Inactivation of caspase-8 on mitochondria of Bcl-xL expressing MCF7-Fas cells: Role for the BAR protein

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Running title: Sequestration of active caspase-8 subunits by mitochondria
Abstract

Apoptosis induction through CD95 (APO-1/Fas) critically depends on generation of active caspase-8 at the death-inducing signaling complex (DISC). Depending on the cell type, active caspase-8 either directly activates caspase-3 (Type I cells) or relies on mitochondrial signal amplification (Type II cells). In MCF7-Fas cells that are deficient for pro-caspase-3, even high amounts of caspase-8 produced at the DISC cannot directly activate downstream effector caspases without mitochondrial help. Overexpression of Bcl-xL in these cells renders them resistant to CD95 mediated apoptosis. However, activation of caspase-8 in control (vector) and Bcl-xL transfectants of MCF7-Fas cells proceeds with similar kinetics, resulting in a complete processing of cellular caspase-8. Most of the cytosolic caspase-8 substrates are not cleaved in the Bcl-xL protected cells, raising the question of how Bcl-xL-expressing MCF7-Fas cells survive large amounts of potentially cytotoxic caspase-8. We now demonstrate that active caspase-8 is initially generated at the DISC of both MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells and that the early steps of CD95 signaling such as caspase-8-dependent cleavage of DISC bound c-FLIPL, caspase-8-dependent clustering and internalization of CD95 as well as processing of pro-caspase-8 bound to mitochondria are very similar in both transfectants. However, events downstream of mitochondria, such as release of cytochrome c, only occur in the vector-transfected MCF7-Fas cells and no in vivo caspase-8 activity can be detected in the Bcl-xL-expressing cells. Our data suggest that in Bcl-xL-expressing MCF7-Fas cells, active caspase-8 is sequestered on the outer mitochondrial surface presumably by association with the protein "bifunctional apoptosis regulator" (BAR) in a way that does not allow substrates to be cleaved. Mitochondria of MCF7-Fas-Bcl-xL cells bind and inactivate caspase-8 active subunits generated in response to the triggering of CD95, identifying a novel mechanism of regulation of apoptosis sensitivity by mitochondrial Bcl-xL.
Introduction

CD95 (APO-1/Fas) is a member of a family of death receptors that regulates apoptosis in many different tissues. Other members of this family include TNF-R1, DR3 (TRAMP, wsl-1, APO-3, LARD), TRAIL-R1 (DR4, APO-2), TRAIL-R2 (DR5, TRICK, KILLER) and DR6 (for review see 1). Induction of apoptosis through CD95 involves activation of caspase-8 at the death-inducing signaling complex (DISC\textsuperscript{4}). The DISC is comprised of oligomerized CD95, the adapter FADD/Mort1 and the cysteine protease caspase-8, activation of which initiates the caspase cascade for apoptosis (2-4). In addition, we have identified the caspase-8-like molecule c-FLIP as a general component of the DISC (5).

Apoptosis in certain types of cells (Type II cells) depends in part on caspase-8-mediated cleavage and activation of the BH3-domain-containing Bcl-2 family member Bid (6-8) and can be inhibited by Bcl-2 or Bcl-x\textsubscript{L} (9). However, Bcl-x\textsubscript{L} has been shown to act on intracellular organelles such as mitochondria and does not interfere with formation or activity of the CD95 DISC (10). Type II cells therefore must have the ability to survive the active caspase-8 that is generated upon binding of death ligands to their receptors.

MCF7-Fas cells were chosen to investigate how Type II cells survive, since these particular cells are devoid of caspase-3 and therefore cannot respond like Type I cells (9). We have previously shown that transient expression of pro-caspase-3 in MCF7-Fas cells converted them from Type II to Type I cells, where in Bcl-x\textsubscript{L} could not protect them from CD95-mediated apoptosis (9). We now show that all of the initial CD95 signaling events such as cleavage of DISC-bound c-FLIP\textsubscript{L} and pro-caspase-8 and clustering and internalization of CD95 are found in both vector transfected and Bcl-x\textsubscript{L}-expressing MCF7-Fas cells. However, shortly after generation at the DISC, active caspase-8 subunits become inactivated in the Bcl-x\textsubscript{L}-expressing cells, at a time when this protease can be
detected on mitochondria. We also demonstrate that the mitochondrial protein BAR, which has been shown to simultaneously bind caspase-8 and Bcl-2 (11), also binds active caspase-8 in a way similar to heavy membrane fractions isolated from Bcl-xL-expressing cells. The data point to a model in which Bcl-xL gives mitochondria of MCF7 cells the ability to sequester and neutralize active caspase-8 through binding to BAR.
Experimental Procedures

Cell Lines- MCF7-Fas-vector (Vec) or Bcl-x<sub>L</sub> transfectants (12) were maintained in RPMI-1640 media (Cellgro) supplemented with 10% FCS (Sigma), 2 mM L-glutamine (Cellgro), 100 I.U./ml penicillin (Cellgro), 100 µg/ml streptomycin (Cellgro) (RPMI/10), 200 µg/ml G418 (Cellgro) and 150 µg/ml hygromycin B (Sigma). MCF7 cells reconstituted with pro-caspase-3 (13) were maintained in RPMI/10 with 1 mg/ml G418. 293T cells were maintained in DMEM media supplemented with 10% FCS (Sigma), 2 mM L-glutamine (Cellgro), 100 I.U./ml penicillin (Cellgro), and 100 µg/ml streptomycin (Cellgro). Transient transfections were performed using the calcium phosphate precipitation method as previously described (14).

