Caspase-3 is Essential for Procaspase-9 Processing and Cisplatin-Induced Apoptosis of MCF-7 Breast Cancer Cells[1]

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ABSTRACT

In this study we sought to investigate in more detail the role of caspase -3 in apoptotic processes in cultured cells and in cell-free extracts of breast cancer cells. We present evidence that apoptosis of caspase -3-deficient MCF-7 breast cancer cells is defective in response to cisplatin treatment, as determined by chromatin condensation, nuclear fragmentation, DNA-fragmentation and release of cytochrome c from mitochondria. Reconstitution of MCF-7 cells by stable transfection of a *CASP*-3 cDNA restores all these defects and results in an extensive apoptosis after cisplatin treatment. We further show that in extracts from caspase-3 deficient MCF-7 cells, procaspase-9 processing is strongly impaired after stimulation with either cytochrome c or recombinant caspase -8. Reconstitution of MCF-7 cell extracts with procaspase -3 corrects this defect, resulting in an efficient and complete processing of procaspase -9. Together, our data define caspase -3 as an important integrator of the apoptotic process in MCF -7 breast cancer cells and reveal an essential function of caspase-3 for procaspase-9 processing.

INTRODUCTION

Apoptosis or programmed cell death is essential for organogenesis during development, proper function of the immune system, elimination of genetically instable cells, and maintenance of tissue homeostasis in the adult (1). Apoptosis is also the main response of cells to chemotherapeutic agents (2). Apoptosis results from activation of members of the caspase family of aspartate-specific proteases (3, 4). Caspases form a proteolytic network within the cell whereby upstream initiator caspases are activated early in the apoptotic process (e.g. caspase-8, caspase-9) and then activate other downstream caspases (e.g. caspase-3, caspase-7). The downstream caspases are largely responsible for cleavage of many other cellular proteins, leading to the morphological manifestations of apoptosis.

In some cell types, the caspase -9 enzyme is found in the mitochondrial intermembrane space and is released into the cytosol together with cytochrome c after rupture of the outer mitochondrial membrane (5, 6). Once released, caspase -9 interacts with and is activated by the apoptosis activating factor Apaf -1 in a cytochrome c- and dATP-regulated manner (7-10). Subsequently, procaspase -3 is recruited to the Apaf-1/caspase-9 complex and undergoes proteolysis and activation (10, 11). Interestingly, caspase -9 contains a caspase -3 cleavage site at position 330 and it has been shown that procaspase -9 is also a substrate of caspase-3 during apoptosis (12). Indeed, using a cell-free system of apoptosis, Slee and co-workers (13) showed that caspase-9 processing can be enhanced by caspase -3. Furthermore, these authors showed that cytochrome c mediates a hierarchical activation of numerous caspases in addition to caspase -9 and caspase-3, such as caspase-2, -6, -7, -8 and -10 in a complex proteolytic cascade. Once activated, caspase -9 translocates to the nucleus where it may participate in the nuclear dismantling during apoptosis (5). Studies including the investigation of Apaf -1 and caspase-9 deficient animals show that the release of mitochondrial cytochrome c and the subsequent Apaf -1-dependent activation of caspase -9 and caspase-3 represents a common pathway used by many apoptosis-inducing stimuli and which is important for tumor suppression by p53 (14-18).

Recent evidence suggests that caspase -3 plays an important role for several key events during apoptosis such as nuclear fragmentation, DNA fragmentation and membrane blebbing, in a cell-type specific and stimulus specific manner (19). Furthermore, caspase-3 was reported to play a role as an amplifier of the apoptotic signals i.e. by the cleavage of Bcl-2 (20, 21). The role of caspase -3 was extensively studied in caspase -3 deficient animals (22, 23). These animals exhibit massively impaired developmental apoptosis in the brain whereas programmed cell death in other organs occurs normally. Caspase -3 deficient embryonic stem cells are resistant to UV and sorbitol-induced cell death, whereas ?-irradiation-induced cell death occurs normally. Similar results were obtained in MCF -7 cells that harbour a spontaneous deletion of 47 base pairs within exon 3 of the *CASP*-3 gene (24).

This mutation introduces a premature stop-codon and leads to a complete absence of caspase-3 protein and activity. A comparison of MCF-7 cells and *CASP*-3-transfected MCF-7 cells revealed that DNA fragmentation and membrane blebbing were severely affected after TNF or staurosporine treatment (24).

In this study we extend our previous observations by showing that cisplatin-induced cytochrome c release, nuclear fragmentation, and fragmentation of genomic DNA were all strongly enhanced by restoring caspase-3 in MCF-7 cells. Cytochrome c- and caspase-8-mediated procaspase-9 processing were highly dependent on caspase-3, placing this caspase in a central position as a regulator and amplifier of essential apoptotic pathways in breast cancer cells.

