Nuclear localization of procaspase-9 and processing by a caspase-3-like activity in mammary epithelial cells

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Running Title: nuclear localization of caspase-9

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Abbreviations: CP cisplatin - DAPI 4',6-Diamidin-2'-phenylindol-dihydrochlorid - DMSO Dimethyl sulfoxide - hsp60 heat-shock protein 60 - PARP Poly(ADP-ribose) polymerase

Keywords: apoptosis, caspases, mammary epithelial cells, mitochondria

Abstract
Caspases are aspartate-specific proteases that are specifically activated by numerous death stimuli. Caspase activation is thought to play a major role for the execution of apoptosis. Inactive caspase-9 zymogen is known to be localized within the mitochondrial intermembrane space where it is involved in monitoring mitochondrial damage associated cytochrome c release and subsequent activation of procaspase-3. Here we show that in mammary epithelial cell lines a significant fraction of caspase-9 proform is associated with discrete structures in the nucleus. Stimulation of cells with chemotherapeutic agents leads to the processing of nuclear procaspase-9 and to the accumulation of nuclear and cytoplasmic caspase activity. Using cell-free extracts from caspase-3 deficient MCF-7 cells we show that caspase-8 mediated processing of nuclear procaspase-9 requires caspase-3. In caspase-3 expressing breast cancer cells, cytochrome c induced processing of nuclear procaspase-9 is blocked by the caspase inhibitors z-VAD and DEVD but not by YVAD. Purified active caspase-3 is sufficient to cleave nuclear caspase-9 zymogen. These results suggest that, in addition to the mitochondrial localization, caspase-9 proform is found within the nucleus and its processing can be regulated by caspase-3.

Introduction
Apoptosis is essential for organogenesis, tissue homeostasis and the elimination of cells that are potentially harmful. Apoptosis was first defined on a morphological basis by Kerr and co-workers (Kerr et al., 1972). In many cases it depends on the caspase family of aspartate-specific cysteine-proteases (Kerr et al., 1972; Kidd, 1998; Salvesen and Dixit, 1997). To date about 14 different members of caspases have been described and many of them are expressed as inactive zymogens in normal cells. Caspases share typical structural and functional features such as an N-terminal pro-domain that is variable in length and sequence and two catalytic subunits (Kidd, 1998; Salvesen and Dixit, 1997). The prodomain mediates subcellular localization and protein/protein interaction; the catalytic subunits include a large ~17-20 kDa and a small ~10 kDa form. During apoptosis caspases are converted to the active enzyme in an ordered fashion by proteolytic cleavage separating the large from the small subunit. Many caspase substrates are subsequently cleaved by limited proteolysis, among them are poly(ADP-ribose) polymerase (PARP) (Tewari et al., 1995), Lamin A (Rao et al., 1996; Takahashi et al., 1996), DNA fragmentation factor (DFF) (Liu et al., 1997; Enari et al., 1998; Liu et al., 1998; Sakahira et al., 1998), U1 associated 70 kDa protein (Casciola-Rosen et al., 1996), ? -fodrin (Martin et al., 1995), MEKK-1 (Cardone et al., 1997), Bid (Li et al., 1998; Luo et al., 1998) and Bcl-2 (Cheng et al., 1997). Caspases are commonly separated into initiator caspases (e.g. caspases-8, 9 and 10) and effector caspases (e.g. caspases-2, 3 and 7) (Salvesen and Dixit, 1997). Initiator caspases are believed to mainly activate effector caspases. Many apoptotic triggers activate caspase-8 or caspase-9 as an early event. Either of these two caspases are able to directly activate caspase-3, a step that often initiates the execution phase of apoptosis (Thornberry and Lazebnik, 1998). Whereas caspase-8 is a cytosolic protein, caspase-9 is known to primarily reside in the mitochondrial intermembrane space (Krajewski et al., 1999; Samali et al., 1999; Susin et al., 1999). The mitochondrial intermembrane space is also the site of numerous other factors that regulate apoptosis such as cytochrome c, apoptosis inducing factor (AIF), caspase-2, caspase-3 and a DNase of unknown nature (Susin et al., 1999). During apoptosis there is a marked change of mitochondrial membranes either by the opening of permeability transition pores or by rupture of the outer mitochondrial membrane (Green and Reed, 1998). The consequence is a release of intermembrane components into the cytosol where cytochrome c and

caspase-9 form a complex with the cytosolic apoptosis activating factor Apaf-1 that eventually leads to the activation of caspase-9 and caspase-3 (Liu et al., 1996; Li et al., 1997; Reed, 1997; Zou et al., 1997).
In the present study we investigated the localization and activation of caspase-9 in different mammary epithelial cells in culture. We found that, besides the mitochondrial localization, procaspase-9 is also localized within the nucleus where it is accessible to active caspase-3 during apoptosis.

Materials and methods

Cell culture, cisplatin treatment and quantification of apoptotic cells

31D (Reichmann et al., 1989) and HC11 (Ball et al., 1988) mouse mammary epithelial cells were kept in DMEM containing 10% fetal calf serum with 200 U/ml penicillin and 200 μg/ml streptomycin, 10 ng/ml epidermal growth factor and 5 μg/ml insulin. ZR-75-1 and MCF-7 human breast cancer cells (ATCC, Manassas, VA) were kept in RPMI 1640 medium containing 10% fetal calf serum and penicillin/streptomycin. Cells were grown at 37°C in a humid 5% CO_2 atmosphere.
Growing cells were stimulated with 10 μg/ml cisplatin (CP, Bristol-Myers Squibb, Baar, Switzerland) for 36 hrs. After CP stimulation, attached and floating cells were pooled, washed with medium containing fetal calf serum to inactivate excess trypsin and washed with PBS. For DAPI staining, a small fraction of cells was fixed in 3.5% paraformaldehyde, stained with 1 μg/ml DAPI (4',6-Diamidin-2'-phenylindol-dihydrochlorid, Roche Diagnostics, Switzerland) and the percentage of nuclei with an apoptotic morphology was determined. For quantification at least 200 cells were counted.