Immunoblot Analysis - For immunoblot detection of proteins, cellular lysates equivalent to 0.4-1x10<sup>6</sup> cells were prepared using a Triton X-100 containing lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and peptide inhibitor cocktail (Sigma), 1 % Triton X-100 (Serva) and 10% glycerol (9)) and separated by 12% or 15% SDS-PAGE. After electrophoresis proteins were transferred to Hybond nitrocellulose membrane (Amersham). Membranes were blocked with 5% milk in PBS/Tween (phosphate-buffered saline with 0.05% (v/v) Tween-20 (Sigma)) for 1 h, washed with PBS/Tween, and incubated with one of the following antibodies: anti-caspase-8 antibodies C15 or N2 (15), anti-FADD, anti-Bcl-x<sub>L</sub>, or anti-Tim23 (Transduction Laboratories), anti-cytochrome c (Pharmingen), anti-Bid (Santa Cruz Biotechnology), anti-PARP (Biomol), anti-cytokeratin 8 or anti-myc (Sigma), or anti-BAR (11). The blots were washed with PBS/Tween and developed with horse radish peroxidase conjugated goat anti-mouse IgG2b, IgG2a, IgG1 (1:10,000-30,000 in 5% milk PBS/Tween), or rabbit-anti-goat IgG (1:5000 in 5% milk Tween/PBS). After washing with PBS/Tween blots were developed using
an enhanced chemoluminescence method following the manufacturer’s protocol (Amersham). Plectin preparation and immunoblots were performed as described previously (16).

**Flow Cytometric Analysis of Mitochondrial Membrane Potential (ΔΨₘ)** - To measure ΔΨₘ, anti-CD95 (2 µg/ml)-treated or -untreated MCF7-Fas-Vec or MCF7-Fas-Bcl-xL cells (5 x 10⁵ per ml) were incubated with 5 µg/ml JC-1 (5, 5’, 6, 6’-tetrachloro-1, 1’, 3, 3’-tetraethylbenzimidazolycarbocyanine iodide) (Molecular Probes). The analysis was performed as described (17).

**Fluorescent Labeling of Antibodies** - For antibody labeling 0.5 mg of antibody was dialyzed against borate buffer (0.05 M Boric acid, 0.2 M NaCl, pH 9.2), and incubated with 50 µg of fluorescein isothiocyanate (FITC) in DMSO for 2 hr at room temperature, followed by extensive dialysis against PBS. FITC conjugation was determined by fluorometry and all antibodies equalized for protein and FITC labeling.

**Immunofluorescence Microscopy** - Cells were plated on POLY-PREP (Sigma) poly-L-lysine coated glass slides at a confluence of 50% and were allowed to become adherent overnight. After washing 3 times with PBS containing 1 mM MgCl₂ (PBS/MgCl₂) the cells were fixed with methanol/acetone 1:1 at -20°C for 4 min. The slides were allowed to dry, rehydrated with PBS containing 4% BSA, incubated 1 hr in a humidified chamber followed by incubation for 1 hr with FITC-labeled monoclonal antibody C5 (IgG2a) in PBS/4% BSA (15). After washing 3 times with PBS/MgCl₂ slides were rinsed in water and dehydrated in 100% ethanol. After drying, coverslips were mounted on the slides, using Vectashield (Vector Laboratories) mounting medium. For costaining the cells were incubated with 20 nM MitoTracker Red (Molecular Probes) for 15 min at
37°C, 5% CO₂ prior to washing and fixation, or stained with anti-Bcl-x<sub>L</sub> rabbit polyclonal (Transduction Laboratories) for 45 min, washed 3 times in PBS/MgCl<sub>2</sub> and incubated for 45 min with phycoerythrin conjugated goat anti-rabbit secondary antibodies. Photographs for colocalization were obtained using confocal microscopy (LSM 510, Zeiss, Jena).

**DISC Immunoprecipitation** – Cells were harvested at different times after anti-CD95 stimulation (1 µg/ml), and 10<sup>7</sup> lysed in 1 ml of Triton X-100 lysis buffer as above. As a control, anti-CD95 antibody was added to lysates prepared from the unstimulated cells. Lysate protein content was determined by Bradford assay and equalized prior to immunoprecipitation. Protein A Sepharose beads (Sigma) were added to the samples and tumbled for 2 hours at 4ºC. The beads were washed four times with 40 fold excess of lysis buffer, and either subjected to SDS-PAGE and immunoblotting or a caspase activity assay as described below.

**Preparation of Cytosolic Extracts for Detection of Cytochrome c**- Cells (10<sup>6</sup>) were suspended in 50 µl of 5 µg/ml digitonin/0.25 M sucrose and mixed for 30 seconds. Cells were then centrifuged for 1 minute at 14,000 rpm and 10 µg of supernatant protein was analyzed by SDS-PAGE and Western blotting as above.

