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Molecular Ordering of Apoptosis Induced by Anticancer Drugs in Neuroblastoma Cells[1]

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ABSTRACT

Apoptosis mediated by anticancer drugs may involve activation of death-inducing ligand/receptor systems such as CD95 (APO-1/Fas), cleavage of caspases, and perturbance of mitochondrial functions. We investigated the sequence of these events in SHEP neuroblastoma cells transfected with Bcl-2 or Bcl-X_L using two different drugs, namely, doxorubicin (Doxo), which activates the CD95/CD95 ligand (CD95-L) system, and betulinic acid (Bet A), which does not enhance the expression of CD95 or CD95-L and which, as shown here, directly targets mitochondria. Apoptosis induced by both drugs was inhibited by Bcl-2 or Bcl-X_L overexpression or by bongkrekic acid, an agent that stabilizes mitochondrial membrane barrier function, suggesting a critical role for mitochondria. After Doxo treatment, enhanced CD95/CD95-L expression and caspase-8 activation were not blocked by Bcl-2 or Bcl-X_L and were found in cells with a mitochondrial transmembrane potential ($\Delta\Psi_m$) that was still normal ($\Delta\Psi_m^{high}$ cells). In marked contrast, after Bet A treatment, caspase-8 activation occurred in a Bcl-2- or Bcl-X_L-inhibitable fashion and was confined to cells that had lost their $\Delta\Psi_m$ ($\Delta\Psi_m^{low}$ cells). Mitochondria from cells treated with either Doxo or Bet A induced cleavage of both caspase-8 and caspase-3 in cytosolic extracts. Thus, caspase-8 activation may occur upstream or downstream of mitochondria, depending on the apoptosis-initiating stimulus. In contrast to caspase-8, cleavage of caspase-3 or poly(ADP-ribose)polymerase was always restricted to $\Delta\Psi_m^{low}$ cells, downstream of the Bcl-2- or Bcl-X_L-controlled checkpoint of apoptosis. Cytochrome *c*, released from mitochondria undergoing permeability transition, activated caspase-3 but not caspase-8 in a cell-free system. However, both caspases were activated by apoptosis-inducing factor, indicating that the mechanism of caspase-8 activation differed from that of caspase-3 activation. Taken together, our findings demonstrate that perturbance of mitochondrial function constitutes a central coordinating event in drug-induced cell death.

INTRODUCTION

Cytotoxic drugs with distinct primary intracellular targets induce apoptosis in chemosensitive cells (1). Activation of apoptosis pathways may involve ligand-/receptor-driven amplifier systems such as the CD95 system. Upon CD95-L[3]/receptor interaction, the receptor proximal caspase-8 (FLICE/MACH) is cleaved, leading to activation of a downstream caspase cascade involving caspase-3 (CPP32/Yama; Refs. 2–11). Induction of CD95-L and up-regulation of CD95 after treatment with cytotoxic drugs such as Doxo have been observed in a variety of tumor cells (12–18). Blockade of CD95/CD95-L interaction by antagonistic antibodies inhibits drug-induced cell death (12–18). However, drug-triggered apoptosis may also occur independently of the CD95/CD95-L system (19–21). Thus, Bet A kills cells without triggering the CD95/CD95-L system, apparently by directly perturbing mitochondrial functions (19).

Alterations of mitochondrial functions such as PT have been found to play a major role in the apoptotic process, including cell death induced by chemotherapeutic agents (22–28). Mitochondria undergoing PT release apoptogenic proteins such as cyt *c* or AIF from the mitochondrial intermembrane space into the cytosol, where they can activate caspases and endonucleases (22, 23, 26, 29–32). The connection of mitochondrial PT to activation of the caspase cascade appears to be complex (22, 23, 33, 34). On one hand, recombinant caspases can induce PT, probably via a direct effect on the PT pore complex (23, 35). On the other hand, caspases are activated by mitochondrial intermembrane proteins (23, 31), suggesting that caspases can act either upstream or downstream of mitochondria.

Mitochondrial function during apoptosis seems to be controlled by the Bcl-2 family of proteins (36, 37). Bcl-2 and several of its homologues have been localized to intracellular membranes, including the mitochondrial membrane (36). Overexpression of the antiapoptotic molecules Bcl-2 and Bcl-X_L has been found to confer resistance to anticancer treatment (38–41). Bcl-2 and Bcl-X_L may inhibit apoptosis through the capacity to prevent PT (25, 26) and/or to stabilize the barrier function of the outer mitochondrial membrane (29, 30, 42, 43). Both Bcl-2 and Bcl-X_L have been shown to prevent opening of the purified PT pore complex reconstituted in liposomes (35). In addition, it has been suggested that Bcl-2 and Bcl-X_L can bind cytosolic caspases via Apaf-1 to the mitochondrial membrane, thereby preventing their activation (44, 45). Bcl-X_L has recently been reported to prevent Apaf-1-dependent caspase-9 activation via interaction with Apaf-1 (46, 47).

The sequences and relative contributions of the different pathways (activation of the CD95/CD95-L system, activation of upstream and downstream caspases, and perturbation of mitochondrial membrane barrier function) participating in drug-triggered apoptosis is unknown. Here, we investigated the functional relationship of these signaling events in SHEP neuroblastoma cells transfected with Bcl-2 or Bcl-X_L following treatment with two different cytotoxic drugs: Doxo, which triggers apoptosis via activation of the CD95/CD95-L system (12–18); and Bet A, which induces apoptosis in a CD95-independent fashion (19).

MATERIALS AND METHODS

Drugs. Doxo (Farmitalia, Milan, Italy) and Bet A (Sigma Chemical Co., Deisenhofen, Germany) were provided as pure substances and were dissolved in sterile water (Doxo) or DMSO (Bet A).

Cell Culture. The human neuroblastoma cell line SHEP was kindly provided by Prof. M. Schwab (German Cancer Research Center, Heidelberg, Germany). Cells were maintained in monolayer culture in 75-cm^2 tissue

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[3] The abbreviations used are: CD95-L, CD95 ligand; Doxo, Doxorubicin; Bet A, betulinic acid; PT, permeability transition; cyt *c*, cytochrome *c*; AIF, apoptosis-inducing factor; FACS, fluorescence-activated cell sorting; Z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; BA, bongkrekic acid; moab, monoclonal antibody; RT-PCR, reverse transcription-PCR; PARP, poly(ADP-ribose)polymerase; CFS, cell-free system; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanide iodide; $\Delta\Psi_m$, mitochondrial transmembrane potential; DISC, death-inducing signaling complex.

culture flasks (Falcon, Heidelberg, Germany) in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Conco, Wiesbaden, Germany), 10 mM HEPES (pH 7.4; Biochrom, Berlin, Germany), 100 units/ml penicillin (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.), and 2 mM L-glutamine (Biochrom) and were incubated at 37°C in 95% air/5% CO_2. SHEP neuroblastoma cells stably transfected with Bcl-2, Bcl-X_L, or vector control were cultured in DMEM (Life Technologies, Inc.) containing 500 μg/ml G418 (Geneticin, Life Technologies, Inc.; Refs. 39 and 40).

Determination of Apoptosis. Cells were incubated for indicated times with Doxo, Bet A, or anti-APO-1 and harvested by trypsinization using 0.05% trypsin and 0.02% EDTA without Ca^{2+} and Mg^{2+} (Life Technologies, Inc.). Quantification of DNA fragmentation was performed by FACS analysis of propidium iodide-stained nuclei, as described previously (48), using CELLQuest software (Becton Dickinson, Heidelberg, Germany).

Inhibition of Drug-induced Apoptosis by Z-VAD.fmk or BA. The broad spectrum tripeptide inhibitor of caspases Z-VAD.fmk (Enzyme Systems Products, Dublin, USA) was used at a concentration of 60 μM and the mitochondrion-specific inhibitor BA at a concentration of 50 μM (kindly provided by Dr. J. A. Duine, University of Delft, Delft, the Netherlands).

Analysis of CD95 Expression. Cells were stained with anti-APO-1 (CD95) IgG1 moab (1 μg/ml; Ref. 3) for 45 min at 4°C followed by goat antimouse IgG-FITC (Immunotech, Hamburg, Germany) for 30 min at 4°C. FII23 IgG1 antibody was used as an isotype-matched nonbinding antibody to control for nonspecific binding.