Fractionation of cells

Cells were trypsinized, washed once with PBS and once with hypotonic lysis buffer, HLB (20 mM HEPES, 10 mM KCl and 2 mM EGTA, 1 mM DTT, 100 μM PMSF) and 10 μM cytochalasin B (Sigma) where indicated. Cells were then incubated in HLB on ice for 20 min and lyzed by 15 passages through a 25 Gauge needle. Lysis was controlled under the microscope after staining with acridine orange (10 μg/ml) and ethidium bromide (10 μg/ml) in PBS. A crude nuclear pellet was obtained by centrifugation at 800 g for 10 min at 4°C. The supernatant was removed and cleared by centrifugation at 15'000 g for 30 minutes (Sigma 202 MK centrifuge) at 4°C. The cleared supernatant was used as an organelles-depleted cytosolic fraction and the resulting pellet was dissolved in sample buffer and used as mitochondria-enriched pellet. Nuclei were further purified by resuspending in HLB and centrifugation through a 30% sucrose/HLB cushion containing 10 μM cytochalasin B at 800 g for 15 min at 4°C and the purity was confirmed by microscopy. The resulting nuclei were washed once with HLB and stored in aliquots in nuclear storage buffer (10 mM HEPES pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine containing 50% glycerol).
Nuclear extracts were prepared essentially as described (Marti et al., 1994). In brief, crude nuclei were incubated in 20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 100 μM PMSF and 25% glycerol on ice for 30 min and centrifuged at 15'000 g for 30 min at 4°C (Sigma 202 MK). The supernatant was stored as nuclear extract and the nuclear pellet was resuspended in sample buffer. All samples were kept at –70°C until use. Where indicated nuclei or nuclear extracts were prepared in the presence of a protease inhibitor mix consisting of 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml antipain, 5 μg/ml chymostatin, 0.5 μg/ml pepstatin, 5 mM benzamidin, 1 mM aminohexanoic acid.

Processing of nuclear procaspase-9, Western blot analysis and fluorometric assays

Cytosolic extracts (100 μg) were activated with recombinant active caspase-3 or caspase-8, or with cytochrome c (10 μM) and dATP (1 mM) in a total volume of 20 ? l by incubation for 30 min at 30°C. Purified nuclei were added to the activated cytosol and incubated for 30 min at 30°C in the absence or presence of 50 μM z-VAD-fmk (Bachem), DEVD-fmk (Promega) or YVAD-fmk (Promega) dissolved in DMSO. Nuclei that were incubated without cytosolic extracts were used as control. After incubation 2 μl of each sample were transferred to a new tube for fluorometric assays of caspase activity. The remaining 18 ? l were boiled in sample buffer and processed for Western blot analysis.

Protein samples (~ 25 μg) were run on 12% SDS Polyacrylamide gels and transferred to a nitrocellulose membrane overnight. To prevent unspecific binding during Western analysis membranes were pre-incubated in PBS containing 5% nonfat dry milk, 0.05% Tween 20 at room temperature for 60 min. Membranes were then incubated with the first antibody for 60 min at room temperature on an orbital shaker, washed for 60 min with PBS containing 0.05% Tween 20. The washing solution was changed three times. The membranes were incubated with the secondary antibody for 60 min at room temperature and washed again for 60 min with PBS containing 0.05% Tween 20. The specific protein signal was detected by using the Western Blot Chemiluminescence Reagent Plus (NEN, Life Science Products, Boston, USA) following the instructions of the manufacturer.

Anti-hsp60 (a gift of G. Schatz, Basel, Switzerland) was used at a dilution of 1:2000 in PBS containing 5% nonfat dry milk, anti-caspase-9 (C9/Bur49, polyclonal rabbit), anti-PARP (sc-7150, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-cytochrome c (mouse monoclonal 7H8.2C12, Pharmingen, San Diego, CA) were used at a dilution of 1:1000. Secondary antibodies (goat anti-rabbit HRP and goat anti-mouse HRP, Bio-Rad Laboratories, Hercules, CA) were diluted 1:3000.

For fluorometric analyses of caspase activity, cytosolic or nuclear extracts (20 μg protein) were combined with 32 μl ICE-like Enzyme Assay Buffer (Promega, Madison, WI), 2 μl DMSO, 1 μl 1 M DTT, 62 μl H_2O and 1 μl substrate (100 μM DEVD-amc or 100 μM LEHD-afc, Calbiochem, La Jolla, CA), shortly mixed and emitting fluorescence at 30°C was kinetically measured for 50 min with a Spectramax Gemini Fluorometer (Molecular Devices, Sunnyvale, CA). The relative cleavage was determined by calculating the slope of the accumulation of amc or afc fluorochrome during the assay.

Immunofluorescence and confocal microscopy

Intracellular localization of cytochrome c and caspase-9 were determined by confocal microscopy. In brief, cells were washed twice with PBS and fixed on the plates with 4% paraformaldehyde in PBS for 10 min. The plates were washed twice with PBS for 5 min and stored under 50% ethanol or processed directly. The cells were incubated in methanol containing 0.6% H_2O_2 at room temperature for 30 min and washed with TBS containing 0.1% BSA for 10 min. Non-specific binding was blocked with goat serum (KPL, Kirkegaard and Perry, Gaithersburg, MD) at room temperature for 30 min followed by incubation with the first antibody at dilutions 1:50 (anti-caspase-9, C9/Bur73) and 1:100 (anti-cytochrome c, mouse monoclonal 7H8.2C12, Pharmingen, San Diego, CA) in goat serum at 4°C over night. Cells were washed with TBS containing 0.1% BSA for 10 min before incubation with FITC-labeled secondary antibody at dilutions of 1:80 (anti-mouse antibody, Cappel, Turnhout, Belgium) or 1:150 (anti-rabbit, Cappel, Turnhout, Belgium) in TBS containing 0.1% BSA at room temperature for 60 min. Cells were then incubated in the dark with propidium iodide (1 μg/ml) (Sigma) and heat inactivated RNase A (30 μg/ml) for 30 minutes at room temperature, rinsed with water and covered with Moviol. Analysis was performed with

a confocal microscope (Bio-Rad, Microradiance AG-2 using a Nikon Eclipse TE300 inverted microscope and a 20x objective with 0.5 numerical aperture).