**Subcellular Fractionation and in vitro Caspase Activity Assays** - MCF7-Fas cells (5 x 10<sup>7</sup>) were stimulated with anti-CD95 (2 µg/ml) for the indicated times and washed with PBS. The fractionation procedure was performed so that cells were homogenized using a glass douncer until a degree of lysis of 60 % was reached. Subcellular fractionation to produce heavy membrane and cytosolic fractions was performed as described elsewhere (18). For the determination of caspase activity in cytoplasmic or heavy membrane fractions, MCF7-Fas-Vec or MCF7-Fas-Bcl-x<sub>L</sub> (10<sup>7</sup> each) were stimulated with anti-CD95 (2 µg/ml) for 3 hrs. Heavy membrane as well as cytoplasmic fractions were incubated in cleavage buffer with 40 µM of amino trifluoromethyl coumarin (AFC)
labeled caspase-8 specific peptide zIETD (Bachem) for 1 h at 37°C. Caspase activities were determined fluorometrically using a fluorescence plate reader (Bio-Tek Instruments, Inc.). Values obtained with unstimulated cells were taken as background and subtracted from those obtained with stimulated cells.

Caspase-8 Localization on the Outer Mitochondrial Membrane - MCF7 heavy membranes were treated with trypsin in trypsin assay buffer (20 mM HEPES/KOH, pH 7.4, 0.25 M sucrose) for 20 min at 4°C. Proteins (10 µg) were separated by 12.5 % SDS-PAGE and proteins were detected by immunoblotting as described above. To induce processing of mitochondrial pro-caspase-8, MCF7 heavy membranes were isolated as described above and treated from 10^7 cells with recombinant caspases (active concentration 20 µM) for 15 minutes at 37°C. The membranes were then subjected to SDS-PAGE, blotted and probed with the N2 antibody (15) recognizing the prodomain of caspase-8.

In vitro Binding of ^35^S-labeled Caspase-8 to Isolated Heavy Membranes and GST-BAR-DED-L

- An in vitro binding assay using in vitro transcribed/translated proteins was performed as previously described (16). In brief ^35^S-labeled caspase-8, caspase-3, N-CASP-8 (AA 1-216, containing the prodomain), C-CASP-8 (AA 224-479, containing the catalytic domain), or processed C-CASP-8 was incubated with heavy membranes from MCF7-Fas-Bcl-xL cells for 15 min at 37°C in the presence of 50 µM zVAD-fmk. Pellets and supernatants were analyzed by 12.5% SDS-PAGE. Active caspase-8 was generated by incubating C-CASP-8 with recombinant caspase-8 (active concentration 40 µM) for 16 hrs at room temperature. This treatment resulted in a complete processing of C-CASP-8 into p10 and p18 subunits. A larger p18 subunit (p18*) was also generated, derived from cleavage of CASP-8*. The identity of the fragments was confirmed by immunoprecipitation using subunit-specific anti-caspase-8 mAbs. To test in vitro binding of
caspase-8 isoforms described above to BAR a GST-BAR-DED-L protein (residues 167-358) recently described (11) was immobilized on 20 µl of glutathione-Sepharose beads and incubated with 10 µl of the indicated 35S labeled in vitro translated (TNT reticulate lysates, Promega) caspase-8 proteins in 100 µl of the following binding buffer: 1 mg/ml BSA 142.2 mM KCl, 5 mM MgCl₂, 10 mM HEPES pH 7.4, 0.5 mM EGTA, 0.2% NP-40, 1 mM DTT, supplemented with 12.5 mM β-glycerolphosphate, 2 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF and 1x protease inhibitor mix (Roche, Mannheim). After incubation for 16 hrs at 4°C beads were washed three times with binding buffer and analyzed by SDS-PAGE and autoradiography.

*In vivo Caspase Activity* - The cell permeable in vivo caspase inhibitor substrate CaspaTag 8, Carboxyfluorescein-LETDA-fluoromethyl ketone (Intergen Co, Purchase, NY), was used according to manufactures’ instructions. Briefly, MCF7-Fas-Vec or Bcl-xL cells were plated and allowed to become adherent overnight. The cells were then stimulated with 1 µg/ml anti-CD95 for the indicated times. CaspaTag 8 (1x working dilution) was added to the samples 1 hour before harvest. The cells were washed twice and analyzed by flow cytometry.

*CD95 Surface Clustering* – Cells were detached from flasks by trypsination, and resuspended at 10⁶/ml. For pretreatment with zIETD-fmk (Bachem) cells were incubated at 37°C for 45 min with 50 µM zIETD-fmk. Cells were then stained at 4°C with anti-CD95 antibodies for 45 min, washed and stained with FITC conjugated goat anti-mouse IgG. After washing cell were resuspended in media and warmed to 37°C for 2 hours or kept on ice (0 time). Cells were plated for 5 min at 37°C on poly-L-lysine (Sigma) coated plates, and fixed in methanol/acetone (1:1). Slides were washed, rinsed with water, and coverslips mounted with Vectashield mounting media. Quantification of receptor internalization was done as previously described (19).
**Cell Permeabilization** – Cells (8x10^6) were resuspended in PBS and permeabilized with 200-250 µg/ml of lysophosphatidylcholine (LPC) as determined by trypan blue exclusion as previously described (20). The reagent was added until greater than 99% of cells were permeabilized. Cell pellets and supernatant were then separated by centrifugation at 14,000 rpm for 1 min. Protein content was determined and protein amounts equalized. 40 µg (corresponding to approximately 10% of the protein fractions) of protein was subjected to SDS-PAGE and immunoblotting, the remaining fraction was subjected to a caspase-8 activity assay as described above.