RT-PCR for CD95 and CD95-L mRNA. RT-PCR for CD95 and CD95-L mRNA was performed as described previously (13).

Western Blot Analysis. Western blot analyses for caspase-3 and caspase-8, PARP, CD95-L, CD95, and cyt *c* proteins were performed as described previously (13, 49, 50).

Preparation of Mitochondria, Cytosolic Extracts, Nuclei, and AIF. Mitochondria were isolated as described previously by ultracentrifugation at 100,000 × *g* for 1 h at 4°C (50). Cytosolic extracts, nuclei, and AIF-containing mitochondrial supernatant were prepared as reported previously (23, 50).

CFS of Apoptosis. Nuclear fragmentation was determined as described previously (50). For determination of caspase activation, cytosolic extracts (2 μg/μl; all concentrations are final) were incubated with mitochondria (1 μg/μl), cyt *c* (10 μM) or AIF-containing mitochondrial supernatant (0.5 μg/μl) in buffer D [10 mM HEPES (pH 7.4), 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine kinase, and 10 μM cytochalasin B) for 2 h at 37°C. Proteins were separated by 15% SDS-PAGE, and Western blot analysis was performed as described above.

Determination of Mitochondrial Membrane Potential. For determination of mitochondrial membrane potential, cells (5×10^5/ml) were incubated with $DiOC_6(3)$ (40 nM; Molecular Probes, Inc., Eugene, OR) for 15 min at 37°C and analyzed on a flow cytometer (FACScan; Ref. 25). For cell sorting, cells were stained with $DiOC_6(3)$ and sorted into $DiOC_6(3)^{high}$ and $DiOC_6(3)^{low}$ cells on a cytofluorometer (FACS Vantage; Becton Dickinson). As a control, cells were treated with the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (200 μM; Sigma).

RESULTS

Doxo- and Bet A-induced Apoptosis Depends on Mitochondrial PT and Caspase Activation. We initially analyzed Doxo- and Bet A-induced apoptosis by determining nuclear fragmentation and disruption of the $\Delta\psi_m$. Doxo and Bet A induced loss of $\Delta\Psi_m$ and nuclear fragmentation (Fig. 1, *A* and *B*) at doses previously shown to be comparable with respect to cytotoxicity (87 ± 3% specific apoptosis for 0.5 μg/ml Doxo and 78 ± 7% specific apoptosis for 10 μg/ml Bet A; Refs. 13 and 19). Both the mitochondrial and the nuclear manifestations of apoptosis were blocked by overexpression of Bcl-2 and Bcl-X_L (Fig. 1, *A* and *B*). To determine whether apoptosis involved opening of the mitochondrial PT pore, we tested the effect of BA, a specific inhibitor of this pore (24, 25). Addition of BA inhibited drug-triggered nuclear fragmentation and $\Delta\Psi_m$ loss, indicating that mitochondrial alterations involved opening of PT pores (Fig. 1, *A* and *B*). In Doxo-treated cells, nuclear fragmentation and loss of $\Delta\Psi_m$ were both inhibited by the broad range caspase inhibitor Z-VAD.fmk (Fig. 1, *A* and *B*). In contrast, Z-VAD.fmk affected only the Bet A-induced nuclear fragmentation; it had no effect on Bet A-induced $\Delta\Psi_m$ dissipation (Fig. 1, *A* and *B*). To test whether drug-induced mitochondrial alterations were sufficient to cause nuclear fragmentation, mitochondria isolated from drug-treated cells were incubated with nuclei in a CFS, and nuclear DNA loss was measured by flow cytometry. Mitochondria from Doxo- or Bet A-treated cells induced nuclear DNA fragmentation (Fig. 1*C*). This effect was blocked by overexpression of Bcl-2 or Bcl-X_L, as well as by treatment of cells with Z-VAD.fmk or BA (Fig. 1*C*). Taken together, these experiments suggest that nuclear apoptosis induced by Doxo or Bet A indistinguishably depends on mitochondrial PT and caspase activation. However, the mechanism leading to the $\Delta\Psi_m$ loss depends on which anticancer agent is used. In the case of Doxo, it requires the activation of Z-VAD.fmk-inhibitable caspases. In sharp contrast, Bet A triggers $\Delta\Psi_m$ dissipation in a caspase-independent fashion.

Bet A Directly Triggers Mitochondrial PT. To investigate whether Bet A has a direct effect on mitochondria, we analyzed $\Delta\Psi_m$ in isolated mitochondria treated with Bet A. Bet A caused a loss in $\Delta\Psi_m$ in isolated mitochondria (Fig. 1*D*). This loss of $\Delta\Psi_m$ was inhibited by Bcl-2 or Bcl-X_L overexpression or by BA (Fig. 1*D*), indicating that the $\Delta\Psi_m$ loss involved PT. However, the decline in $\Delta\Psi_m$ was not blocked by Z-VAD.fmk (Fig. 1*D*), suggesting that Bet A can trigger PT through a direct effect on mitochondria.

Doxo Induces CD95-L and CD95 Upstream of Mitochondria. We previously found that Doxo induces the expression of CD95-L and CD95 in neuroblastoma cells (13). To determine whether up-regulation of CD95-L and CD95 in response to Doxo is modulated by mitochondrial PT, we analyzed the mechanism of Doxo-induced expression of CD95-L and CD95. Both CD95-L and CD95 were induced at the mRNA and protein level in Neo-transfected control cells and Bcl-2- or Bcl-X_L-overexpressing cells (Fig. 2). No significant differences were observed with respect to the kinetics of CD95-L and CD95 induction (data not shown). A similar increase in CD95 and CD95-L expression was observed following treatment with cisplatinum or VP-16, irrespective of the expression of Bcl-2 or Bcl-X_L (data not shown). These findings suggest that up-regulation of CD95-L and CD95 upon Doxo treatment occurs independently and upstream of mitochondria. In contrast, no up-regulation of CD95-L or CD95 was found at any time point following treatment with Bet A (Fig. 2, *A* and *B*), indicating that Bet A triggers apoptosis independently of the CD95-L/receptor system.

Connection of $\Delta\Psi_m$ Disruption to Activation of the Caspase Cascade. Available data suggest that caspases can act either upstream or downstream of mitochondria (22, 23, 26, 35, 43). To investigate the functional relationship between mitochondrial events and activation of the caspase cascade, we monitored the effect of Bcl-2 and Bcl-X_L on activation of the receptor-proximal caspase-8 and the downstream caspase-3 by Western blot analysis in cells treated with Doxo or Bet A. Upon treatment with Doxo, caspase-8 was cleaved into p43 and p41 intermediates and the active p18 subunit, regardless of Bcl-2 or Bcl-X_L overexpression (Fig. 3*A*). In contrast, proteolytic processing of caspase-3 and PARP was inhibited in Bcl-2- and Bcl-X_L-transfected cells (Fig. 3*A*). Similar data were obtained when cisplatinum or VP-16 were used as chemotherapeutic agents (data not shown). In contrast, when apoptosis was triggered by Bet A, processing of both caspase-8 and caspase-3 was inhibited by Bcl-2 or Bcl-X_L. These data indicate that caspase-8 is cleaved upstream of a Bcl-2/Bcl-X_L-controlled checkpoint after treatment with Doxo but downstream of this checkpoint following incubation with Bet A. In both cases, however, processing of caspase-3 and PARP was prevented by Bcl-2 or Bcl-X_L.