Results

Procaspase-9 has a mitochondrial and a nuclear localization in mammary epithelial cells

31D and HC11 cells are non-tumorigenic mouse mammary epithelial cells which have retained the capacity to differentiate *in vitro* upon stimulation with lactogenic hormones (Ball et al., 1988; Doppler et al., 1989; Reichmann et al., 1989). In order to investigate expression and localization of caspase-9 in these mammary epithelial cells, we used previously characterized polyclonal caspase-9 antibodies (Krajewski et al., 1999). Fig. 1 shows an immunofluorescence analysis of growing, untreated 31D cells using confocal microscopy. Many nuclei stain positive for caspase-9 which appears to be associated with discrete structures in the nucleus (panel A). In addition, a punctate staining is observed throughout the cytoplasm which is consistent with caspase-9 being localized in mitochondria. Nuclei were visualized with propidium iodide (panel B). Panel C represents an overlay of panels A and B confirming that caspase-9 is both nuclear and cytoplasmic. Mitochondria were stained with a cytochrome c specific antibody (panel D) resulting in a punctate staining throughout the cytoplasm, typical for mitochondrial proteins. No nuclear staining was observed with the cytochrome c specific antibody as shown by a double staining with propidium iodide (panel F). In the absence of primary antibody no nuclear or mitochondrial signals were detected (panels G and I). A similar distribution of procaspase-9 was found in HC11 cells or with a different anti-caspase-9 antibody (data not shown).

Western blot analyses confirmed the nuclear localization of procaspase-9. An organelles-depleted fraction (Fig. 2, lane 1), a mitochondria-enriched fraction (lane 2) and a nuclear fraction (lane 3) were separately tested for the presence of procaspase-9. In the nuclear and mitochondria-enriched fractions a 49 kDa band was detected indicative for procaspase-9 corroborating the results obtained by confocal microscopy. Only residual amounts of procaspase-9 were detected in the organelles-depleted fraction. To evaluate the purity of the subcellular fractions, mitochondrial heat-shock protein 60 (hsp60) and cytochrome c were analyzed. Expression of hsp60 and cytochrome c was restricted to the mitochondria-enriched fraction (lane 2). Similar results were obtained with HC11 mammary epithelial cells (data not shown).

Processing and activation of procaspase-9 by cisplatin

Next we investigated whether nuclear procaspase-9 was processed when cells were exposed to a chemotherapeutic agent. 31D cells were treated with cisplatin and extensive apoptosis was observed 36 hours later as judged by nuclear morphology (data not shown). An organelles-depleted and a nuclear fraction were prepared in the presence of protease inhibitors. The nuclear fraction was further subdivided into a soluble nuclear extract and an insoluble nuclear membrane preparation (nuclear pellet). Fig. 3A shows that procaspase-9 is associated with the nuclear pellet preparation in untreated cells (lane 3, upper panel) whereas no procaspase-9 was detected in nuclear extracts (lane 5) or in organelles-depleted fractions (cytosolic extracts, lane 1). Treatment of cells with cisplatin leads to a complete loss of procaspase-9 from the insoluble nuclear pellet (lane 4).

Caspase activity was directly determined in cytosolic and nuclear extracts using LEHD-afc, a synthetic substrate for caspase-9 (Fig. 3B) and DEVD-amc, a substrate primarily for caspase-3-like caspases (Fig 3C). Both, LEHD-afc and DEVD-amc

cleavage activity were significantly induced after cisplatin treatment in cytosolic (lanes 1 and 2) and nuclear extracts (lanes 5 and 6). These results suggest that active caspases accumulate after cisplatin treatment not only in the cytosol but also in the nucleus.
To further document caspase-mediated changes after cisplatin treatment, poly(ADP-ribose) polymerase (PARP) cleavage was measured (Fig. 3A, lower panel). Intact PARP is predominantly found as a ~110 kDa protein within the soluble nuclear fraction of untreated cells (lane 5) and is readily cleaved to the characteristic 85 kDa form after cisplatin treatment (lane 6).