**BAR/Bcl-xL Mediated Suppression of Apoptosis** – 293T cells were transfected with different combinations of plasmids encoding the following proteins: wild-type BAR (BARwt) (1 µg DNA), Bcl-xL (0.2 µg DNA), and a CD8:caspase-8 fusion protein (0.8 µg DNA) which produces large amounts of active caspase-8 and induces apoptosis (21). The cells were harvested and subjected to an apoptosis assay as previously described (9) using propidium iodide staining and flow cytometry.

**BAR/Bcl-xL Mediated Inactivation of Caspase-8** – 293T cells were transiently transfected with the indicated constructs (BAR∆R (11), 5 µg DNA or Bcl-xL, 3 µg DNA). Heavy membranes were isolated and incubated with extracts of CD95 stimulated (3 hr) or unstimulated MCF7-Fas-Vec cells, prepared by LPC permeabilization for 1 hr at room temperature. Membranes were then removed by centrifugation and the supernatants tested for caspase-8 activity as above using IETD-AFC. Depletion was determined by subtraction from control lysates. Heavy membranes were tested for expression of transfected proteins using immunoblotting.

**In vitro Binding of ^35^S-labeled Caspase-8 to Trypsin-Treated Heavy Membranes** – Isolated heavy membranes were treated with 80 µg/ml trypsin in trypsin assay buffer as described above, or with trypsin inactivated with a two fold excess (over that required to inhibit this amount of trypsin) of trypsin inhibitor (Sigma). Active trypsin was then inhibited with a two fold excess of trypsin...
inhibitor. These membranes were pelleted and resuspended with $^{35}$S-labeled, *in vitro* translated caspase-8 and incubated for 15 min at 37°C. The membranes were then pelleted and pellets and supernatants were analyzed by SDS-PAGE. Western blots of membrane proteins were done to control for trypsin treatment.
Results

The breast carcinoma cell line MCF7-Fas behaves as a Type II cell in that over-expression of Bcl-xL protects these cells from CD95-induced apoptosis (12,10) and blocks most of the associated features such as cell detachment (10), DNA fragmentation (10), decline of mitochondrial transmembrane potential (ΔΨm) and release of cytochrome c from mitochondria (Fig. 1A). In contrast, processing of caspase-8 was not reduced in the presence of Bcl-xL (Fig. 1B). In fact, after 16 hrs, most of the cellular pro-caspase-8 had been converted to active caspase-8, with no resulting loss of cell viability. Unexpectedly, however, cleavage of poly (ADP-ribose) polymerase (PARP) and Bid was inhibited by Bcl-xL (Fig. 1B and C). Furthermore, degradation of plectin, another direct in vivo substrate of caspase-8 (16), was also inhibited (Fig. 1C). These data demonstrate the ability of MCF7-Fas-Bcl-xL cells to tolerate large quantities of active caspase-8. It therefore appears that in these cells, active caspase-8 subunits are either inactivated and/or prevented from reaching their cytosolic targets.

We have recently shown that in MCF7 cells pro-caspase-8 is mainly localized on mitochondria (16). In contrast, using confocal microscopy analysis of cells costained for caspase-8 and phosphodiesterase, an enzyme of the endoplasmic reticulum (ER), we did not observe a significant localization of caspase-8 to the ER (16). The localization of caspase-8 to mitochondria in these cells is not due to the absence of caspase-3, since caspase-8 is still localized mainly on mitochondria in MCF7 cells reconstituted with pro-caspase-3 (Fig. 2A). MCF7 cells therefore express caspase-8 at two intracellular locations, in the cytosol and on mitochondria. This raises the question of how mitochondrial pro-caspase-8 is processed. Active caspase-8 may move from the DISC to mitochondria and directly process mitochondrial pro-caspase-8 or mitochondrial pro-caspase-8 may be recruited to the cell surface and become activated at the DISC.
Since no significant cleavage of Bid could be detected in the Bcl-xL-expressing MCF7-Fas, we tested whether caspase-8 itself might directly process mitochondrial pro-caspase-8. To test this hypothesis we incubated isolated heavy membranes (HM) from MCF7-Fas-Bcl-xL cells with various recombinant caspases and examined which of these proteases are capable of processing HM-associated pro-caspase-8 (Fig. 2B). Among the caspases tested, caspase-8 most efficiently cleaved pro-caspase-8 in this assay. Since all of the pro-caspase-8 in these cells becomes activated after triggering CD95, we postulate that a small quantity of active caspase-8 generated at the DISC might directly activate mitochondrial pro-caspase-8 independently of the apoptogenic activity of mitochondria. Since this activity is blocked by Bcl-xL in vivo (Fig. 1A) but processing of HM bound pro-caspase-8 occurs, this suggests that caspase-8 is neutralized at the level of mitochondria assuming that active caspase-8 is generated at the DISC in similar amounts in both transfectants. To directly test this assumption we immunoprecipitated the DISC and determined its IETD-cleaving activity in a fluorogenic assay (Fig. 2C). Early after receptor triggering, the activity of caspase-8 in the DISC was the same in both transfectants confirming that the block in the apoptosis pathway in MCF7-Fas-Bcl-xL cells must be located downstream of the DISC.