MATERIALS AND METHODS

Cell culture, cisplatin treatment and apoptosis analysis. ZR-75-1 and MCF-7 are human breast cancer cell lines (derived of American Type Culture Collection). MCF-7 cells lack functional caspase-3 (24, 25). MCF-7 *CASP*-3 and MCF-7 vc cells were were obtained by stable transfection of caspase-3 cDNA or empty vector and selection with G418 as described (24, 25). All cells were maintained at a subconfluent state in RPMI 1640 medium containing 10% FCS and 200 U/ml penicillin and 200 µg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For cisplatin treatment, MCF-7 cells were seeded at 60% confluency in 9 cm dishes. The next day 10 ? g/ml cisplatin (500 ? g/ml stock, Bristol-Myers Squibb, Baar, Switzerland) was added and culture was continued for 48 h or 72 h. Cells were harvested by trypsinization and either analyzed for apoptotic morphology after DAPI staining or for DNA fragmentation after isolation of total DNA. Experiments were repeated three to five times. DNA fragmentation was analyzed essentially as described in (26). For DAPI staining (4', 6'-Diamidin-2'-phenylindol-dihydrochlorid, Roche Diagnostics, Switzerland), cells were fixed with 3% formalin and stained with 1 ? g/ml DAPI in PBS for 10 min. Cells were mounted on glass slides, covered and analyzed using fluorescent microscopy. For statistical analysis of each experiment, five to ten fields (magnification 400x) were counted per stimulation and cell type (between 400 and 700 cells in total). Standard deviation and mean was calculated and displayed as bar graph. T-test analysis was performed and p values for each of the corresponding pairs were calculated using Microsoft Excel program.

Preparation, activation and reconstitution of cell-free extracts. Cell-free extracts and mitochondrial enriched fractions were prepared essentially as described (27, 28). Protein concentration was determined using the Pierce BCA protein assay kit. Extracts were activated either by the addition of 10 µM bovine heart cytochrome c (Sigma) in combination with 1 mM dATP (Sigma) or by the addition of purified recombinant active caspase-3 or caspase-8 as described (27). MCF-7 cell extracts were reconstituted by the addition of recombinant procaspase-3. For normalization, the amount of procaspase-3 that was added to extracts was checked by Immunoblot analysis. Extracts were either directly activated as described above or pre-incubated for 30 minutes at 37°C, prior to activation.

Immunoblot analysis. Protein samples were separated by 12% SDS polyacrylamide gel electrophoreses and subjected to Immunoblot analysis as described (28). Protein detection was performed using the Immunoblot 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-3 (polyclonal rabbit) 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.

DEVD-amc cleavage assay. For fluorometric assays equal amounts of cytosolic extracts (~ 40 μg protein) were combined with 32 μl ICE-like Enzyme Assay Buffer (Promega, Madison, WI), 2 μl DMSO, 1 μl 1 M DTT, 60 μl H_2O and 1 μl synthetic Asp-Glu-Val-Asp-amc (DEVD-amc) caspase-3-substrate (100 ?M stock of DEVD-amc in DMSO, Calbiochem, La Jolla, CA), briefly mixed and emitted fluorescence at 30°C was kinetically measured over 50 min using a Spectramax Gemini Fluorometer (Molecular Devices, Sunnyvale, CA). The relative cleavage activity was determined by calculating the slope of the accumulation of amc fluorochrome during the linear portion of the reaction.

Analysis of caspase processing with *in vitro* generated caspase-9 proteins. 1 ?g of pET21 plasmids encoding full length procaspase-9 (29) was *in vitro* transcribed and translated in the presence of [^{35}S]L-methionine or [biotin]L-methionine using the coupled transcription/translation TNT kit (Promega) according to the manufacturer's instructions. Proteins were desalted and exchanged into buffer A with Bio-spin P-6 columns (Bio-Rad). 0.5 ?g labeled procaspase-9 was added to 9.5 ?l ZR-75-1 or MCF-7 extracts that were activated as described with cytochrome c, recombinant active caspase-8 or recombinant active caspase-3. After incubation, proteins were separated on a 12% SDS polyacrylamide gel. For detection of [^{35}S]L-methionine labeled caspase-9, gels were fixed in 25% isopropanol / 10% acetic acid for 30 min. For amplification of the signal, gels were incubated in 1 M Na-salicylate (Sigma) for 15 min. Gels were dried and exposed to a X-ray film (Kodak). For detection of the [biotin]L-methionine labeled caspase-9, proteins were transferred to a nitrocellulose membrane overnight. Membranes were incubated in PBS containing 0.05% Tween 20 at room temperature for 1 h and caspase-9 was visualized according to the manufacturer's instructions using horse radish peroxidase coupled streptavidin (Promega) diluted 1:10,000 in PBS containing 0.05% Tween 20.