Nuclear procaspase-9 is cleaved by caspase-3
In order to investigate whether processing of nuclear procaspase-9 is mediated by caspases, we made use of a cell-free system. Purified nuclei were incubated with organelles-depleted extracts that were activated with recombinant active caspase-8 or caspase-3 (Fig. 4). When nuclei were incubated with untreated ZR-75-1 breast cancer cell extracts, no procaspase-9 processing was observed (Fig. 4, lane 1, top panel). Incubation of nuclei with ZR-75-1 cell extracts that were pre-incubated with recombinant active caspase-8 (lane 2) or caspase-3 (lane 3) resulted in a complete loss of nuclear procaspase-9. To further document caspase activity, PARP cleavage was determined from the same fractions. Fig. 4 (lower panel) shows that PARP was cleaved after incubation of nuclei with extracts that were stimulated with caspase-8 (lane 2) or caspase-3 (lane 3). No PARP cleavage was observed when nuclei were incubated with untreated extracts (lane 1).
In order to investigate in more detail the role of caspase-3 for caspase-9 processing, extracts of MCF-7 cells were used that are known to be deficient in caspase-3 expression (Li et al., 1997; Janicke et al., 1998). Cytosolic extracts derived of MCF-7 cells were stimulated with recombinant active caspase-3 or caspase-8 prior to the addition of purified nuclei. Only active caspase-3 was able to mediate procaspase-9 cleavage (lane 6, upper panel). Addition of active caspase-8 failed to mediate nuclear procaspase-9 cleavage under these conditions (lane 5), although it mediated directly or indirectly a partial PARP cleavage in the absence of caspase-3 (lane 5, lower panel). Procaspase-9 was only detectable in samples containing purified nuclei (Fig. 4, top panel). Only residual amounts of caspase-9 were observed in organelles-depletet cytosolic extracts derived of ZR-75-1 or MCF-7 cells (middle panel). The caspase-3-like activity that was monitored by a DEVD-amc cleavage assay correlated well with procaspase-9 processing (Fig. 4).
To extend these studies, organelles-depleted cytosolic cell extracts were activated with cytochrome c (Fig. 5A, lanes 4 to 8) and purified nuclei were added in the absence (lanes 4 and 5) or presence (lanes 6 to 8) of caspase inhibitors. After cytochrome c stimulation, nuclear procaspase-9 and PARP were cleaved in the absence of caspase inhibitors (lanes 4 and 5). The peptide based caspase inhibitor YVAD-fmk (lane 8) was without any effect with respect to DEVD cleavage activity and caspase-9 or PARP cleavage. In contrast, z-VAD-fmk (lane 6) and DEVD-fmk (lane 7) almost completely blocked nuclear procaspase-9 and PARP processing as well as DEVD cleavage activity. Again, no procaspase-9 was detected in organelles-depleted fractions in the absence of nuclei (middle panel). As a control, purified nuclei were also incubated without cellular extracts in the absence (lane 1) or presence of cytochrome c (lane 2). No DEVD-amc cleavage activity was generated and neither procaspase-9 nor PARP were cleaved under these conditions (lane 2).
The data obtained with caspase-3 deficient MCF-7 cell extracts and caspase-inhibitors indicate a role of caspase-3-like caspases for the processing of nuclear proceaspase-9. To further investigate this role of caspase-3 for procaspase-9 processing, organelles-depleted cell extracts were incubated with recombinant active caspase-3 prior to the addition of purified nuclei which resulted in a processing of nuclear procaspase-9 and PARP cleavage (Fig. 5B, lane 3). To test whether

caspase-3 mediated cleavage of nuclear procaspase-9 was dependent on cytosolic components, purified nuclei were incubated with recombinant active caspase-3 in the absence of cellular extracts. As shown in lane 5, active caspase-3 was sufficient to cleave nuclear procaspase-9. Caspase activity was again monitored by a DEVD cleavage assay in the same extracts and it correlated with procaspase-9 processing and PARP cleavage. Lower levels of caspase-3-like activity in samples lacking cytosolic cell extracts were due to a loss of recombinant caspase-3 activity in the presence of low protein concentrations (lane 5). Together, these data identify caspase-3 as a regulatory caspase of nuclear procaspase-9.

Discussion

In this study we present morphological and biochemical evidence of a nuclear localization of procaspase-9 in non-tumorigenic mammary epithelial cells. Processing of nuclear procaspase-9 correlates with caspase activation during apoptosis and recombinant caspase-3 is sufficient to mediate processing of procaspase-9 in purified nuclei.

During apoptosis caspase-9 is activated by cytochrome c and Apaf-1 (Li et al., 1997; Reed, 1997; Green and Reed, 1998; Zou et al., 1999). Recent data obtained by a number of groups suggest that procaspase-9 is predominantly a mitochondrial protein (Krajewski et al., 1999; Samali et al., 1999; Susin et al., 1999). Activation of caspase-9 may be an important requirement for the subsequent activation of caspase-3, a caspase that is central for the execution and amplification of the apoptotic response within cells (Porter and Janicke, 1999). Using purified components, caspase-9 was also described as a caspase-3 substrate (Srinivasula et al., 1996) and Stennicke and co-workers found that processing of caspase-9 was dependent on the presence of caspase-3 during TNF induced apoptosis (Stennicke et al., 1998). Caspase-9 contains two segments rich in basic amino acids suggesting that it may also associate with nuclei. Indeed, Krajewski and co-workers (Krajewski et al., 1999) described an association of caspase-9 with nuclei using nerve cells or cardiomyocytes. This nuclear association was shown to be the consequence of a translocation of mitochondrial caspase-9 to the nucleus during apoptosis. Our own results extend these observations and suggest a constitutive nuclear localization of a subpopulation of procaspase-9 in non-transformed mammary epithelial cells.

The localization of procaspase-9 may be cell type specific since Samali and co-workers (1999) did not find any procaspase-9 localized to the nucleus in Jurkat cells. Furthermore, we have evidence that the nuclear localization of procaspase-9 in mammary epithelial cells may be proliferation dependent (P.R., A.M. and R.J., unpublished observation). Based on morphological analyses and cellular fractionations our data reveal that procaspase-9 is associated with discrete structures of unknown nature within the nucleus. Treatment of cells with cisplatin induces a strong cytoplasmic and nuclear activation of caspase-3- and caspase-9-like caspases that correlates with the loss of procaspase-9 from the nucleus. However, it is presently unknown whether processing of nuclear procaspase-9 contributes to the generation of biologically active nuclear or cytoplasmic caspase-9-like activity. Using caspase-3 positive and caspase-3 negative cell lines we present direct evidence that processing of nuclear procaspase-9 depends on caspase-3. The nuclear localization of procaspase-9 and its regulation by caspase-3 may extend the repertoire of potential sites of action of this caspase and may help to define potentially novel targets and activation pathways. Caspase-9 substrates may include nuclear lamins that contain potential caspase-9 cleavage sites (for theoretical caspase-9 cleavage sites see Thornberry et al., 1997). It will be important to evaluate the molecular details that involve nuclear translocation, nuclear localization and activation of nuclear procaspase-9 during apoptosis. It remains to be shown whether nuclear