To identify the step in the CD95 pathway where caspase-8 is inactivated in the MCF7-Fas-Bcl-xL cells, we directly compared the time-course of processing of pro-caspase-8 at the DISC and on HMs in both vector control and Bcl-xL transfectants (Fig. 3A). Stimulation of CD95 in both transfectants resulted in rapid recruitment of caspase-8 and its processing at the DISC (Fig. 3A). The activity of the DISC is reflected by the early appearance of the caspase-8 cleavage intermediates p43/p41. We further found evidence that caspase-8 is initially active when converted at the DISC in both transfectants, as the cleaved p43 form of c-FLIPL is present in the DISC of both vector-control and Bcl-xL-expressing cells. Pretreating cells with the caspase-8-selective inhibitor
zIETD-fmk inhibited processing of c-FLIP<sub>L</sub>. Interestingly, most of the cytosolic c-FLIP<sub>L</sub> was cleaved very early after triggering CD95<sup>5</sup>. Thus c-FLIP<sub>L</sub> and caspase-8 are among the few caspase-8 substrates in MCF7-Fas cells we detected whose cleavage is not blocked by Bcl-x<sub>L</sub>. The appearance of p43/p41 partially processed pro-caspase-8 on isolated HM fractions seemed to be delayed when compared to its appearance in the DISC, consistent with the initiation of the death signal at the plasma membrane. However, no difference was observed in the rate of loss of full-length pro-caspase-8 in HM fractions when comparing vector- and Bcl-x<sub>L</sub>-transfectants, despite the fact that release of cytochrome c from mitochondria could only be detected in the MCF7-Fas-Vec cells (Fig. 3A). The data therefore suggest that the block in the CD95 apoptosis pathway in the Bcl-x<sub>L</sub>-expressing MCF7-Fas cells resides at a step between proteolytic processing of mitochondria bound pro-caspase-8 and mitochondrial dysfunction (as measured by cytochrome c release and drop in ΔΨ<sub>m</sub>).

Our data suggested that caspase-8 was inhibited in Bcl-x<sub>L</sub>-expressing cells either by a soluble cytosolic caspase inhibitor or by being bound and sequestered at intracellular membranes. To distinguish between these two possibilities, we permeabilized MCF7-Fas cells using lysophosphatidylcholine (LPC), separated supernatant (cytosol) from pellet (membranes) and determined by immunoblot analysis whether active caspase-8 subunits was released or retained in cells (Fig. 3B). Tim23, a protein of the inner mitochondrial membrane, was analyzed to demonstrate that the supernatant was not contaminated with mitochondrial membranes. In addition, we determined the caspase-8 protease activity of supernatants and pellets using a fluorogenic substrate (Fig. 3B, bottom). When MCF7-Fas-Vec cells were tested, the caspase-8 p18 subunit was detected in the supernatant in an active form (Fig. 3B, lane 2). In contrast the caspase-8 cleavage intermediates p41 and p43 were found in the pellet fraction of MCF7-Fas-Vec cells, indicating that
they are bound to intracellular membranes and that activation of caspase-8 takes place in a membrane context. In contrast to MCF7-Fas-Vec cells, no fully processed active caspase-8 was released into the supernatant of MCF7-Fas-Bcl-xL cells, suggesting that active caspase-8 is not freely soluble in these cells. Also, we could not reproducibly detect the caspase-8 p18 subunits associated with the membrane fraction of the Bcl-xL-transfected cells, likely due to secondary degradation of the protein after detergent lysis. When taken together, these data predict that after generation at the DISC, active caspase-8 is neutralized by binding to intracellular membranes in a Bcl-xL-dependent fashion in MCF7-Fas cells. The finding that the rate of processing of pro-caspase-8 was very similar in both the vector and the Bcl-xL transfectants while cytochrome c release was blocked by Bcl-xL strongly suggested that the block in the apoptosis pathway in MCF7-Fas-Bcl-xL cells was located downstream of caspase-8 bound to intracellular membranes and upstream of mitochondrial cyt c release.

To further explore at which step the apoptosis pathway was blocked by expression of Bcl-xL, we tested the activity of caspase-8 in these cells in vivo during the course of CD95-mediated apoptosis. We used the membrane permeable fluorescein-conjugated peptide-based caspase substrate CaspaTag 8 that becomes covalently coupled to caspase-8 when it is cleaved by the protease. This compound labels active caspase subunits directly in living cells. Cells were incubated for different times with anti-CD95 and one hour before stopping the reaction the fluorescent compound was added. CaspaTag 8 specifically labeled apoptosing MCF7-Fas-Vec cells (Fig. 4A). Three independent experiments are shown. Caspase-8 activity in MCF7-Fas-Vec cells increased over the entire time-course of anti-CD95 treatment. In contrast, we only detected a small but reproducible caspase-8 activity in the apoptosis-resistant Bcl-xL-expressing cells 4 hrs after stimulation (Fig. 4A). This activity was not detectable 24 hrs after stimulation, despite full processing of pro-caspase-8,
suggesting that processed caspase-8 was inactivated in these cells at a relatively early time after CD95 stimulation.

We wished to probe in more detail the issue of when caspase-8 becomes neutralized in Bcl-xL overexpressing cells. In this regard, we have recently found that one of the earliest events during CD95-mediated apoptosis occurring shortly after formation of the DISC, is CD95 surface clustering. Furthermore, we have shown that this CD95 clustering and subsequent internalization of the receptor is caspase-8 dependent (19). To test whether Bcl-xL affects this early event associated with caspase-8 activity, we incubated MCF7-Fas-Vec and Bcl-xL expressing cells with anti-CD95 mAb and a FITC-conjugated goat anti-mouse IgG antibody and monitored clustering of CD95 by fluorescence microscopy. For this experiment detached cells were used, since the clustering effect was more pronounced than in attached cells. In contrast to the diffuse staining of unstimulated cells, a 60 minute stimulation with anti-CD95 caused clustering of the receptor in both vector and Bcl-xL transfectants. Consistent with recent data, this effect was inhibited by the caspase-8 selective inhibitor zIETD (19). We also examined internalization of CD95 which is dependent on active caspase-8 (19). We found that the small amount of caspase-8 formed in the apoptosis-resistant Bcl-xL-expressing cells was sufficient to efficiently internalize CD95 (Fig. 4C), consistent with our conclusion that caspase-8 generated at the DISC of Bcl-xL-expressing cells is initially active, thus allowing early caspase-8-regulated steps in CD95-signaling to occur. However, caspase-8 presumably becomes inactivated shortly thereafter.