RESULTS

Caspase-3 is required for cisplatin-induced apoptosis of breast cancer cells

Human breast cancer cell lines were used to characterize the requirement for caspase-3 in cisplatin-mediated apoptosis. MCF-7 breast cancer cells that harbor a spontaneous mutation in the *CASP*-3 gene were stably transfected with a caspase-3 expression vector (MCF-7 *CASP*-3) or with an empty vector (MCF-7 vc) (30). ZR-75-1 breast cancer cells that express endogenous caspase-3 were used as control. Fig. 1 *A* shows results of a DAPI staining, visualizing the extent of nuclear fragmentation before stimulation (upper panels) and 72 h after cisplatin treatment (lower panels). Quantification of nuclear changes revealed that MCF-7 vc cells were highly resistant to cisplatin-mediated apoptosis (mean of about 4% apoptotic nuclei 72 h after cisplatin treatment) whereas ZR-75-1 were sensitive (mean of about 40% apoptotic nuclei 72 h after cisplatin treatment) (Fig. 1 *B*). Transfection of *CASP*-3 cDNA into MCF-7 cells converted resistant cells to cisplatin sensitive cells (MCF-7 *CASP*-3, mean of almost 60% after 72 h of cisplatin treatment). Cisplatin-mediated apoptosis was significantly induced in MCF-7 *CASP*-3 cells as compared to MCF-7 vc cells ($p = 0.0041$ after 48 h, $p = 0.0016$ after 72 h). Panel *C* demonstrates that cisplatin induces DNA fragmentation in ZR-75-1 and MCF-7 *CASP*-3 cells, while MCF-7 vc cells were resistant to oligonucleosomal DNA-fragmentation.

To determine in more detail the level at which caspase-3 deficiency interferes with the apoptotic process, release of cytochrome c from mitochondria was monitored. Fig. 2 *A* demonstrates that cytochrome c is retained within mitochondria for at least 48 h in MCF-7 vc cells (lane 2) and is only released after 72 h (lane 3). In MCF-7 *CASP*-3 cells, cytochrome c release was already complete 48 h after cisplatin stimulation (lane 5). In contrast, levels of the mitochondrial matrix protein Hsp60 remain similar in mitochondria after cisplatin treatment (Fig. 2 *B*). These results indicate that cisplatin-mediated cytochrome c release is accelerated in caspase-3 expressing cells.

Processing and activation of caspase-3 by cytochrome c in breast cancer cell extracts

The data presented in Fig. 1 and 2 document a prominent role for caspase -3 during cisplatin-mediated apoptosis including an enhancement of the mitochondrial cytochrome c release. To further investigate the connection between cytochrome c and caspase -3, cell-extracts from ZR-75-1 cells, untransfected MCF -7 cells, control transfected MCF -7 cells (MCF-7 vc) and *CASP*-3 transfected MCF -7 cells (MCF-7 *CASP*-3) were analyzed. Caspase -3 expression and processing was determined in extracts before and after stimulation with purified cytochrome c and dATP (Fig. 3 *A*). As previously described for other cell lines (7, 27), caspase-3 was efficiently processed in ZR -75-1 derived cell extracts after stimulation with cytochrome c and dATP (lanes 1, 2, 3). No caspase -3 protein was detected in MCF -7 cells (lanes 5 to 8) and MCF -7 vc cells (lanes 9, 10). However, in MCF -7 *CASP*-3 cell extracts, expression of caspase -3 (lane 11) and proc essing by the cytochrome-mediated pathway was fully restored (lane 12).

Caspase-3 activity was measured from the same cell extracts by analyzing the cleavage of the synthetic caspase -3 peptide-substrate Asp-Glu-Val-Asp-amc (DEVD-amc) (Fig. 3 *B*). DEVD based peptides have previously been shown to be specifically recognized and cleaved by active caspase -3 and to a lesser extent by active caspase -2 and -7 (31, 32). A significant induction of cytochrome c-mediated DEVD -amc cleavage activity was o nly detected in ZR-75-1 (lanes 2, 3) and MCF -7 *CASP*-3 cell extracts after cytochrome c stimulation (lane 12) and it correlated well with caspase -3 expression and processing.