procaspase-9 activation is also dependent on a CARD domain mediated recruitment of procaspase-9 into a high molecular weight complex similar to the apoptosome that was previously described (Li et al., 1997; Reed, 1997; Green and Reed, 1998; Zou et al., 1999). Our data indicate that nuclear procaspase-9 can not be directly regulated by cytochrome c since addition of cytochrome c to purified nuclei failed to mediate nuclear procaspase-9 processing. Moreover, Stennicke and co-workers (1999) showed that caspase-9 displays the non-typical property that its activity is primarily regulated by cytosolic factors and not necessarily by proteolytic processing adding an additonal level of complexity to the regulation of procaspase-9. Further studies will be required to elucidate in more detail the biological significance of the nuclear localization and processing of procaspase-9.

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References

Ball, R. K., Friis, R. R., Schönenberger, C. A., Doppler, W., Groner, B. (1988): Prolactin regulation of b-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J. **7,** 2089-2095.

Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., Frisch, S. M. (1997): The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. Cell **90,** 315-323.

Casciola-Rosen, L., Nicholson, D. W., Chong, T., Rowan, K. R., Thornberry, N. A., Miller, D. K., Rosen, A. (1996): Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. J. Exp. Med. **183,** 1957-1964.

Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., Hardwick, J. M. (1997): Conversion of Bcl-2 to a Bax-like death effector by caspases. Science **278,** 1966-1968.

Doppler, W., Groner, B., Ball, R. K. (1989): Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat b-casein gene promoter constructs in a mammary epithelial cell line. Proc. Natl. Acad. Sci. USA **86,** 104-108.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., Nagata, S. (1998): A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature **391,** 43-50.

Green, D. R., and Reed, J. C. (1998): Mitochondria and apoptosis. Science **281,** 1309-1312.

Janicke, R. U., Sprengart, M. L., Wati, M. R., Porter, A. G. (1998): Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem. **273,** 9357-9360.

Kerr, J. F. R., Wyllie, A. H., Currie, A. R. (1972): Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. Br. J. Cancer **26,** 239-257.

Kidd, V. J. (1998): Proteolytic activities that mediate apoptosis. Annu. Rev. Physiol. **60,** 533-573.

Krajewski, S., Krajewska, M., Ellerby, L. M., Welsh, K., Xie, Z., Deveraux, Q. L., Salvesen, G. S., Bredesen, D. E., Rosenthal, R. E., Fiskum, G., Reed, J. C. (1999): Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. Proc. Natl. Acad. Sci. USA **96,** 5752-5757.

Li, F., Srinivasan, A., Wang, Y., Armstrong, R. C., Tomaselli, K. J., Fritz, L. C. (1997): Cell-specific induction of apoptosis by microinjection of cytochrome c. Bcl-XL has activity independent of cytochrome c release. J. Biol. Chem. **272,** 30299-30305.

Li, H., Zhu, H., Xu, C. J., Yuan, J. (1998): Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94,** 491-501.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., Wang, X. (1997): Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell **91,** 479-489.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., Wang, X. (1996): Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86,** 147-157.

Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W. T., Wang, X. (1998): The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. Proc. Natl. Acad. Sci. USA **95,** 8461-8466.

Liu, X., Zou, H., Slaughter, C., Wang, X. (1997): DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell **89,** 175-184.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., Wang, X. (1998): Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell **94,** 481-490.

Marti, A., Jehn, B., Costello, E., Keon, N., Ke, G., Martin, F., Jaggi, R. (1994): Protein kinase-A and AP-1 (c-Fos/JunD) are induced during apoptosis of mouse mammary epithelial cells. Oncogene **9,** 1213-1223.

Martin, S. J., O'Brien, G. A., Nishioka, W. K., McGahon, A. J., Mahboubi, A., Saido, T. C., Green, D. R. (1995): Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. J. Biol. Chem. **270,** 6425-6428.

Porter, A. G., and Janicke, R. U. (1999): Emerging roles of caspase-3 in apoptosis. Cell Death Differ. **6,** 99-104.

Rao, L., Perez, D., White, E. (1996): Lamin proteolysis facilitates nuclear events during apoptosis. J. Cell Biol. **135,** 1441-1455.

Reed, J. C. (1997): Cytochrome c: can't live with it--can't live without it. Cell **91,** 559-562.

Reichmann, E., Ball, R., Groner, B., Friis, R. R. (1989): New mammary epithelial and fibroblastic cell clones in coculture form structures competent to differentiate functionally. J. Cell Biol. **108,** 1127-1138.

Sakahira, H., Enari, M., Nagata, S. (1998): Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature **391,** 96-99.

Salvesen, G. S., and Dixit, V. M. (1997): Caspases: intracellular signaling by proteolysis. Cell **91,** 443-446.

Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P., Orrenius, S. (1999): Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. EMBO J. **18,** 2040-2048.

Srinivasula, S. M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R. C., Wang, L., Trapani, J. A., Tomaselli, K. J., Litwack, G., Alnemri, E. S. (1996): The Ced-3/interleukin 1beta converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2alpha are substrates for the apoptotic mediator CPP32. J. Biol. Chem. **271,** 27099-27106.

Stennicke, H. R., Deveraux, Q. L., Humke, E. W., Reed, J. C., Dixit, V. M., Salvesen, G. S. (1999): Caspase-9 can be activated without proteolytic processing. J. Biol. Chem. **274,** 8359-8362.

Stennicke, H. R., Jurgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., Salvesen, G. S. (1998): Pro-caspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. **273,** 27084-27090.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M. C., Alzari, P. M., Kroemer, G. (1999): Mitochondrial release of caspase-2 and -9 during the apoptotic process. J. Exp. Med. **189,** 381-394.