To directly detect caspase-8 active subunits in cells treated with anti-CD95, we compared the localization of active caspase-8 in the MCF7-Fas-Vec cell with the MCF7-Fas-Bcl-xL cells, using the p10-specific anti-caspase-8 antibody C5 for laser scanning microscopy analysis. Pro-caspase-8 was localized at mitochondria in non-apoptotic MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells (Fig.
Co-staining for caspase-8 and Bcl-xL showed that both Bcl-xL and caspase-8 colocalized almost entirely on mitochondria of the MCF7-Fas-Bcl-xL cells (Fig. 5B). Consistent with our previous report (16), upon induction of apoptosis through CD95, the staining for caspase-8 in most MCF7-Fas control cells changed from a mitochondrial pattern to a more diffuse meshwork-like pattern, which we have recently identified as the cytoskeletal protein plectin (16) (Fig. 5A). In contrast, following treatment of MCF7-Fas-Bcl-xL cells with an anti-CD95 antibody for 16 hrs, we still detected only a mitochondrial staining of caspase-8 active subunits (Fig. 5B). The C5 antibody recognizes both full-length and the active subunit p10 of caspase-8 and it therefore also stains procaspase-8 in unstimulated cells. We have recently established that in both transfectants, the prodomain of caspase-8 stays bound to mitochondria following cleavage (16). Since the processing of caspase-8 in this experiment showed a very similar degree of conversion of caspase-8 between vector and Bcl-xL transfected cells (Fig. 5C), the difference between the staining patterns seen in cells stimulated with anti-CD95 indicates that the p10 subunits in MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells were at different intracellular locations. The data gave rise to the hypothesis that mitochondria of MCF7-Fas cells have the ability to bind and inactivate caspase-8 when they express Bcl-xL.

To directly demonstrate that endogenous caspase-8 bound to Bcl-xL-expressing HMs, we isolated cytoplasmic and HM fractions from MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells and subjected them to a caspase-8 activity assay (Fig. 5D). After stimulation of MCF7-Fas control cells for 3 hrs, caspase-8 activity in the cytoplasm was high whereas no caspase-8 activity was detected in the HM fraction. In contrast, in Bcl-xL-overexpressing MCF7-Fas cells, significant caspase-8 activity was found associated with HM, whereas the caspase-8 activity in the cytosolic fraction was
reduced when compared to control cells. Extensive work demonstrated that during the incubation of 1 h at 37˚C for fluorogenic assays, inactive caspase-8 regains activity by either dissociating from HM fractions or by undergoing a conformational change\(^5\). The data suggest that Bcl-x\(_L\)-expressing intracellular membranes have the capacity to actively recruit caspase-8 from the cytoplasm when Bcl-x\(_L\) is overexpressed in MCF7-Fas cells.

We predicted a binding partner for active caspase-8 on intracellular membranes that would bind and neutralize active caspase-8 in a Bcl-x\(_L\) dependent fashion. Recently, a novel protein, bifunctional apoptosis regulator (BAR), was described that binds both pro-caspase-8 and Bcl-2 and is localized to mitochondria \(^{(11)}\). Endogenous BAR protein was found associated with HMs of MCF7 cells and it was present at very high levels in MCF7-Fas when compared to other tumor cell lines\(^5\) \(^{(11)}\). Thus, we initiated experiments to explain the role of BAR in the caspase-8 sequestration phenomenon. Our first task was to deal with the issue of where BAR, Bcl-x\(_L\) and caspase-8 reside when associated with mitochondria since if BAR is involved it must have the same topological orientation on intracellular membranes as caspase-8 and Bcl-x\(_L\). To determine the localization of caspase-8, Bcl-x\(_L\) and BAR on HMs of MCF7-Fas-Bcl-x\(_L\) cells, we treated HM fractions of these cells with trypsin (Fig. 6A). Membrane-associated pro-caspase-8, Bcl-x\(_L\) and BAR were all determined to be protease sensitive, whereas an integral transmembrane protein of the inner mitochondrial membrane, Tim23, was resistant to the trypsin treatment. Addition of Triton X-100 detergent to solubilize membranes resulted in sensitivity of Tim23 to tryptic degradation. These experiments suggest that Bcl-x\(_L\), BAR and caspase-8 are located on the outer-mitochondrial membrane, oriented toward the cytosol.