Caspase-9 processing after stimulation with cytochrome c and caspase-8

Major death signals are initiated within the cells by the release of mitochondrial cytochrome c which initially activates procaspase -9 or by death receptors which initially activate procaspase-8. Both pathways are likely to contribute to chemotherapy-induced cell dea th (33-35). To analyze processing of procaspase -9 mediated by either cytochrome c or caspase-8, extracts derived from MCF -7 and ZR-75-1 cells were incubated with ^{35}S-methionine-labeled *in vitro* synthesized procaspase -9 and treated with cyt ochrome c (Fig. 4*A*) or active caspase -8 (Fig. 4*B*). This method has previously been shown to be very accurate for analyzing the fate of procaspase -9 (29). In ZR-75-1 cell extracts, both treatments resulted in an efficient and complete processing of ^{35}S-methionine-labeled procaspase-9 (Fig. 4*A*, lane 2 and Fig. 4 *B*, lane 2). In contrast, no processing of ^{35}S-methionine-labeled procaspase -9 was observed when caspase -3 deficient MCF-7 cell extracts were treated with cytochrome c or caspase -8 (Fig. 4*A*, lane 4 and Fig. 4 *B*, lane 4). As a control, incubation of ^{35}S-methionine-labeled procaspase -9 with cytochrome c in the absence of cellular extracts did not result in any processing of *in vitro* translated procaspase-9 (Fig. 4*A*, lane 6). Similarly, incubation of ^{35}S-methionine-labeled procaspase -9 with caspase-8 in the absence of extracts resulted in only a very minor processing of caspase-9 (Fig. 4*B*, lane 6).

Reconstitution of MCF-7 cell extracts with caspase-3 restores caspase-9 processing

To verify that the impaired procaspase -9 processing in MCF -7 cell extracts was due to the lack of caspase -3 activity, we reconstituted extracts by the addition of bacterially produced and purified inactive procaspase -3. Procaspase-3 reconstituted MCF -7 cell extracts w ere subjected to either cytochrome c or caspase -8 treatment. Fig. 5 shows that incubation of extracts with recombinant caspase -8 induced strong DEVD-amc cleavage activity (lane 3) whereas cytochrome c was rather inefficient in mediating a significant activation of procaspase-3 (lane 2). Interestingly, when extracts were pre-incubated at 37°C for 20 minutes, sensitivity to cytochrome c was much improved (lanes 4, 5). In contrast, caspase -8-mediated induction of caspase -3 activity was independent of the pre-i ncubation at 37°C (lane 6).

After having established the conditions for the reconstitution of MCF -7 cell extracts with recombinant procaspase-3, we next analyzed cytochrome c-mediated procaspase -9 processing in these extracts. *In vitro* synthesized biotinylated procaspase -9 was added to pre-incubated MCF-7 extracts in the absence (Fig. 6, lanes 1 to 4) or presence of recombinant inactive procaspase -3 (lanes 5 to 7) and cell extracts were analyzed untreated (lane 1) or after stimulation with cytochrome c (lanes 2, 6), recombinant active caspase -8

(lanes 3, 7) or recombinant active caspase -3 (lane 4). Stimulation with cytochrome c resulted in a processing of procaspase -9 only after reconstitution of extracts with inactive procaspase-3 (lane 6). Similarly, caspase-8 induced procaspase -9 processing only in the presence of exogenously added procaspase -3 (lane 7) but not in its absence (lane 3). In accordance with previous data, addition of constitutively active recombinant caspase -3 was sufficient to completely process procaspase -9 (lane 4). A similar dependence of procaspase-9 processing on caspase -3 was obtained when extracts from MCF -7 vc and MCF-7 *CASP*-3 cells were compared (data not shown). The same MCF-7 cell extracts were also subjected to a DEVD cleavage assay. As shown in Fig. 6, DEVD cleavage activity closely correlated with the presence of active caspase -3 and with the processing of procaspase-9. These results underscore the tight relationship between the presence or absence of active caspase -3 and procaspase-9 processing.

DISCUSSION

This study documents the specific role of caspase -3 as an amplifier of mitochondrial cytochrome c release, and of morphological changes of nuclei and DNA fragmentation during cisplatin-induced apoptosis in breast cancer cells. Moreover, cytochrome c- and caspase -8-mediated processing of procaspase -9 is strictly dependent on caspase -3 in these cells, suggesting that caspase -3 may be central for the regulation of procaspase -9.

Our results with cisplatin confirm and extend previous results obtained with MCF -7 cells and *CASP*-3 transfected MCF-7 cells (24, 30). In these earlier studies it was shown that during TNF- and staurosporine-induced apoptosis, DNA fragmentation and membrane blebbing were impaired due to the absence of caspase -3 (24). We found that cisplatin-mediated fragmentation of nuclei and the appearance of the DNA ladder are also largely dependent on caspase-3, further generalizing the contribution of caspase -3 to these events.

Cytochrome c release from mitochondria that occurs after cisplatin stimulation is controlled, at least in part, by caspase -3. This is evidenced by the fact that cytochrome c release is strongly delayed in the absence of caspase -3 in MCF-7 cells and transfection of *CASP*-3 resulted in a release of cytochrome c. It may be that caspase -3 substrates influence the mitochondrial status. Bcl-2 and caspase-8 are possible candidates for such substrates. Bcl -2 can be converted to a pro-apoptotic protein by caspase -3 that may no longer protect mitochondria from cytochrome c release (20, 21). Caspase-3-mediated caspase -8 cleavage may result in cleavage of Bid, a pro-apoptotic protein that efficiently induces cytochrome c release (36-38). However, whether cleavage of Bcl -2 or Bid is a requirement for the cytochrome c release after cisplatin treatment in breast cancer cells remains to be determined.