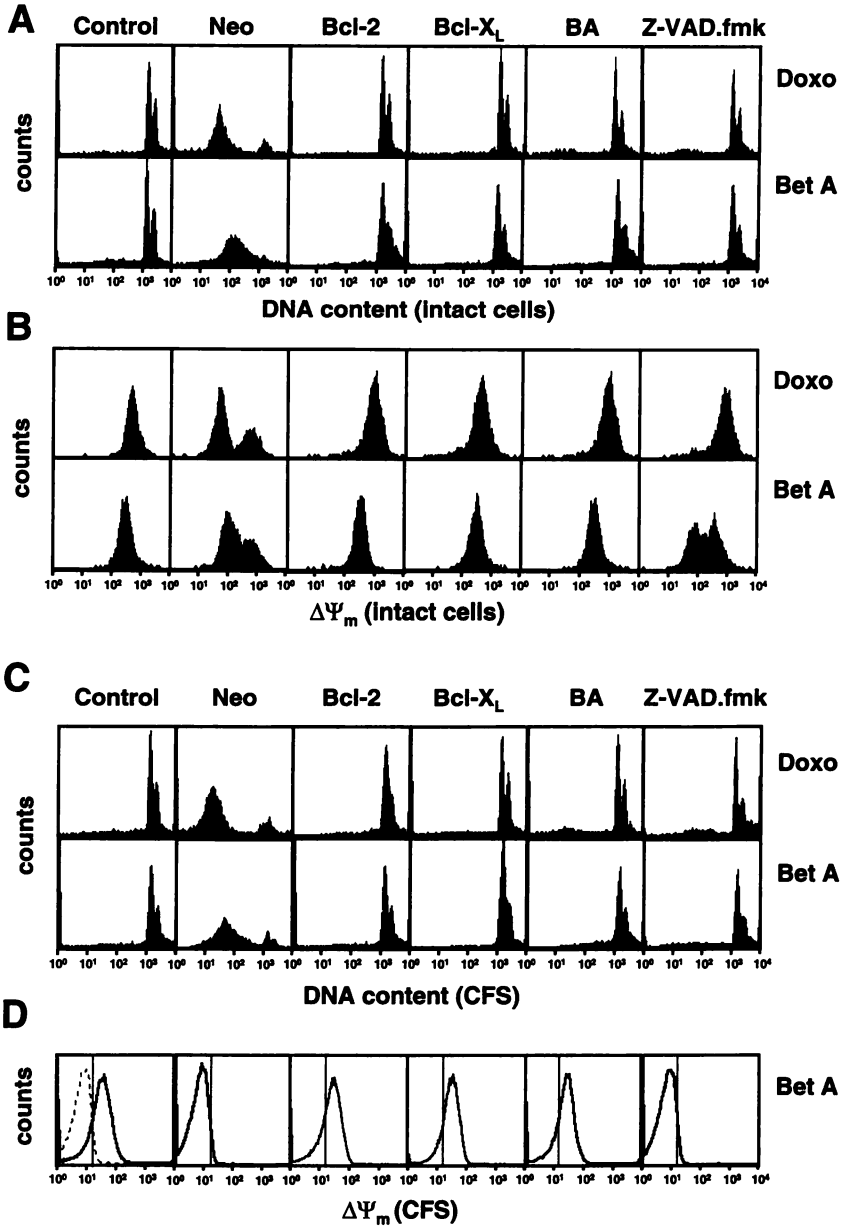

Fig. 1. Drug-induced apoptosis and mitochondrial PT. *A–C*, Doxo- and Bet A-induced apoptosis depends on mitochondrial PT and activation of caspases. SHEP cells transfected with Bcl-2 or Bcl-X_L or a neomycin resistance vector only (*Neo*) were treated with 0.5 μg/ml Doxo or 10 μg/ml Bet A for 48 h (*A*) or 24 h (*B* and *C*). Cells transfected with a neomycin resistance vector only (*Neo*) were treated in the presence or absence of 50 μM BA or 60 μM Z-VAD.fmk. Nuclear apoptosis was determined by FACS analysis of propidium iodide-stained DNA content in intact cells (*A*) or in a CFS incubating isolated nuclei with mitochondria from cells treated with Doxo or Bet A (*C*). $\Delta\Psi_m$ was determined by staining cells with the potential-sensitive fluorochrome $DiOC_6(3)$ (*B*). *D*, Bet A directly induces mitochondrial PT. Mitochondria isolated from SHEP cells transfected with Bcl-2 or Bcl-X_L or a neomycin resistance vector only (*Neo*) were treated with 10 μg/ml Bet A for 0.5 h. Cells transfected with a neomycin resistance vector only (*Neo*) were treated in the presence or absence of 50 μM BA or 60 μM Z-VAD.fmk. $\Delta\Psi_m$ was determined by staining mitochondria with the fluorochrome $DiOC_6(3)$. *D*, the *dotted line* in the first histogram indicates the staining profile obtained in the presence of the $\Delta\Psi_m$ -dissipating agent carbonyl cyanide *m*-chlorophenylhydrazone.

To further delineate the relationship between mitochondrial PT (which represents a Bcl-2-/Bcl-X_L-controlled event), induction of CD95-L, and caspase activation, cells treated with Doxo or Bet A were sorted into cells with still normal $\Delta\Psi_m$ [$DiOC_6(3)^{high}$] and cells with a disrupted $\Delta\Psi_m$ [$DiOC_6(3)^{low}$]. After incubation with Doxo, CD95-L induction and cleavage of caspase-8 were found in both $\Delta\Psi_m{}^{high}$ and $\Delta\Psi_m{}^{low}$ cells (Fig. 3*B*). However, only $\Delta\Psi_m{}^{low}$ cells displayed cleaved caspase-3 and PARP (Fig. 3*B*). A similar pattern of caspase cleavage was seen when cells were stimulated by CD95 cross-linking (Fig. 3*B*). This indicates that initiation of the apoptosis program by up-regulation of CD95/CD95-L and CD95-triggered caspase activation occurs upstream of mitochondria. In contrast, upon incubation with Bet A, only $\Delta\Psi_m{}^{low}$ cells displayed cleaved caspase-3 and caspase-8, in addition to processed PARP (Fig. 3*B*). Thus, caspase-8 activation occurred downstream of the $\Delta\Psi_m$ dissipation in Bet A-induced apoptosis, whereas caspase-8 cleavage and CD95-L induction occurred upstream of the $\Delta\Psi_m$ collapse in Doxo-induced apoptosis.

Caspase-8 Is Activated by Mitochondria Undergoing PT. Caspase-8 has been reported to be cleaved at the activated CD95 DISC upon CD95 cross-linking (10). To test whether caspase-8 could also be activated by mitochondria that have undergone PT after drug treatment, we used a CFS in which mitochondria isolated from drug-treated cells were incubated with cytosolic extracts from untreated cells. Then, processing of caspases was assessed by Western blot analysis. Mitochondria from Doxo- or Bet A-treated vector control cells induced cleavage of caspase-3, caspase-8, and PARP in cytosolic extracts (Fig. 4*A*). This effect was blocked by the broad-range caspase inhibitor Z-VAD.fmk, indicating that protease activity was required for activation of caspase-3 and caspase-8 (Fig. 4*A*). To confirm that cleavage of caspases depended on mitochondria undergoing PT, similar experiments were performed using Bcl-2- or Bcl-X_L-transfected cells in which mitochondria fail to undergo PT subsequent to Doxo or Bet A treatment (Fig. 1). Mitochondria from Bcl-2- or Bcl-X_L-overexpressing cells treated with Doxo or Bet A did not induce caspase cleavage in cytosolic extracts (Fig. 4*A*).