Takahashi, A., Alnemri, E. S., Lazebnik, Y. A., Fernandes-Alnemri, T., Litwack, G., Moir, R. D., Goldman, R. D., Poirier, G. G., Kaufmann, S. H., Earnshaw, W. C. (1996): Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. Proc. Natl. Acad. Sci. USA **93,** 8395-8400.

Tewari, M., Quan, L. T., O Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., Dixit, V. M. (1995): Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA- inhibitable protease that cleaves the death substrate poly(ADP- ribose) polymerase. Cell **81,** 801-809.

Thornberry, N. A., and Lazebnik, Y. (1998): Caspases: enemies within. Science **281,** 1312-1316.

Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., Nicholson, D. W. (1997): A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J. Biol. Chem. **272,** 17907-17911.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A., Wang, X. (1997): Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell **90,** 405-413.

Zou, H., Li, Y., Liu, X., Wang, X. (1999): An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. **274,** 11549-11556.

Figure legends

Fig. 1 Nuclear localization of caspase-9 in mammary epithelial cells. 31D cells were incubated with caspase-9 (C9/Bur73) (panel A) or cytochrome c specific antibodies (panel D). Bound primary antibodies were visualized with FITC-labeled secondary antibodies. Panel G represents an FITC staining in the absence of primary antibody. After immunocytochemistry, cells were stained with propidium iodide (panels B, E, and H). Panels C, F and I represent overlays of the FITC and the corresponding propidium iodide stainings. Pictures were taken with confocal laser scanning microscope using a 20x objective with 5x zoom. The bar represents 20 μm.

Fig. 2 Procaspase-9 is associated with mitochondrial and nuclear fractions in mammary epithelial cells. 31D cells were lyzed and organelles-depleted (lane 1), mitochondria-enriched (lane 2) and nuclear fractions (lane 3) were generated in the presence of cytochalasin B as described in Materials and methods. The fractions were examined for the presence of caspase-9, hsp60 or cytochrome c by Western blot analyses as indicated.

Fig. 3 (A) Processing of nuclear procaspase-9 after treatment of mammary epithelial cells with cisplatin. 31D cells were either treated with cisplatin for 36 hours or left untreated as indicated. Cytosolic extracts (lanes 1 and 2), nuclear pellets (lanes 3 and 4) and nuclear extracts (lanes 5 and 6) were analyzed for procaspase-9 processing (upper panel) or PARP cleavage (lower panel) by immunoblotting. (B, C) Induction of caspase activity by cisplatin. Cytosolic and nuclear extracts were prepared and tested for LEHD-afc (B) or DEVD-amc cleavage activity (C) in a fluorometer and relative activity was determined as described in Materials and methods. The nuclear pellet fractions were dissolved in sample buffer and caspase activity could not be determined (N.D.)

Fig. 4 Processing of nuclear procaspase-9 depends on caspase-3. Purified HC11 nuclei were incubated with cytosolic extracts derived from ZR-75-1 (lanes 1 to 3) or MCF-7 cells (lanes 4 to 6) in the absence (lanes 1 and 4) or presence of recombinant active caspase-8 (lanes 2 and 5) or caspase-3 (lanes 3 and 6). Procaspase-9 and PARP cleavage were analyzed by immunoblotting. Procaspase-9 was also analyzed in cytosolic extracts in the absence of purified nuclei (putative position at 49 kDa is indicated). Extracts in the presence (black bars) or absence (gray bars) of purified nuclei were analyzed for DEVD-amc cleavage activity using a fluorometer.

Fig. 5 (A) Cytochrome c induced processing of nuclear procaspase-9 is inhibited by z-VAD-fmk and DEVD-fmk. Purified HC11 nuclei were incubated without (lanes 1, 2) or with organelles-depleted cellular extracts (lanes 3 to 8) that were either left untreated (lanes 1, 3) or activated with cytochrome c and dATP (lanes 2, 4, 5, 6, 7, 8). DMSO (lane 5), z-VAD-fmk (lane 6), DEVD-fmk (lane 7) or YVAD-fmk (lane 8) were added to the extracts prior to the addition of nuclei. Procaspase-9 and PARP cleavage were visualized by immunoblotting. Procaspase-9 was also anlayzed in cytosolic extracts in the absence of purified nuclei (putative position at 49 kDa is indicated). Extracts in the presence (black bars) or absence (gray bars) of purified nuclei were analyzed for DEVD-amc cleavage activity using a fluorometer. (B) Caspase-3 is sufficient to cleave nuclear procaspase-9. Purified HC11 nuclei were either incubated with cellular extracts (lanes 1, 2, 3) or with buffer A (lanes 4, 5). Procaspase-9 and PARP were measured by Western blot analyses prior to incubation (lane 1) or after incubation at 30°C (lanes 2 to 5) in the absence (lanes 1, 2 and 4) or presence (lanes 3 and 5) of recombinant active caspase-3. Procaspase-9 was also analyzed in cytosolic extracts in the absence of purified nuclei (middle panel, putative position at 49 kDa is indicated). Extracts in the presence (black bars) or absence (gray bars) of purified nuclei were analyzed for DEVD-amc cleavage activity using a fluorometer.