We have recently shown that isolated HMs fractions from MCF7-Fas-Bcl-x\(_L\) cells can bind pro-caspase-8 in vitro \(^{(16)}\). Since BAR was exquisitely sensitive to trypsin treatment, we treated HMs
with trypsin to test whether HMs loose their activity to bind caspase-8 in the absence of BAR (Fig. 6B). 35S-labeled pro-caspase-8 bound very efficiently to isolated HMs before trypsin-treatment (Fig. 6B, lane 2). In contrast, after trypsin-treatment of HMs caspase-8 did not bind. Trypsin-treatment also resulted in a complete loss of BAR correlating with the complete loss of caspase-8 binding to these membranes (Fig. 6B, lane 4). In comparison, trypsin-treatment only partially reduced the amount of Bcl-xL on these HM fractions, and it had no effect on the inner membrane protein Tim23 (Fig. 6B, lane 7).

To directly test which domain of caspase-8 binds to HMs from MCF7 cells, we performed an in vitro binding assay in which in vitro translated pro-caspase-8 was added to isolated HM fractions from Bcl-xL-expressing MCF7-Fas cells, essentially as described (16). When pro-caspase-3 and pro-caspase-8 were mixed and applied to HM fractions, only pro-caspase-8 was quantitatively bound (Fig. 6C, lane 2), demonstrating the specificity of this binding. Comparisons of full-length caspase-8 were then made with various fragments of this protein to map the relevant domains. The prodomain of caspase-8 (N-CASP-8; corresponding to amino acids 1-216) bound to HMs (Fig. 6C, lane 5) but not as effectively as full-length caspase-8 (based on comparisons of the relative amounts of material in the "bound" versus "unbound" fractions. The C-terminal domain of caspase-8 (C-CASP-8, corresponding to the unprocessed catalytic domain, residues 224-479) also bound to HM fractions (Fig. 6C, lane 8). Note that an aberrant C-CASP-8 translation product (C-CASP-8*) failed to bind HMs (Fig. 6C, lane 9). To test whether fully-processed active caspase-8 would also bind to HMs, we generated active caspase-8 p18/p10 subunits by treating in vitro translated C-CASP-8 with recombinant caspase-8 (Fig. 6C, lane 10). Though processed caspase-8 is notoriously difficult to detect by SDS-PAGE/immunoblotting, making signals weak, these binding experiments suggest
that the processed caspase-8 p18 (large) and p10 (small) catalytic subunits also are capable of associating with HM fractions (Fig. 6D, lane 11).

If BAR is the protein that binds active caspase-8 on the surface of mitochondria, then we expected its binding properties to be similar to those of HMs isolated from BAR-expressing MCF7-Fas-Bcl-xL cells and therefore performed in vitro protein binding assays using a GST fusion protein containing the death effector domain-like (DED-L) from BAR (11) and testing its ability to interact with in vitro translated N-CASP-8, C-CASP-8, and processed p18/p10 caspase-8 (Fig. 6D). Similar to HM fractions, GST-BAR-DED-L bound pro-caspase-8 but did not bind pro-caspase-3 (Fig. 6D, lane 2 and 3) thus confirming the specificity of this result. The prodomain of caspase-8 (N-CASP-8) as well as the unprocessed catalytic domain (C-CASP-8) bound to GST-BAR-DED-L (Fig. 6D, lane 6 and 9) but not to GST control protein (Fig. 6D, lane 5 and 8) and not to GST-FADD. Similar to the binding of caspase-8 to HM fractions, GST-BAR-DED-L also bound specifically processed p18/p10 caspase-8 subunits (Fig. 6D, lane 12). Control GST (Fig. 6D, lane 11) as well as GST-FADD5 did not bind processed caspase-8 thus confirming the specificity of this result. These experiments identify BAR as a protein that binds both unprocessed and active caspase-8 making it a potential candidate for explaining the sequestration of active caspase-8 on the surface of Bcl-xL-expressing mitochondria.

We next tested whether BAR in combination with Bcl-xL could protect cells from caspase-8 induced apoptosis. To mimic the situation in MCF7 cells, we induced apoptosis in 293T cells by transiently transfecting them with a plasmid encoding a CD8:caspase-8 fusion protein (21). Expression of this plasmid results in a robust generation of active caspase-8 through induced proximity (21). When 293T cells were transfected with this plasmid, DNA fragmentation could be detected, which was somewhat reduced by expression of either BAR or Bcl-xL (Fig. 7A). However,
when BAR was co-expressed with Bcl-xL, DNA fragmentation was reduced almost to background levels. Since both BAR (Fig. 7B) and Bcl-xL (Fig. 7C) are present on HMs when expressed in 293T cells, the data are consistent with a model in which mitochondrial BAR can collaborate with Bcl-xL to bind active caspase-8. Consistent with previous data, we found that BAR lacking the N-terminal RING domain was much more efficiently expressed than wild-type BAR (Fig. 7B) (11). Hence, BAR protein content appears to be regulated at a post translational level, consistent with the observation that the N-terminal RING domain of BAR has an E3 ubiquitin ligase activity that might regulate the turnover of this protein (11).