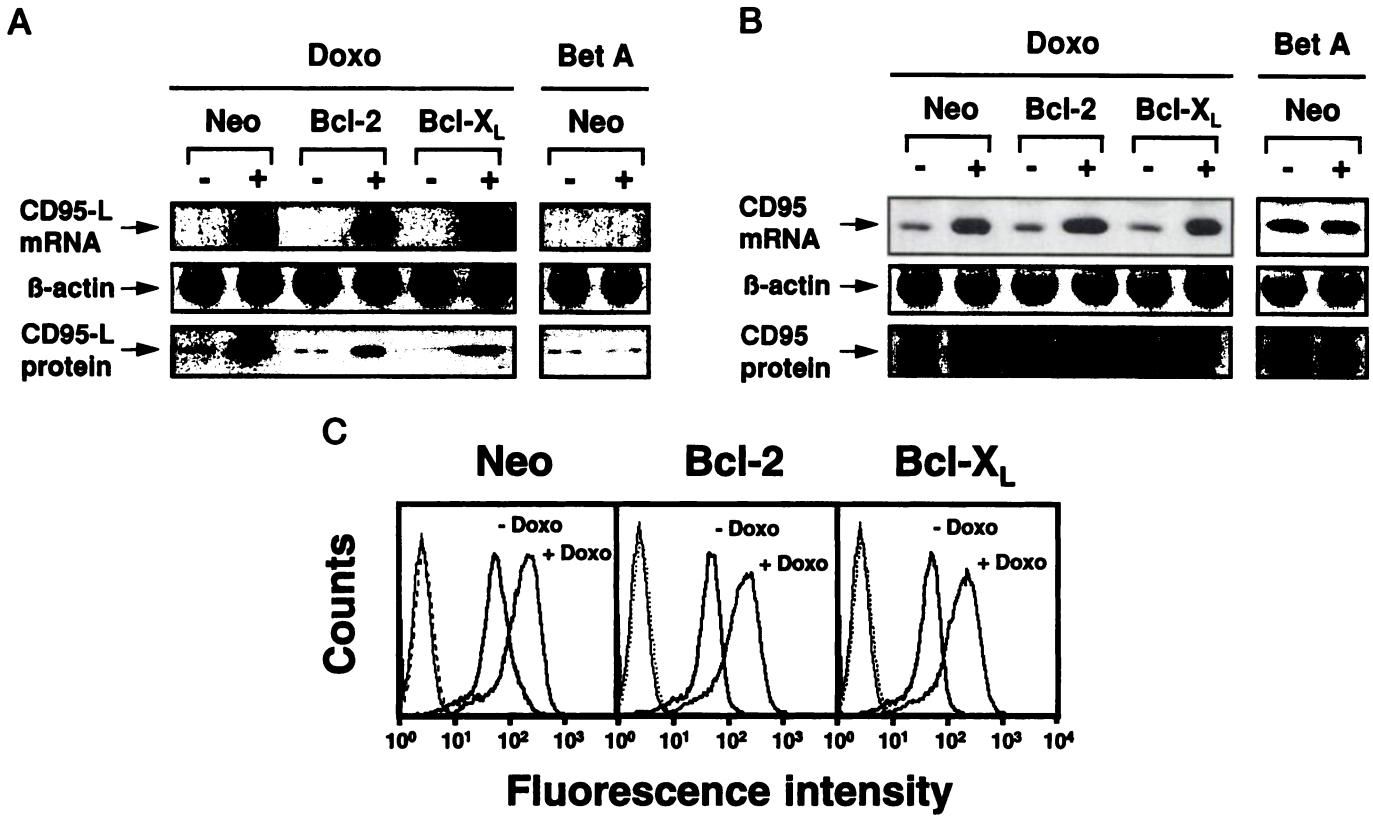

Fig. 2. Activation of the CD95 system by Doxo occurs upstream of mitochondria. *A*, induction of CD95-L. SHEP cells transfected with Bcl-2 or Bcl-X_L or a neomycin resistance vector only (*Neo*) were treated (*Lanes* +) with 0.5 μg/ml Doxo or 10 μg/ml Bet A for 24 h. CD95-L mRNA expression was determined by RT-PCR. Expression of β-actin was used to control RNA integrity and equal gel loading. For Western blot, 40 μg protein of cell lysates per lane were separated by 12% SDS-PAGE. CD95-L protein was detected as a M_r 37,000 band by mouse anti-CD95-L moab and enhanced chemiluminescence. *B* and *C*, induction of CD95. SHEP cells transfected with Bcl-2 or Bcl-X_L or a neomycin resistance vector only (*Neo*) were treated (*Lanes* +) with 0.5 μg/ml Doxo or 10 μg/ml Bet A for 24 h. CD95 mRNA expression was determined by RT-PCR. Expression of β-actin was used to control RNA integrity and equal gel loading. For Western blot analysis (*B*), 40 μg protein of cell lysates per lane were separated by 12% SDS-PAGE. Immunodetection of CD95 protein was performed by mouse anti-CD95 moab and enhanced chemiluminescence. For FACS analysis of CD95 protein expression (*C*), cells were stained with mouse anti-APO-1 moab followed by FITC-conjugated antimouse IgG antibody and analyzed by flow cytometry. Similar results were obtained in three separate experiments.

In addition to interfering with mitochondrial functions, Bcl-2 and Bcl-X_L have been suggested to bind cytosolic pro-caspases via Apaf-1 to the mitochondrial membrane, thereby preventing their activation (27–34). Bcl-X_L has recently been shown to prevent caspase-9 activation by interacting with Apaf-1 (46, 47). To test whether the inhibitory effect of Bcl-2 or Bcl-X_L on caspase cleavage was due to a retention of caspases in the mitochondrial rather than cytosolic compartment, we performed mixing experiments. Mitochondria from vector control cells were incubated with cytosolic extracts of Bcl-2- or Bcl-X_L-overexpressing cells and *vice versa*. Using mitochondria from vector control cells, cleavage of caspases was found in cytosolic extracts of Bcl-2- or Bcl-X_L-transfected cells (Fig. 4*B*). However, processing of caspases was inhibited when mitochondria from Bcl-2- or Bcl-X_L-overexpressing cells were used in combination with cytosolic extracts of vector control cells (Fig. 4*B*). This indicates that deficient cleavage of caspases in Bcl-2- or Bcl-X_L-overexpressing cells is directly related to blocked mitochondrial function rather than to direct or indirect effects, including sequestration of caspases from the cytosol.

Caspase-8 Is Cleaved by AIF-containing Mitochondrial Supernatant but not by cyt *c*. Upon PT, mitochondria have been reported to release cyt *c* from the intermembrane space into the cytosol (33, 34). cyt *c* activates additional cytosolic factors (Apaf-1 and caspase-9) to trigger the activation of caspase-3 (31, 32, 44). After stimulation with Doxo or Bet A, mitochondrial cyt *c* levels declined while the concentration of ectopic, cytosolic cyt *c* increased (Fig. 5*A*). Enforced expression of Bcl-2 or Bcl-X_L blocked the mitochondrial release of cyt *c* (Fig. 5*A*) in agreement with previous studies (29, 30, 38). We then tested whether cyt *c* could actually induce caspase activation. Addition of purified cyt *c* to cytosolic extracts resulted in the processing of caspase-3 and PARP in both vector control and Bcl-2-overexpressing cells (Fig. 5*B*), again indicating that the apoptogenic activity was preserved in the cytosol of Bcl-2-overexpressing cells. However, addition of cyt *c* to cytosolic extracts did not induce caspase-8 activation (Fig. 5*B*). Because mitochondria undergoing PT also release AIF that can proteolytically activate caspase-3 (23), we next investigated the effect of AIF on cytosolic extracts *in vitro*. Incubation of cytosolic extracts with AIF-containing mitochondrial supernatant resulted in cleavage of caspase-3, caspase-8, and PARP (Fig. 5*B*). Thus, different mitochondrial proteins released during PT activate distinct caspases involved in the apoptosis machinery.

DISCUSSION

Mitochondrial PT Constitutes a Central, Coordinating Event of Drug-induced Apoptosis. Several nonexclusive mechanisms have been proposed to play a key role in drug-induced apoptosis: activation of death-inducing ligand/receptor systems such as CD95, activation of caspases, or alterations of mitochondrial functions (12–19, 22–28). However, the sequence and functional relationship of these events have not been defined. The data presented here define a sequence between induction of the CD95 system, activation of the caspase cascade, and mitochondria in the regulation of drug-induced apoptosis by showing that mitochondrial PT is a central, coordinating event.