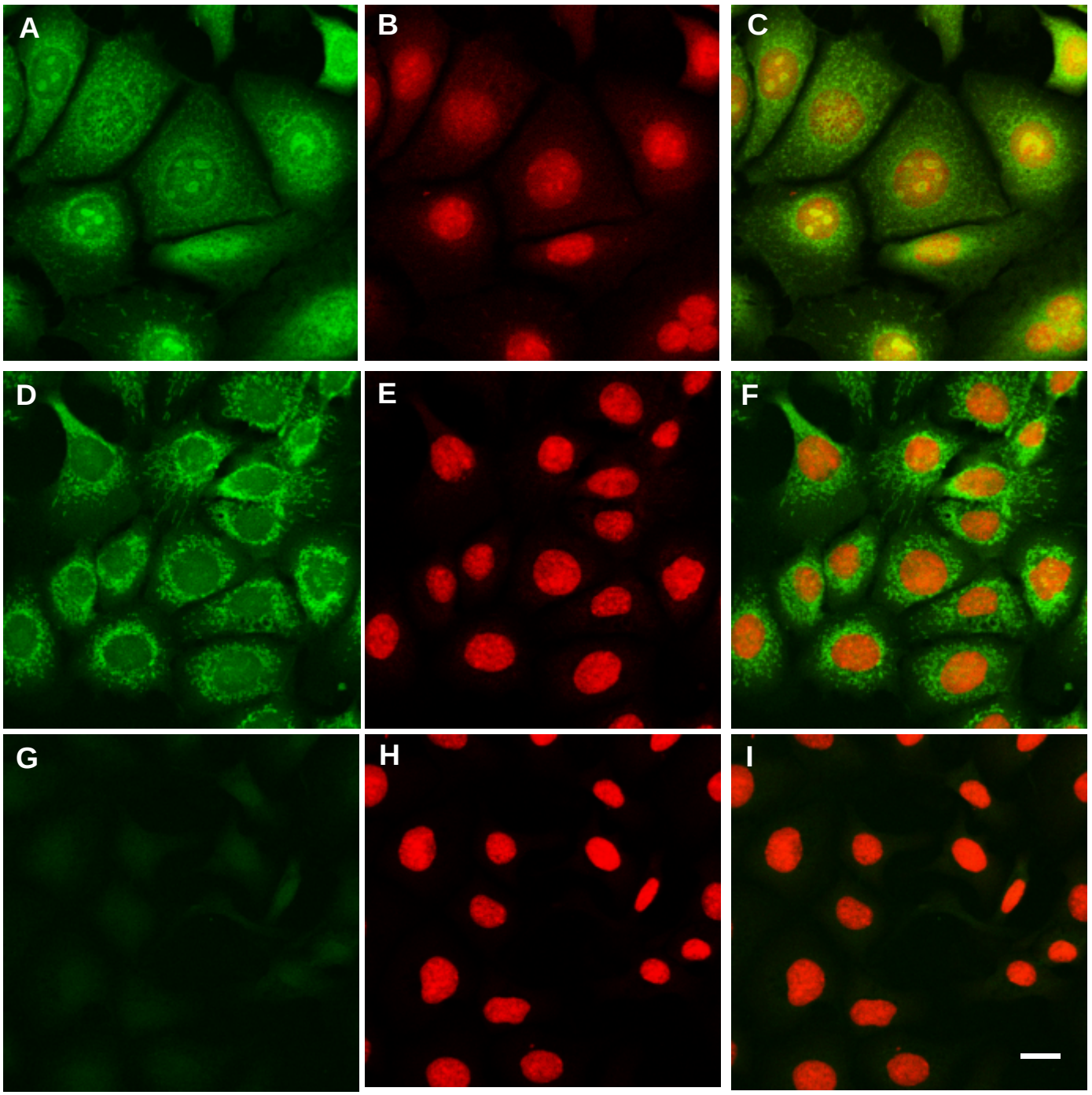

Figure 1

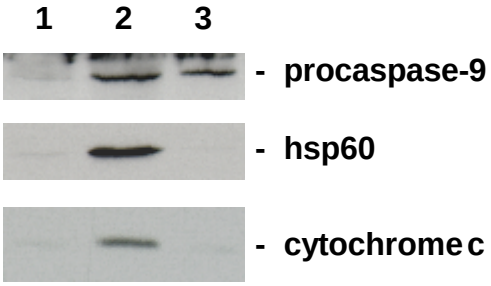

Figure 2

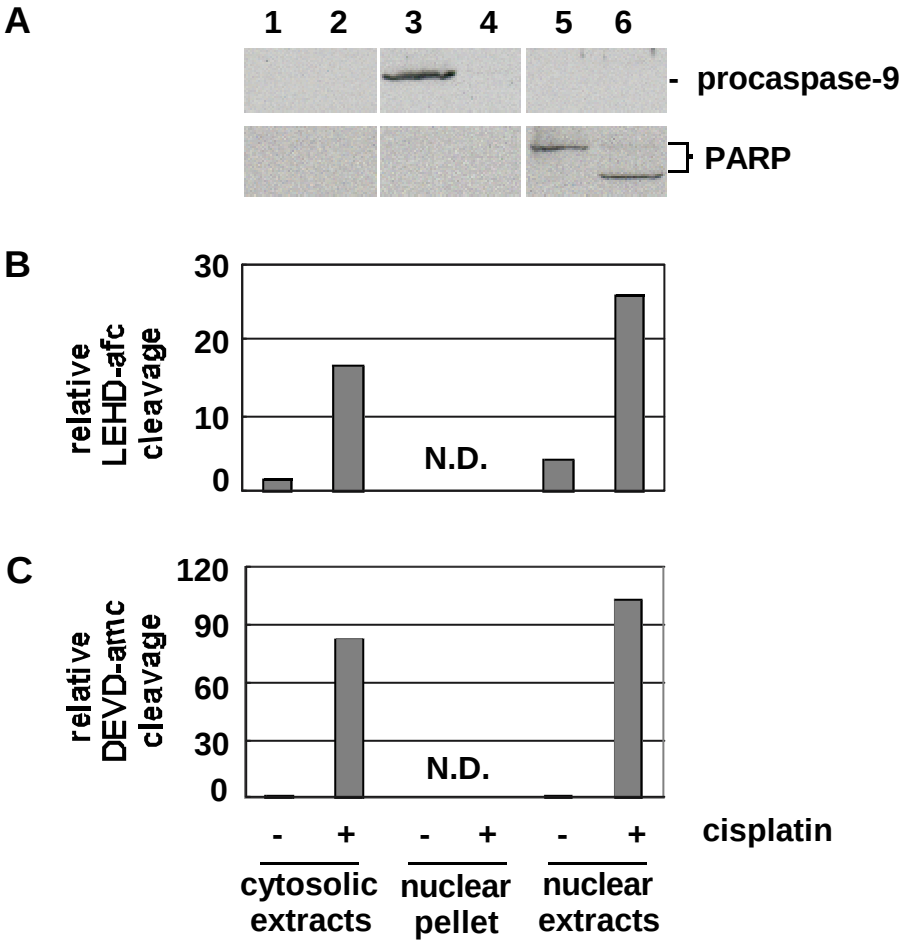