Cytochrome c release from mitochondria and caspase -8 activation by Fas have both been implicated in chemotherapy-induced apoptosis (33-35). Our data obtained with cellular extracts indicate that cytochrome c- and caspase -8-mediated processing of procaspase -9 are both strongly dependent on the presence of caspase -3. These findings extend earlier studies that describe a dependence of procaspase -9 processing on caspase -3 during TNF- and granzyme B-mediated apoptosis (39, 40). The dependence of procaspase -9 processing on caspase-3 after cytochrome c stimulation also sheds new light on the simple hierarchical relationship whereby caspase -9 is placed upstream of caspase -3 in the cytochrome c pathway. Our data indicate that processing of procaspase -9 needs caspase -3 activity, most likely in parallel with Apaf -1. This relationship needs now to be re-examined in cells derived from caspase-3 and caspase-9 knockout animals (15, 16, 22). A defect in caspase -3 leads to an inhibition of cytochrome c-mediated procaspase -9 processing and to an impairment of cisplatin-, TNF- and granzyme B-mediated apoptosis. Caspase -3 inactivation may generally promote tumorigenesis and may have contributed to the development of the breast cancer from which MCF-7 cells are derived.

Interestingly, in procaspase -3 reconstituted MCF -7 cell extracts, a significant induction of caspase-3 activity by cytochrome c was only observed after prolonged incubation of extracts at 37°C. This observation suggests that additional reversible defects in cytochrome c-

mediated caspase activation may exist in MCF -7 cells. The molecular basis for this observation remains to be explored, but recently reported mechanisms of post-mitochondrial apoptosis regulation provide a starting point for future investigations (41).

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FIGURE LEGENDS

Fig. 1. Cisplatin induced apoptosis in breast cancer cells. *A*, ZR-75-1, MCF-7 vc and MCF-7 *CASP*-3 cells were left untreated or were treated for 48 h and 72 h with cisplatin. Cells were stained with DAPI and representative fields of one out of three independent experiments are shown. *B*, Quantification of the percentage of apoptotic nuclei before and after 48 h and 72 h of cisplatin stimulation (mean +/- SD; n = 3). *C*, ZR-75-1 (lanes 1 to 3), MCF -7 vc (lanes 4 to 6) and MCF -7 *CASP*-3 cells (lanes 7 to 9) were left untreated or were treated for 48 h and 72 h with cisplatin. Total DNA was isolated and separated on a 1.5% agarose gel. A 100 bp DNA size marker was used as reference (M). Shown is a representative example of three independent experiments.

Fig. 2. Cisplatin-mediated release of mitochondrial cytochrome c is enhanced by caspase-3. MCF-7 vc cells (lanes 1 to 3) and MCF -7 *CASP*-3 cells (lanes 4 to 6) were treated with cisplatin as indicated. Mitochondrial fractions were analyzed for the presence of cytochrome c (*A*) or Hsp60 (*B*) by Immunoblot analysis. Positions of size marker proteins are indicated. Shown is a representative example of three independent experiments.

Fig. 3. Induction of caspase -3 processing and activity by cytochrome c in breast cancer cells. Extracts prepared from ZR -75-1 (lanes 1 to 4), MCF -7 (lanes 5 to 8), MC F-7 vc (lanes 9, 10) and MCF -7 *CASP*-3 cells (lanes 11, 12) were incubated at 30°C in the absence (lanes 1, 4, 5, 8, 9, 11) or presence of cytochrome c (lanes 2, 3, 6, 7, 10, 12) for the indicated times. *A*, Caspase-3 processing was monitored by Immunoblot analysis. *B*, Caspase-3 activity was measured in a fluorometer using DEVD-amc as substrate. Shown are representative results out of three independent experiments.

Fig. 4. *In vitro* processing of exogenously added procaspase -9. ^{35}S-methionine labeled *in vitro* translated procaspase -9 (procasp-9) was incubated with ZR -75-1 extracts (lanes 1, 2), MCF -7 extracts (lanes 3, 4) or with buffer supplemented with BSA (lanes 5, 6) in the absence (lanes 1, 3, 5) or presence of cytochrome c (*A*, lanes 2, 4, 6) or recombinant active caspase -8 (*B*, lanes 2, 4, 6). Processing of procaspase-9 was monitored by SDS -PAGE and autoradiograpy. One representative experiment of four independent analyses is shown.