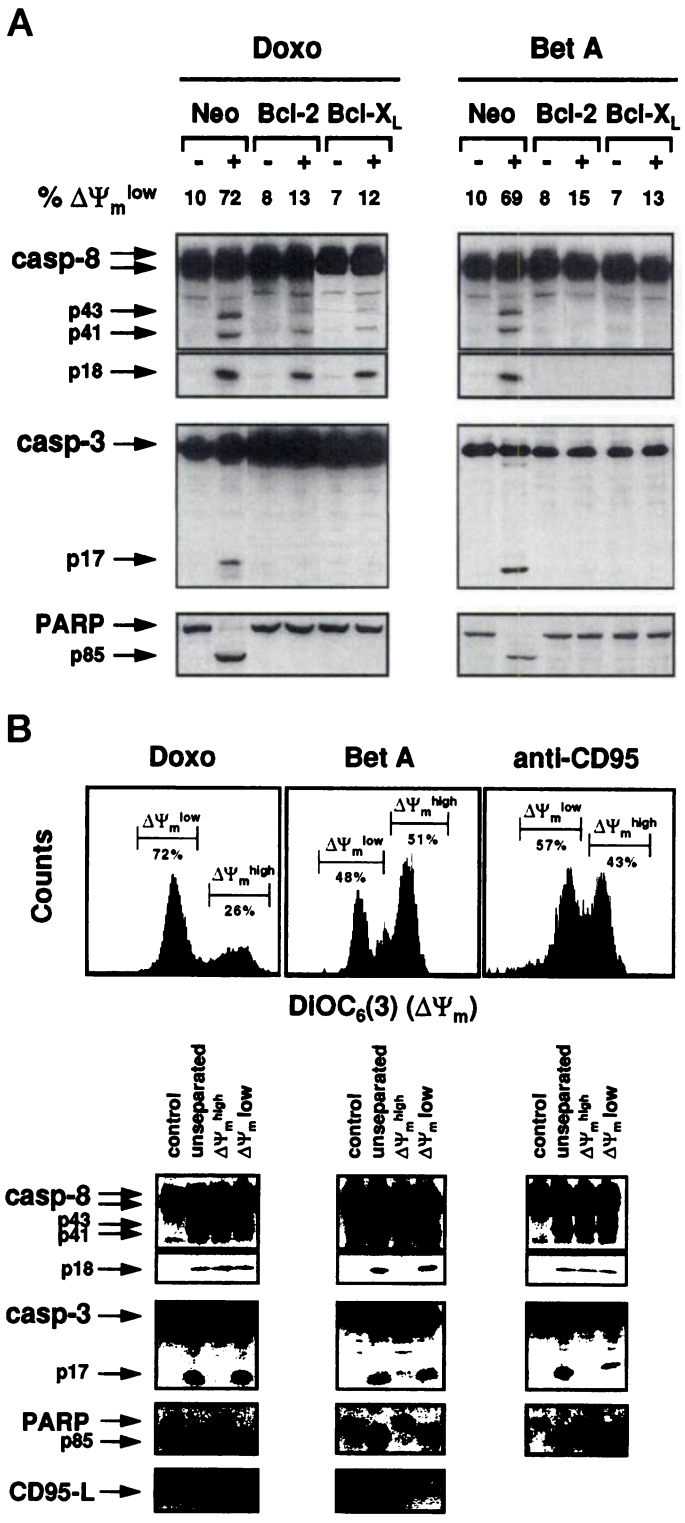

Fig. 3. Functional relationship between $\Delta\Psi_m$ disruption and activation of the caspase cascade. *A*, Bcl-2 and Bcl-X$_L$ block activation of downstream caspases. SHEP cells transfected with Bcl-2 or Bcl-X$_L$ or a neomycin resistance vector only (*Neo*) were treated (*Lanes* +) with 0.5 μg/ml Doxo or 10 μg/ml Bet A for 24 h. The percentage of treated or untreated cells with $\Delta\Psi_m^{low}$ is shown. Forty μg of protein per lane isolated from cell lysates were separated by 15% SDS-PAGE. Immunodetection of caspase-3, caspase-8, and PARP protein was performed by mouse anti-caspase-3 moab, mouse anti-caspase-8 moab, rabbit anti-PARP polyclonal antibody, and enhanced chemiluminescence. *B*, temporal relationship between $\Delta\Psi_m$ disruption and caspase cleavage. SHEP cells were treated with 0.5 μg/ml Doxo, 10 μg/ml Bet A, or 1 μg/ml anti-APO-1 for 18 h, stained with DiOC$_6$(3) and separated on a cytofluorometer into cells with still normal $\Delta\Psi_m$ ($\Delta\Psi_m^{high}$) and cells with a disrupted $\Delta\Psi_m$ ($\Delta\Psi_m^{low}$). Cell sorting was performed according to the regions of $\Delta\Psi_m^{high}$ or $\Delta\Psi_m^{low}$ cells as indicated by the histograms. Western blot analysis of cell lysates was performed as described in *A*.

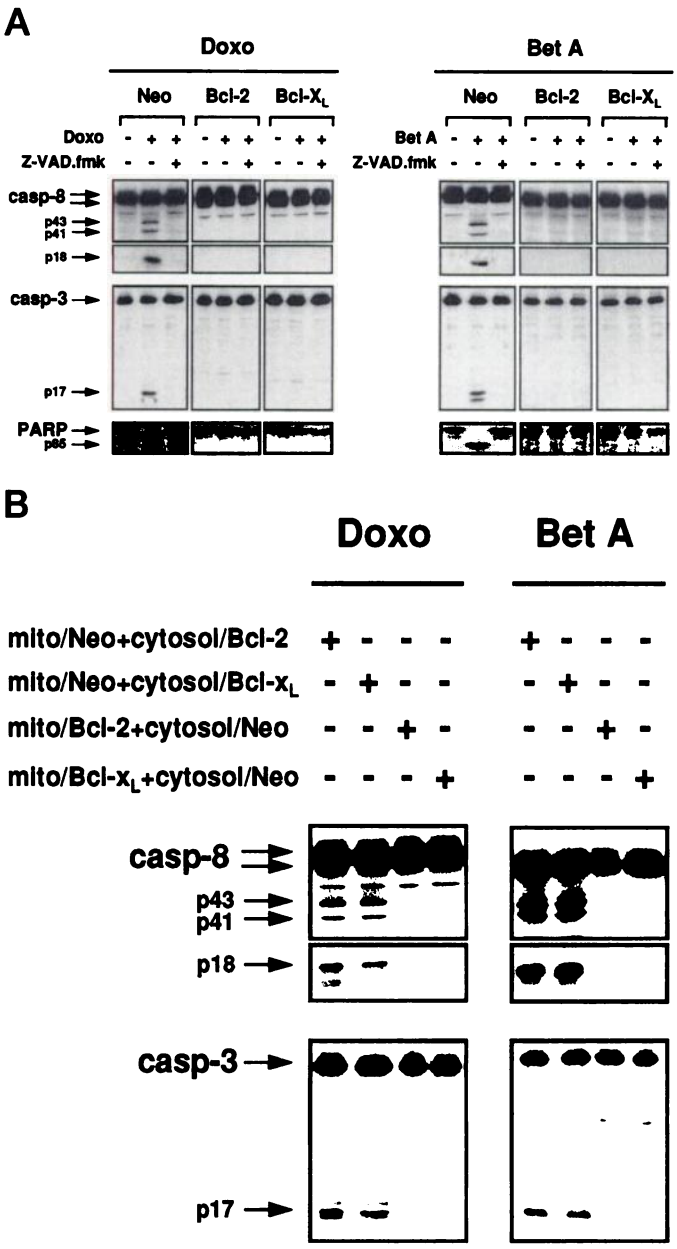

Fig. 4. Activation of caspases in CFS. *A*, caspase-8 is cleaved by mitochondria undergoing PT. SHEP cells transfected with Bcl-2 or Bcl-X$_L$ or a neomycin resistance vector only (*Neo*) were treated (*Lanes* +) with 0.5 μg/ml Doxo or 10 μg/ml Bet A for 16 h. Mitochondria were isolated and incubated with cytosolic extracts for 6 h in the presence or absence of 60 μM Z-VAD.fmk. Western blot analysis was performed as described in Fig. 3*A*. *B*, Bcl-2 and Bcl-X$_L$ inhibit cleavage of caspases at the mitochondrial level. Mitochondria (mito) of Doxo- or Bet A-treated vector control cells (*Neo*) and Bcl-2- or Bcl-X$_L$ overexpressing SHEP cells were incubated with cytosolic extracts of Bcl-2- or Bcl-X$_L$-overexpressing cells and vector control cells, respectively, followed by immunodetection of caspase-3 and caspase-8, as described in Fig. 3*A*.

Inhibition of PT by BA or by Bcl-2 or Bcl-X$_L$ prevented all manifestations of apoptosis at the level of the cytoplasm (caspase-3 activation and disruption of the $\Delta\Psi_m$) and the nucleus (PARP cleavage and loss of nuclear DNA). Different types of drugs, such as Doxo and Bet A, which clearly differ in their initial apoptosis-triggering mechanisms, eventually induce a BA-sensitive $\Delta\Psi_m$ disruption, indicative of PT. Events of the effector/degradation phase of apoptosis such as activation of caspase-3 and cleavage of substrates such as PARP are placed downstream of the PT-mediated $\Delta\Psi_m$ disruption, as indicated by the fact that these changes are only found in cells that have already lost their $\Delta\Psi_m$.