Figure 3

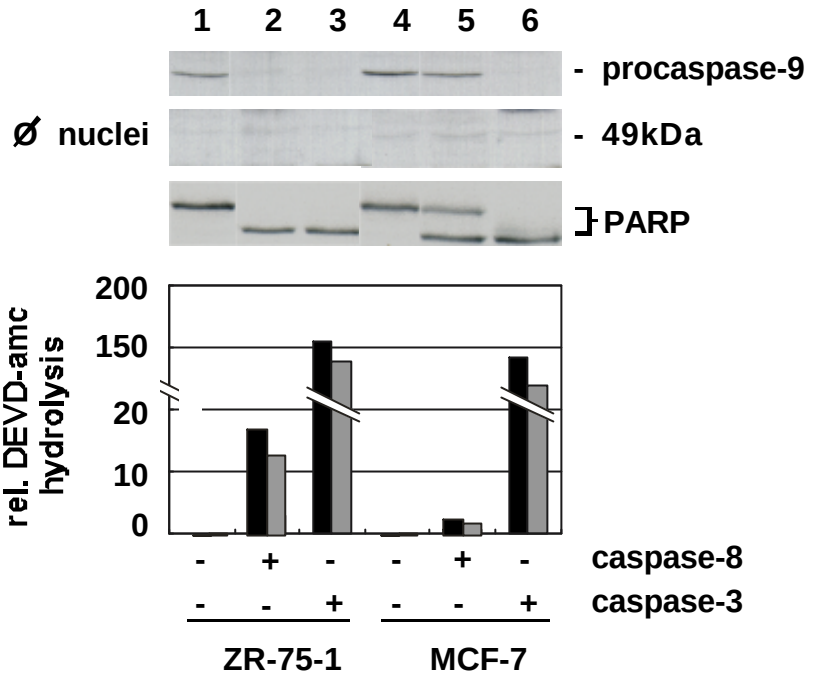

Figure4

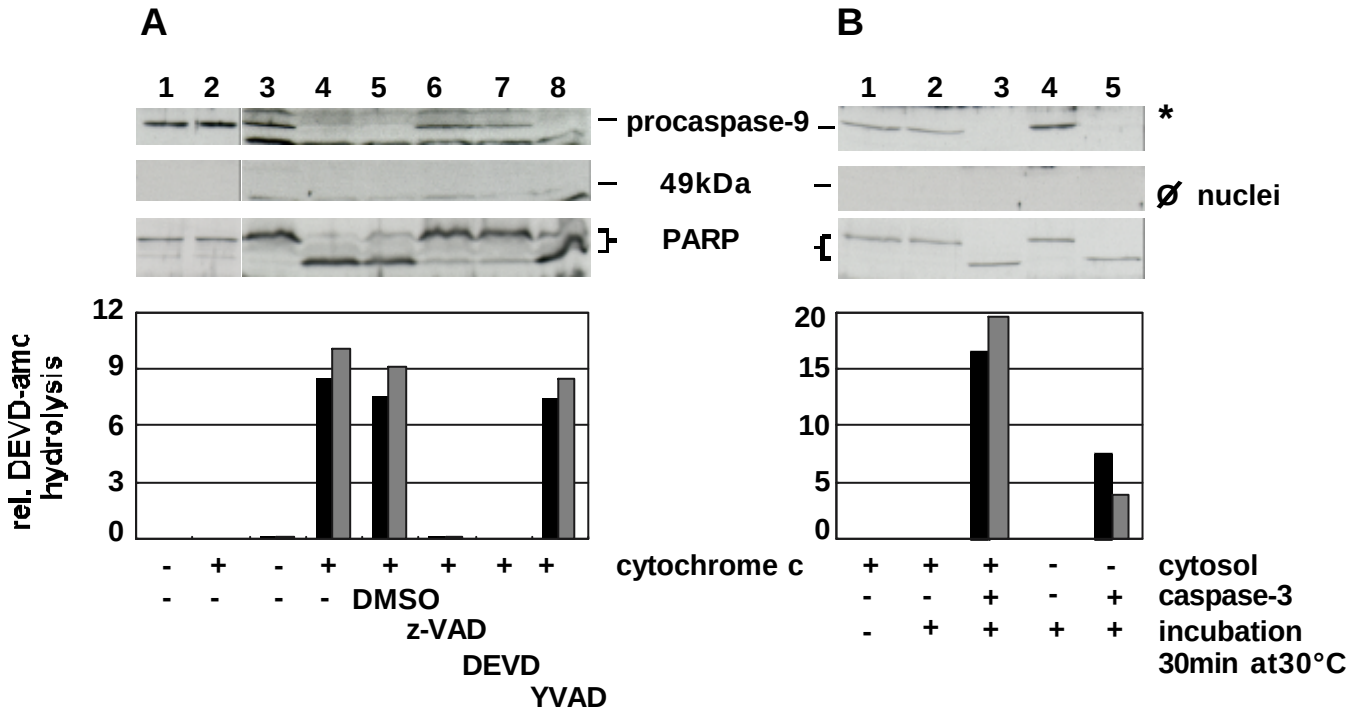

Figure5