Fig. 5. Sensitization of MCF -7 cell extracts for cytochrome c and caspase -8. Recombinant inactive procaspase -3 was added to MCF -7 cell extracts in the absence (lane 1, 4) or presence of cytochrome c (lane 2, 5) or recombinant active caspase -8 (lane 3, 6) and assayed for DEVD -amc cleavage activity. Extracts were either directly use d for the experiment (lanes 1 to 3) or after a pre-incubation for 20 minutes at 37°C (lanes 4 to 6). Shown are mean values and standard deviations from three independent experiments.

Fig. 6. Processing of procaspase -9 in caspase-3 reconstituted MCF -7 cell extracts. Sensitized MCF -7 cell extracts (incubation at 37°C for 20 min) were incubated with biotinylated procaspase -9 in the absence (lanes 1, 3, 4, 5, 7) or presence of cytochrome c (lanes 2, 6), recombinant active caspase -8 (lanes 3, 7) or recombinant active caspase -3 (lane 4). In lanes 5 to 7 extracts were reconstituted with recombinant inactive procaspase -3. Processing of procaspase -9 was monitored after SDS -PAGE as described in Materials and Methods and caspase -3 like activity was determined by DEVD -amc cleavage assay. The asterisk indicates an unspecific band that was recognized by peroxidase coupled streptavidin in extracts and that was independent of biotinylated procaspase -9.

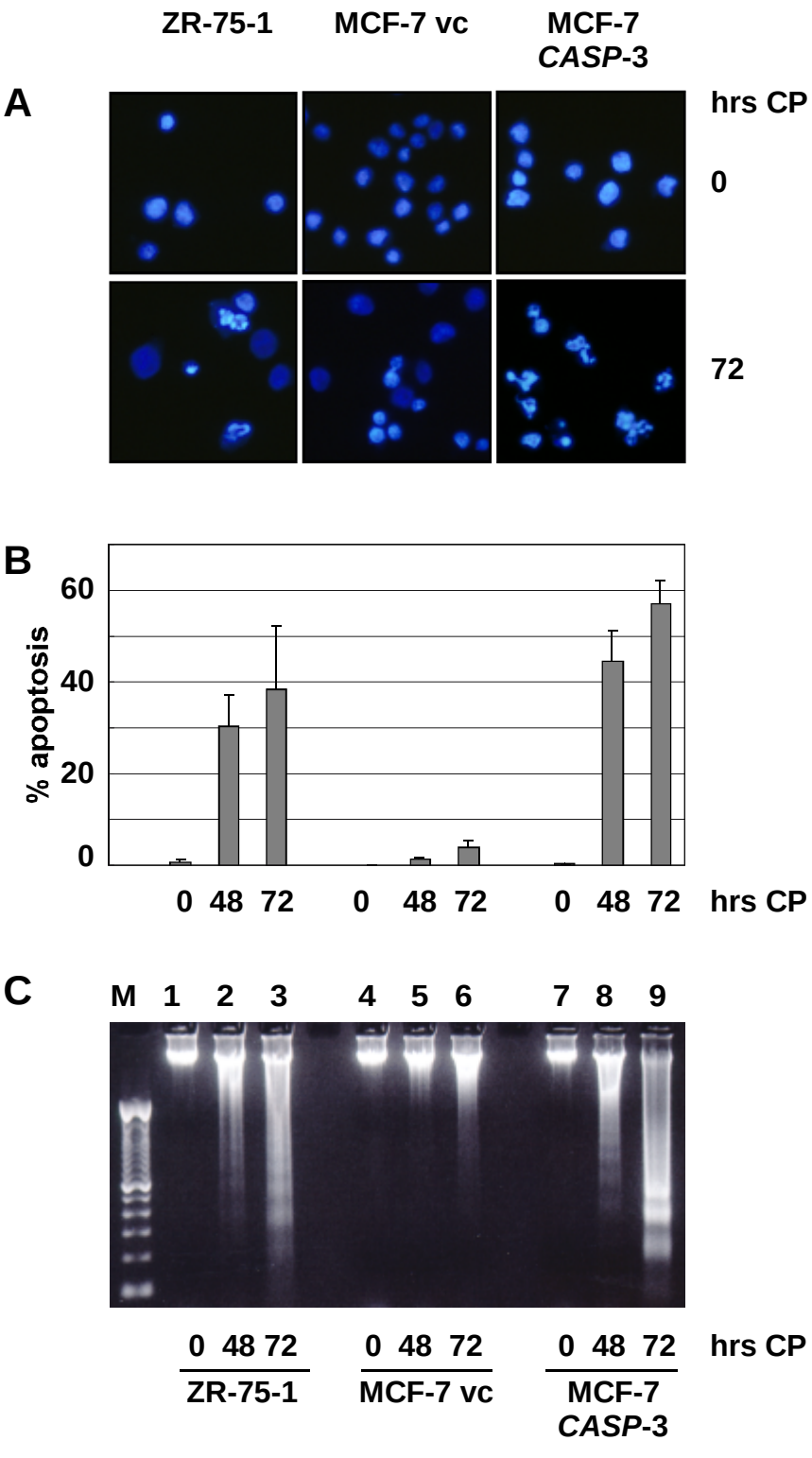
A
ZR-75-1
MCF-7 vc
MCF-7
CASP-3
hrs CP
0
72
B
% apoptosis
60
40
20
0
0 48 72 0 48 72 0 48 72 hrs CP
C
M 1 2 3 4 5 6 7 8 9
0 48 72 0 48 72 0 48 72 hrs CP
ZR-75-1
MCF-7 vc
MCF-7
CASP-3