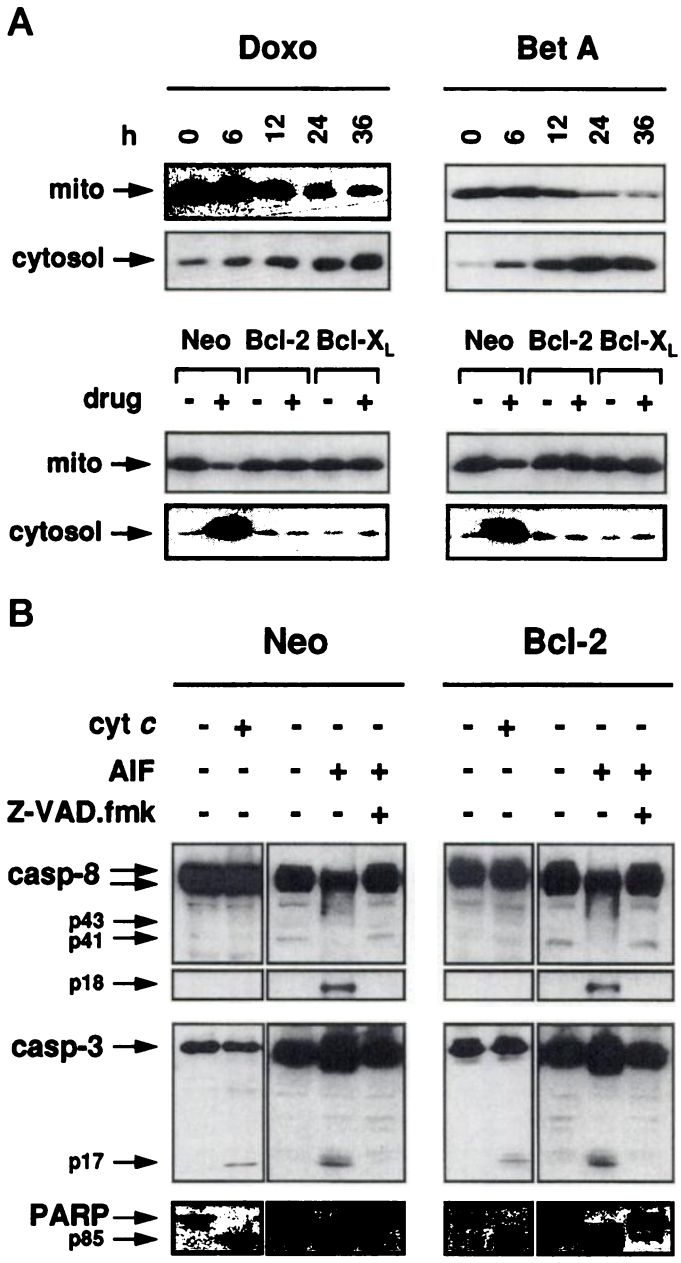

Fig. 5. Cleavage of caspases by cyt *c* and AIF. *A*, drug-induced release of cyt *c* from mitochondria. SHEP cells transfected with Bcl-2 or a neomycin resistance vector only (*Neo*) were treated with 0.5 μg/ml Doxo or 10 μg/ml Bet A for indicated times. Mitochondria and cytosolic extracts (S100 fraction) were prepared as described in "Materials and Methods." Five μg of protein per lane were separated by 15% SDS-PAGE. Immunodetection of cyt *c* was performed by mouse anti-cyt *c* moab and enhanced chemiluminescence. *B*, cleavage of pro-caspase-8 is triggered by AIF but not by cyt *c*. Cytosolic extracts of SHEP cells transfected with a neomycin resistance vector only (Neo) or Bcl-2 were prepared and incubated with 10 μM cyt *c* or AIF-containing mitochondrial supernatant. Immunodetection of caspase-3, caspase-8, and PARP was performed as described in Fig. 3*A*.

Caspases Act Upstream and Downstream of Mitochondria. The hierarchical relationship between mitochondria and activation of the caspase cascade appears to be complex, with several levels of interaction (22, 23, 33, 34, 51–53). First, proteases may act upstream of mitochondria. Cytotoxic drugs such as Doxo cause activation of the CD95 receptor-proximal caspase-8, upstream and independently of mitochondria. Caspase-8 activation was observed in cells with a still normal $\Delta\Psi_m$ and was not inhibited by Bcl-2 or Bcl-X_L. Activated caspase-8 or other caspases may be involved in perturbing mitochondrial functions because Doxo-induced loss of mitochondrial membrane potential was inhibited by the broad-spectrum caspase inhibitor Z-VAD.fmk. The central role of mitochondria in mediating drug-induced cell death was further indicated by the finding that activated caspases, such as caspase-8, located upstream in the cascade, did not directly induce caspase-3 activation but rather involved a Bcl-2-/Bcl-X_L-inhibitable, mitochondrial step. In addition, the data suggest that activation of upstream caspases does not represent the "point of no return" of the apoptotic process and are consistent with recent reports that Bcl-X_L can function downstream of CD95-induced protease activation to prevent loss of $\Delta\Psi_m$ (51–53).

Caspases may also act downstream of mitochondria (22, 23, 33, 34). Thus, cleavage of the downstream caspase-3 was only found in $\Delta\Psi_m^{low}$ cells. In addition, caspase-3 cleavage was induced by mitochondria undergoing PT in a CFS. Moreover, interfering with mitochondrial PT and with the mitochondrial release of cyt *c* and/or AIF by overexpression of Bcl-2 or Bcl-X_L inhibited caspase-3 activation both in intact cells and in a CFS (23, 26, 29, 30, 38).

Caspase-8 cleavage was found both upstream and downstream of mitochondria, in line with recent reports (19, 50). Upon incubation with Doxo or anti-APO-1, caspase-8 is activated upstream of mitochondria, *e.g.*, at the activated CD95 DISC (10), and is already cleaved in cells with a still intact $\Delta\Psi_m$. However, upon treatment with Bet A, caspase-8 is cleaved downstream of mitochondria only in $\Delta\Psi_m^{low}$ cells, consistent with the finding that Bet A triggers apoptosis by a direct effect on mitochondria independently of CD95-L/receptor interaction (19). Cleavage of caspase-8 may be caused by activated mitochondria in the absence of DISC formation. Apoptogenic proteins released from mitochondria undergoing PT such as cyt *c* and AIF have been shown to induce cleavage of caspases (23). Our findings demonstrate that AIF can induce caspase-8 activation, possibly by a direct effect on caspase-8, because partially purified AIF can cleave recombinant caspase-8 (data not shown). However, cyt *c* did not cleave caspase-8, although it did induce caspase-3 activation, indicating that the mechanism of caspase-8 activation downstream of mitochondria differed from that of caspase-3. Moreover, because activated caspases may act on mitochondria (23), caspase-8, which is processed downstream of mitochondria, might engage in a self-amplifying apoptotic process by inducing mitochondrial PT, thereby locking the cell in an irreversible stage of apoptosis. Our findings also suggest a hierarchical order in the cascade of caspase activation (5, 6, 54). Thus, activated caspase-3, although capable of processing PARP, did not induce caspase-8 activation in a CFS (Fig. 5*B*). Caspases might appear to be dispensable for mediating DNA fragmentation because we found fragmentation of nuclei upon incubation with isolated mitochondria consistent with previous findings that AIF can directly induce DNA fragmentation. However, these preparations may still contain small amounts of cytosolic fractions that may mediate activation of downstream targets such as DNA fragmentation factor or caspase-activated desoxyribonuclease (55, 56).

