C-terminal portion of POM121 is believed to be located in the central spoke region separating the smaller peripheral channels of the NPC believed to allow diffusion of smaller proteins between the cytoplasmic and nucleoplasmic compartments (Hinshaw et al., 1992). Elimination of POM121 would thus be expected to facilitate increased diffusion. Pore membrane proteins are believed to function in pore formation and anchoring peripheral NUPs, and are thought to be important for NPC stability. Thus, an initial degradation of POM121 in apoptosis may serve to destabilize the NPC and perhaps allow nuclear entry of effector caspases and nucleases. The latter model would be consistent with a recent study, showing that disruption of the nucleocytoplasmic barrier is dependent on caspase-9 and precedes activation of caspase-3 (Faleiro and Lazebnik, 2000).

The sorting determinants of gp210, another vertebrate pore membrane protein, was located to its single transmembrane segment and to its 58 amino acid cytoplasmically exposed C-terminal tail (Wozniak and Blobel, 1992), suggesting that either or both of these domains are able to interact with other pore complex proteins (e.g. POM121), as was proposed (Hallberg et al., 1993). In POM121, a portion (amino acids 129-618) of its cytoplasmically exposed C-terminal domain has been shown to be responsible for targeting to the nuclear pores (Söderqvist et al., 1997). Here we show that gp210 remained associated with nuclear pores of apoptotic cells when POM121 had been eliminated. Our data is consistent with an earlier study (Buendia et al., 1999) showing that gp210 becomes degraded at a step succeeding degradation of NUP153 and lamin B in apoptosis. Our data also suggest that POM121 is not required for keeping gp210 in the pore membrane. Naturally, this does not exclude interaction(s)

between these two proteins in other situations (e.g. during pore formation).

In this paper we have shown that overexpressed POM121-GFP and endogenous POM121 are degraded synchronously, that POM121 proteolysis is caspase dependent and appears to be a general phenomenon in apoptosis. Furthermore, we have shown that POM121 was one of the earliest proteins of the NE to be eliminated, occurring before many other signs of nuclear apoptosis (i.e. TUNEL assay detectable DNA fragmentation and NPC clustering). It will be interesting to compare elimination of POM121 with other hallmarks of apoptosis (e.g. release of cytochrome c from mitochondria, decreased mitochondrial inner membrane potential or appearance of phosphatidylserine on the cell surface). Non-invasive markers, such as GFP-tagged POM121 and cytochrome c (Goldstein et al., 2000), makes it possible to study propagation of apoptosis in individual living cells by time-lapse microscopy and are thus valuable tools for studies of mechanistic and temporal aspects of cell death progression. This is especially true in light of the recent discovery that reversible epigenetic factors may influence apoptotic development (Jones, 2001).

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