Figure 1

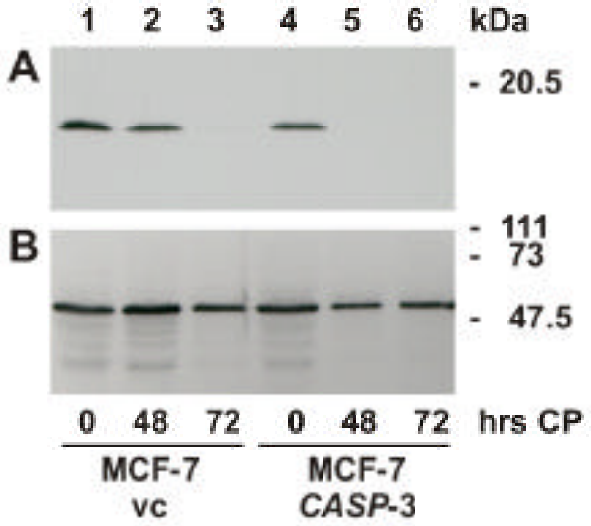
1 2 3 4 5 6 kDa
A
- 20.5
B
- 111
- 73
- 47.5
0 48 72 0 48 72 hrs CP
MCF-7
vc
MCF-7
CASP-3

Figure 2.cdr

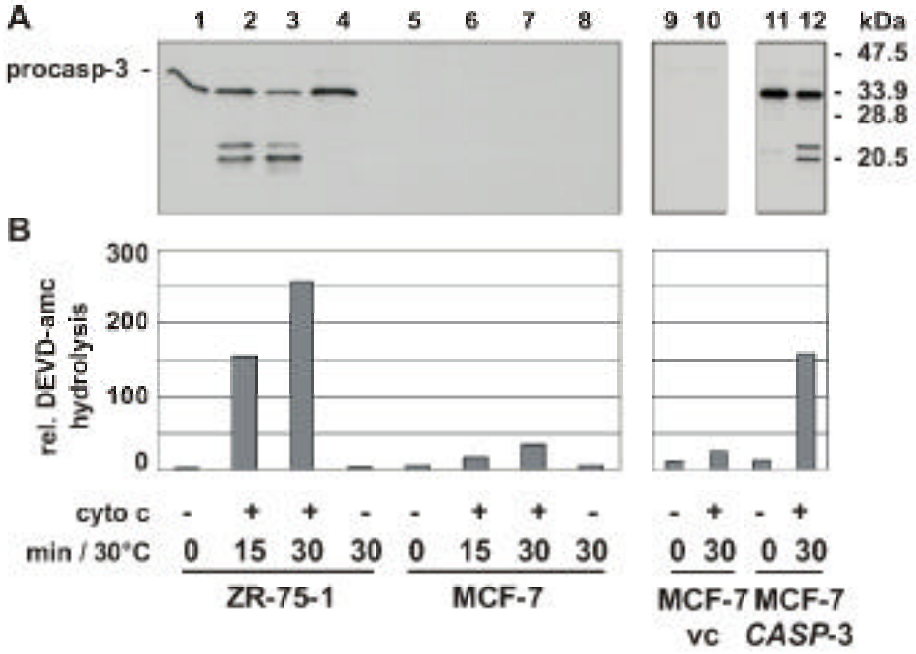
A
1 2 3 4 5 6 7 8 9 10 11 12 kDa
procasp-3
47.5
33.9
28.8
20.5
B
rel. DEVD-amc hydrolysis
300
200
100
0
cyto c - + + - - + + - - + - +
min / 30°C 0 15 30 30 0 15 30 30 0 30 0 30
ZR-75-1
MCF-7
MCF-7 vc
MCF-7 CASP-3