Activation of the CD95 System Occurs Upstream and Independently of Mitochondria. Induction of CD95-L and CD95 occurred upstream of mitochondria because it was not inhibited by Bcl-2 or Bcl-X_L and was also detected in $\Delta\Psi_m^{high}$ cells. At present, the molecular events regulating CD95 or CD95-L expression in response to cytotoxic drug treatment are only partially understood and may be mediated by ceramide generated through activation of sphingomyelinases and the c-*jun* NH_2-terminal kinase/stress-activated protein kinase-dependent stress pathway (57, 58). Intracellular reduced glutathione levels, previously found to be involved in modulation of

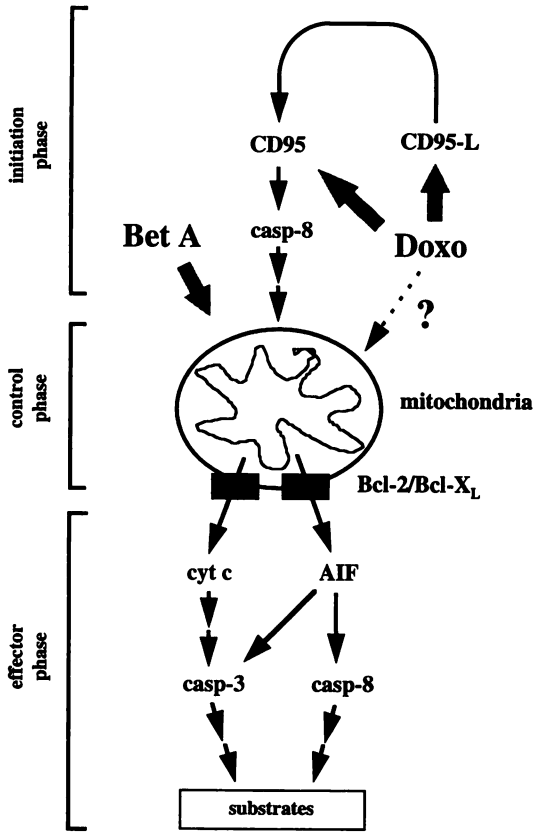

Fig. 6. Hypothetical scenario for drug-induced apoptosis in neuroblastoma cells. See text for details. Doxo, Bet A, CD95, CD95-L, mitochondria, cyt *c*, AIF, caspase-3, and caspase-8 are indicated. *Multiple arrows*, putative intermediate steps.

CD95-L expression,[4] were similar in vector control and Bcl-2- or Bcl-X_L-overexpressing cells, consistent with similar expression levels of CD95-L (data not shown). Also, wild-type p53, which has been implicated in up-regulation of CD95 expression (59), similarly accumulated upon Doxo treatment regardless of Bcl-2 or Bcl-X_L overexpression (data not shown), leading to a similar increase in CD95 expression in these cells.

Hypothetical Scenario for Drug-induced Apoptosis. This work, together with our previous studies (12–19, 22–28), suggests the following scenario for activation of apoptosis programs in drug-induced cytotoxicity of neuroblastoma cells (Fig. 6). The scheme prototypically highlights two different entry sites that may overlap in certain cell types with different drugs. During the initiation phase of apoptosis, different classes of drugs may enter the apoptotic machinery at different entry sites before passing the mitochondrial checkpoint, where they converge into the common effector phase of apoptosis. Entry into the apoptosis program may be triggered through activation of ligand-/receptor-driven amplifier systems such as CD95/CD95-L by drugs such as Doxo, cisplatinum, or VP-16. Through CD95 cross-linking, pro-caspase-8 is cleaved at the activated CD95 DISC upstream of mitochondria. Activated caspase-8 or caspase-8-dependent signaling events, including ceramide generation (60), might then participate in perturbing mitochondrial functions. In contrast, another class of cytotoxic drugs exemplified by Bet A may directly trigger mitochondrial PT and apoptosis without activation of ligand-/receptor-driven amplifier systems resulting in caspase-8 activation downstream of mitochondria. Despite different entries into the apoptosis programs, cytotoxic drugs eventually induce a Bcl-2-/Bcl-X_L-controlled $\Delta\Psi_m$ disruption that marks the initiation of a common effector phase of apoptosis. Upon mitochondrial PT, apoptogenic proteins such as cyt *c* and AIF are released from mitochondria into the cytosol, where they induce activation of caspases, leading to cleavage of downstream targets such as PARP and cell death executioners, such as DNA fragmentation factor and caspase-activated desoxyribonuclease (55, 56). Release of cyt *c* from mitochondria may also occur independently of PT (29, 43).

Thus, mitochondria play a central role in the regulation of drug-induced apoptosis by controlling activation of downstream effector proteases. The molecular ordering of drug-induced apoptosis defined in our studies may also have implication for tumor therapy directed toward activation of apoptosis effector systems. Triggering of CD95/CD95-L interaction and activation of caspase-8 in cells overexpressing Bcl-2-related oncoproteins seems to be largely inefficient for induction of the apoptosis program. On the other hand, direct activation of mitochondrial PT leading to cyt *c* and AIF release, as exemplified by Bet A, may be sufficient for induction of apoptosis in cancer cells. In addition, signals that lead to direct activation of caspase-3 might bypass the requirement for upstream signaling and mitochondrial function. Thus, our findings may be useful to define the pro-apoptotic mode of action of chemotherapeutic agents, to understand the mechanisms of chemoresistance, and to develop strategies to improve the efficacy of anticancer therapy.

[4] C. Friesen, S. Fulda, and K-M. Debatin, Activation of the CD95 system by doxorubicin is modulated by the redox state in chemosensitive- and drug-resistant tumor cells, submitted for publication.

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REFERENCES

1. Fisher, D. E. Apoptosis in cancer therapy: crossing the threshold. Cell, *78:* 539–542, 1994.
2. Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Li-Weber, M., Richards, S., Dhein, J., Trauth, B. C., Ponstingl, H., and Krammer, P. H. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. J. Biol. Chem., *267:* 10709–10715, 1992.
3. Trauth, B. C., Klas, C., Peter, A. M. J., Matz, S., Möller, P., Falk, W., Debatin, K-M., and Krammer, P. H. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science (Washington DC), *245:* 301–305, 1989.
4. Debatin, K-M. Disturbances of the CD95 (APO-1/Fas) system in disorders of lymphohaematopoietic cells. Cell Death Differ., *3:* 185–189, 1996.
5. Nagata, S. Apoptosis by death factor. Cell, *88:* 355–365, 1997.
6. Peter, M. E., Kischkel, F. C., Hellbrandt, S., Chinnaiyan, A. M., Krammer, P. H., and Dixit, V. M. CD95 (APO-1/Fas)-associating signaling proteins. Cell Death Differ., *3:* 161–170, 1996.
7. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A. N. J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell, *85:* 817–827, 1996.
8. Chinnaiyan, A. M., Teppert, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbrandt, S., Peter, M. E., and Dixit, V. M. FADD/MORT-1 is a common mediator of CD95 (APO-1/Fas) and tumor necrosis factor receptor-induced apoptosis. J. Biol. Chem., *271:* 4961–4965, 1996.
9. Kischkel, F. C., Hellbrandt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins (CAP) form a death-inducing signaling complex (DISC) with the receptor. EMBO J., *14:* 5579–5588, 1995.
10. Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A. N. J., Mann, M., Krammer, P. H., and Peter, M. E. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J., *16:* 2794–2804, 1997.
11. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Liwack, G., and Alnemri, E. S. Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5