Figure 3.cdr

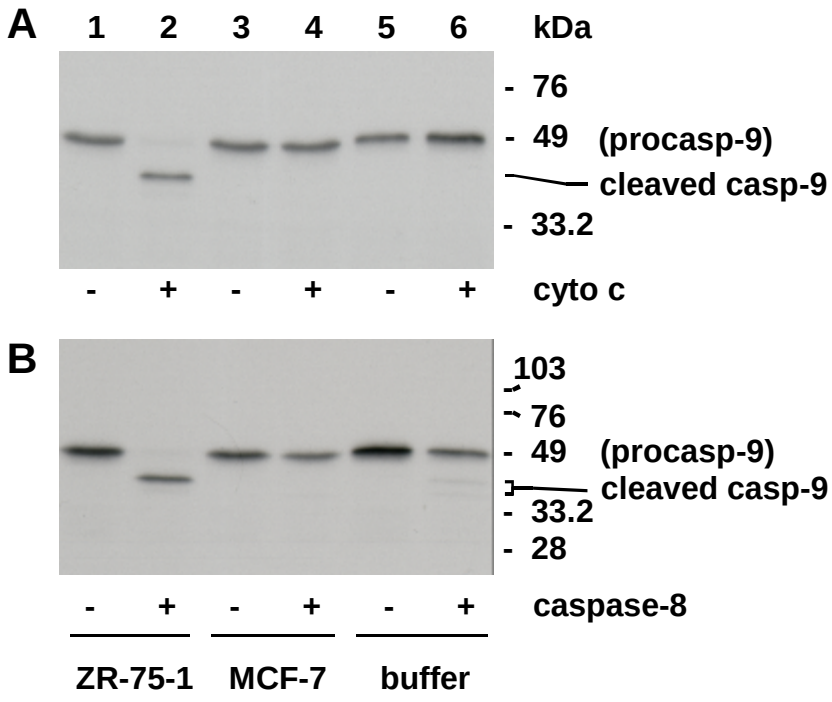
A
1 2 3 4 5 6
kDa
- 76
- 49
(procasp-9)
cleaved casp-9
- 33.2
- + - + - +
cyto c
B
103
76
- 49
(procasp-9)
cleaved casp-9
- 33.2
- 28
- + - + - +
caspase-8
ZR-75-1
MCF-7
buffer

Figure 4

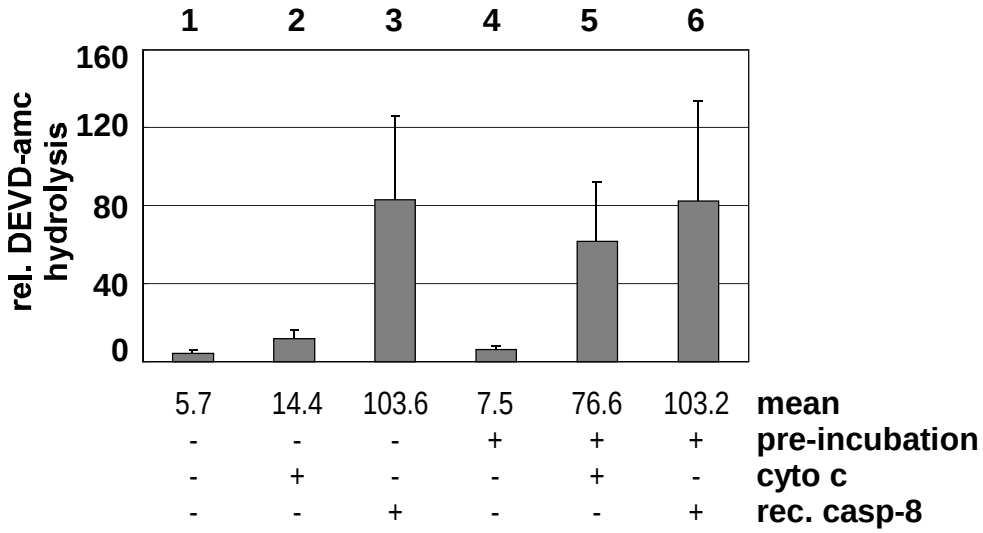
1
2
3
4
5
6
160
120
80
40
0
rel. DEVD-amc hydrolysis
5.7
14.4
103.6
7.5
76.6
103.2
mean
- - - + + + pre-incubation
- + - - + - cyto c
- - + - - + rec. casp-8

Figure 5

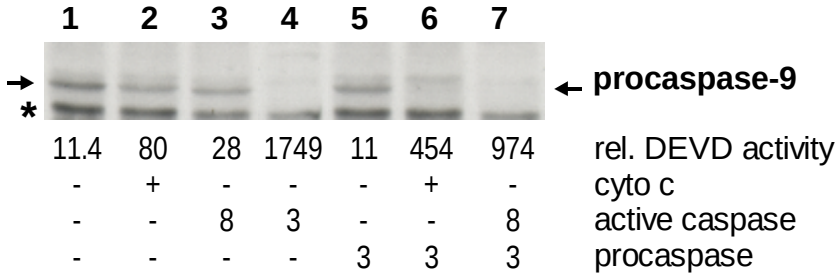
1 2 3 4 5 6 7
procaspase-9
*
11.4 80 28 1749 11 454 974 rel. DEVD activity
- + - - - + - cyto c
- - 8 3 - - 8 active caspase
- - - - 3 3 3 procaspase

Figure 6