is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc. Natl. Acad. Sci. USA, *93:* 14486–14491, 1996.
12. Friesen, C., Herr, I., Krammer, P. H., and Debatin, K-M. Involvement of the CD95 (APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat. Med., *2:* 574–577, 1996.
13. Fulda, S., Sieverts, H., Friesen, C., Herr, I., and Debatin, K-M. The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. Cancer Res., *57:* 3823–3829, 1997.
14. Fulda, S., Los, M., Friesen, C., and Debatin, K-M. Chemosensitivity of solid tumor cells is associated with activation of the CD95 system. Int. J. Cancer, *76:* 105–114, 1998.
15. Los, M., Herr, I., Friesen, C., Fulda, S., Schulze-Osthoff, K., and Debatin, K-M. Cross-resistance of CD95- and drug-induced apoptosis as a consequence of deficient activation of caspases (ICE/Ced-3 proteases). Blood, *90:* 3118–3129, 1997.
16. Friesen, C., Fulda, S., and Debatin, K-M. Deficient activation of the CD95 (APO-1/Fas) system in drug-resistant cells. Leukemia (Baltimore), *11:* 1833–1841, 1997.
17. Debatin, K-M. Anticancer drugs, programmed cell death and the immune system: defining new roles in an old play. J. Natl. Cancer Inst. (Bethesda), *89:* 750–751, 1997.
18. Müller, M., Strand, S., Hug, H., Heinemann, E-M., Walczak, H., Hofmann, W. J., Stremmel, W., Krammer, P. H., and Galle, P. R. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J. Clin. Invest., *99:* 403–413, 1997.
19. Fulda, S., Friesen, C., Los, M., Scaffidi, C., Mier, W., Benedict, M., Nuñez, G., Krammer, P. H., Peter, M. E., and Debatin, K-M. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Res., *57:* 4956–4964, 1997.
20. Eischen, C. M., Kottke, T. J., Martins, L. M., Basi, G. S., Tun, J. S., Earnshaw, W. C., Leibson, P. J., and Kaufmann, S. H. Comparison of apoptosis in wild-type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand interactions. Blood, *90:* 935–943, 1997.
21. Villunger, A., Egle, A., Kos, M., Hartmann, B., Geley, S., Kofler, R., and Greil, R. Drug-induced apoptosis is associated with enhanced Fas (APO-1/CD95) ligand expression but occurs independently of Fas (APO-1/CD95) signaling in human T-acute lymphatic leukemia cells. Cancer Res., *57:* 3331–3334, 1997.
22. Kroemer, G., Zamzami, N., and Susin, S. A. Mitochondrial control of apoptosis. Immunol. Today, *18:* 44–51, 1997.
23. Susin, S. A., Zamzami, N., Castedo, M., Daugas, E., Wang, H. G., Geley, S., Fassy, F., Reed, J. C., and Kroemer, G. The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. J. Exp. Med., *186:* 25–37, 1997.
24. Marchetti, P., Castedo, M., Susin, S. A., Zamzami, N., Hirsch, T., Macho, A., Haeffner, A., Hirsch, T., Geuskens, M., and Kroemer, G. Mitochondrial permeability transition is a central coordinating event of apoptosis. J. Exp. Med., *184:* 1155–1160, 1996.
25. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. Mitochondrial control of nuclear apoptosis. J. Exp. Med., *183:* 1533–1544, 1996.
26. Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med., *184:* 1331–1341, 1996.
27. Decaudin, D., Geley, S., Hirsch, T., Castedo, M., Marchetti, P., Macho, A., Kofler, R., and Kroemer, G. Bcl-2 and Bcl-X_L antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. Cancer Res., *57:* 62–67, 1997.
28. Susin, S. A., Zamzami, N., Larochette, N., Dallaporta, B., Marzo, I., Brenner, C., Hirsch, T., Petit, P. X., Geuskens, M., and Kroemer, G. A cytofluorometric assay of nuclear apoptosis. Application to ceramide-induced apoptosis. Exp. Cell Res., *236:* 397–403, 1997.
29. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science (Washington DC), *275:* 1132–1136, 1997.
30. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science (Washington DC), *275:* 1129–1132, 1997.
31. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell, *86:* 147–157, 1996.
32. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S., Ahmad, M., Alnemri, A. S., and Wang, X. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell, *91:* 479–489, 1997.
33. Kantrow, S. P., and Piantadosi, C. A. Release of cytochrome *c* from liver mitochondria during permeability transition. Biochem. Biophys. Res. Commun., *232:* 669–671, 1997.
34. Ellerby, H. M., Martin, S. J., Ellerby, L. M., Naiem, S. S., Rabizadeh, S., Salvesen, G. S., Casiano, C. A., Cashman, N. R., Green, D. R., and Bredesen, D. E. Establishment of a cell-free system of neuronal apoptosis: comparison of premitochondrial, mitochondrial, and postmitochondrial phases. J. Neurosci., *17:* 6165–6178, 1997.
35. Marzo, I., Brenner, C., Zamzami, N., Susin, S. A., Beutner, G., Brdiczka, D., Remy, R., Xie, Z-H., Reed, J. C., and Kroemer, G. The permeability transition pore complex: a target for apoptosis regulation by caspases and Bcl-2 related proteins. J. Exp. Med., in press, 1998.
36. Kroemer, G. The protooncogene *Bcl-2* and its role in regulating apoptosis. Nat. Med., *3:* 614–620, 1997.
37. Reed, J. C. Double identity for proteins of the Bcl-2 family. Nature (Lond.), *387:* 773–776, 1997.
38. Kim, C. N., Wang, X., Huang, Y., Ibrado, A. M., Liu, L., Fang, G., and Bhalla, K. Overexpression of Bcl-X_L inhibits Ara-C-induced mitochondrial loss of cytochrome *c* and other perturbations that activate the molecular cascade of apoptosis. Cancer Res., *57:* 3115–3120, 1997.
39. Dole, M., Nuñez, G., Merchant, A. K., Maybaum, J., Rode, C. K., Bloch, C. A., and Castle, V. P. Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. Cancer Res., *54:* 3253–3259, 1994.
40. Dole, M. G., Jasty, R., Cooper, M. J., Thompson, C. B., Nuñez, G., and Castle, V. P. Bcl-X_L is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. Cancer Res., *55:* 2576–2582, 1995.
41. Ibrado, A. M., Huang, Y., Fang, G., Liu, L., and Bhalla, K. Overexpression of Bcl-2 and Bcl-X_L inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. Cancer Res., *56:* 4743–4748, 1996.
42. Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. Bcl-X_L regulates the membrane potential and volume homeostasis of mitochondria. Cell, *91:* 627–637, 1997.
43. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. Mitochondrial cytochrome *c* release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J., *17:* 37–49, 1998.
44. Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. Apaf-1, a human protein homologous to C.elegans CED-4, participates in cytochrome *c*-dependent activation of caspase-3. Cell, *90:* 405–413, 1997.
45. Hengartner, M. O. Ced-4 is a stranger no more. Nature (Lond.), *388:* 734–735, 1997.
46. Pan, G., O'Rourke, K., and Dixit, V. M. Caspase-9, Bcl-X_L and Apaf-1 form a ternary complex. J. Biol. Chem., *273:* 5841–5845, 1998.
47. Hu, Y., Benedict, M., Wu, D., Inohara, N., and Nuñez, G. Bcl-X_L interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc. Natl. Acad. Sci. USA, *95:* 4386–4391, 1998.
48. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods, *139:* 271–279, 1991.
49. Scaffidi, C., Medema, J. P., Krammer, P. H., and Peter, M. E. FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. J. Biol. Chem., *272:* 26953–26958, 1997.
50. Scaffidi, C., Fulda, S., Li, F., Friesen, C., Srinivasan, A., Tomaselli, K. J., Debatin, K-M., Krammer, P. H., and Peter, M. E. Two CD95 signaling pathways. EMBO J., *17:* 1675–1687, 1998.
51. Medema, J. P., Scaffidi, C., Krammer, P. H., and Peter, M. E. Bcl-X_L acts downstream of caspase-8 activation by the CD95 death-inducing signaling complex. J. Biol. Chem., *273:* 3388–3393, 1998.
52. Srinivasan, A., Li, F., Wong, A., Kodandapani, L., Smidt, R., Krebs, J. F., Fritz, L. C., Wu, J. C., and Tomaselli, K. J. Bcl-X_L functions downstream of caspase-8 to inhibit Fas- and tumor necrosis factor receptor 1-induced apoptosis of MCF7 breast carcinoma cells. J. Biol. Chem., *273:* 4523–4529, 1998.
53. Boise, L. H., and Thompson, C. B. Bcl-X_L can inhibit apoptosis in cells that have undergone Fas-induced protease activation. Proc. Natl. Acad. Sci. USA, *94:* 3759–3764, 1997.
54. Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M. Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. J. Exp. Med., *187:* 587–600, 1998.
55. Liu, X., Zou, H., Slaughter, C., and Wang, X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell, *89:* 175–184, 1997.
56. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature (Lond.), *391:* 43–50, 1997.
57. Herr, I., Boehler, T., Wilhelm, D., Angel, P., and Debatin, K-M. Activation of the CD95 (APO-1/Fas) pathway by ceramide mediates radio- and chemotherapy-induced apoptosis. EMBO J., *16:* 6200–6208, 1997.
58. Faris, M., Kokot, N., Latinis, K., Kasibhatla, S., Green, D. R., Koretzky, G. A., and Nel, A. The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. J. Immunol., *160:* 134–144, 1998.
59. Owen-Schaub, L. B., Zhang, W., Cusack, J. C., Angelo, L. S., Santee, S. M., Fujiwara, T., Roth, J. A., Deisseroth, A. B., Zhang, W. W., Kruzel, E., and Radinsky, R. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol. Cell. Biol., *15:* 3032–3040, 1995.
60. De Maria, R., Lenti, L., Malisand, F., Agostino, F., Tomassini, B., Zeuner, A., Rippo, M. R., and Testi, R. Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. Science (Washington DC), *277:* 1652–1655, 1997.