To directly test whether mitochondrial BAR has the ability to inhibit caspase-8 in a Bcl-xL-regulated fashion, we isolated HM fractions from 293T cells transiently transfected with BARΔR and/or Bcl-xL and incubated these HM fractions with cellular lysates prepared from MCF7-Fas cells stimulated through CD95 for 3 hrs. We then determined the activity of caspase-8 in the supernatant after removal of HMs by centrifugation (Fig. 7C). In this experiment, only HMs expressing BARΔR in combination with Bcl-xL were able to significantly deplete cellular lysates of active caspase-8. The data suggest that active caspase-8 in MCF7-Fas-Bcl-xL cells is bound and inactivated on the outer mitochondrial membrane with the help of BAR and Bcl-xL.
Discussion

Our original description of the two CD95 signaling pathways (9), one mitochondrial-dependent (Type II) and the other mitochondrial-independent (Type I) is consistent with data from a variety of cell culture models and has been supported by transgenic and gene knock-out experiments in mice. For example, studies of Bcl-2 transgenic mice (22,23), Bid deficient mice (6), and Bax/Bak double knockout mice (24,25), have shown that T lymphocytes and especially thymocytes are Type I cells, whereas hepatocytes represent Type II cells. The main difference between Type I and Type II cells lies in the amount of caspase-8 generated at the DISC after CD95 activation. We have demonstrated that the amount of active caspase-8 generated at the DISC in Type II cells is so small that it cannot be detected by Western blotting (9). Some caspase-8 activity is present, however, as its activity is detectable in a functional assay based on the enzymatic activity of caspase-8 (17). It has been demonstrated in a cell free-system that the amount of active caspase-8 required to induce apoptotic changes in isolated nuclei through direct cleavage of pro-caspase-3 in the absence of mitochondria is orders of magnitudes higher than that required to induce these nuclear changes in the presence of mitochondria (26). These changes can be inhibited by expression of Bcl-2 or Bcl-xL on mitochondria.

Since Bcl-2 and Bcl-xL inhibit the apoptogenic activity of mitochondria without interfering with the activity of the DISC, a confounding question has been the fate of active caspase-8 generated at the level of the death receptors in Bcl-2/Bcl-xL protected cells. To study the biochemical mechanisms underlying this phenomenon, we chose to use MCF7-Fas cells as a model because they produce large Type I-like amounts of caspase-8 at the DISC upon addition of an agonistic anti-CD95 antibody but behave like Type II cells due to the absence of pro-caspase-3.
Most of the pro-caspase-8 in MCF7 cells is localized to mitochondria. Though it has been suggested that a number of caspases, including caspase-8, reside in the intermembrane space of mitochondria (27-31), our data favor a model in which caspase-8 is bound to the outer mitochondrial membrane. This model is also consistent with the finding that isolated HM fractions efficiently bind in vitro translated caspase-8 (16, and present study). We did not succeed in isolating mitochondria from MCF7 cells. However, we repeated this in vitro binding experiment with mitochondria isolated from CEM cells stably transfected with Bcl-xL (9), and these purified mitochondria showed the same binding specificity for caspase-8 and its cleavage products as the HM fractions used in this study\(^5\). These data indicate that mitochondria have a capacity to bind caspase-8. This finding, however, does not exclude the possibility that other intracellular compartments such as the ER might also have this capacity, particularly since the intracellular localization of caspases seems to be tissue-specific.

During CD95-mediated apoptosis in MCF7-Fas cells, all caspase-8, both cytosolic and mitochondrial, is activated. Since the signal is initiated at the cell surface, it is conceivable that a small amount of caspase-8 generated at the DISC travels into the cytoplasm and cleaves mitochondria bound pro-caspase-8. This hypothesis is supported by our observation that addition of recombinant caspase-8 (but not other caspases) resulted in proteolytic processing of pro-caspase-8 when added to HM fractions. It occurs in both the vector transfected as well as the Bcl-xL expressing MCF7-Fas cells consistent with our finding that both DISC-bound and cytosolic caspase-8 (and c-FLIP\(_L\)) are completely cleaved in both transfectants.

Recently, it was demonstrated that inhibition of TRAIL-induced apoptosis of MCF7 cells by transient expression of Bcl-xL could be overcome by co-expression of Smac/Diablo (32). Smac/Diablo has been shown to inhibit the function of apoptosis inhibitor proteins of the IAP
family (33,34). The authors concluded that over-expression of Smac/Diablo converted MCF7 cells from Type II into Type I cells. It is unlikely that Smac/Diablo is responsible for the differences between MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells used in our study. If Smac/Diablo were to regulate the activity of caspase-8 in these cells, it would likely antagonize a caspase-8-specific member of the IAP family. However, no examples of caspase-8 suppression by IAP proteins have been reported (35). In addition, inhibition of caspase-8 by a Smac/Diablo suppressible IAP (33,34) should render even the MCF7-Fas-Vec cells apoptosis resistant. Our finding that active caspase-8 could be found in the supernatant of permeabilized MCF7-Fas-Vec but not MCF7-Fas-Bcl-xL cells argues for a different mechanism. Altogether our data suggest the existence of an inhibitory mechanism that takes place on the outer mitochondrial membrane. Interestingly, we found that caspase-8 is not irreversibly inactivated when bound to HMs, since we could detect significant amounts of caspase-8 activity on HM fractions of Bcl-xL-expressing MCF7-Fas cells using an in vitro caspase assay. This is likely that due to the conditions of this assay (incubation with the substrate for 1 hr at 37°C during which sufficient amounts of caspase-8 became released from inhibitory proteins to reveal caspase-8 enzymatic activity). These data are consistent with a binding of caspase-8 to intracellular membranes in a form in which its access to substrate is restricted.

Neither pro-caspase-8 nor active caspase-8 can bind to Bcl-xL directly (10), suggesting the presence of a protein domain on the surface of mitochondria that specifically binds active caspase-8 and that is regulated by Bcl-2 and Bcl-xL. Recently it was suggested that BAR could mediate binding of pro-caspase-8 to mitochondria in a Bcl-2-regulated fashion (11). Prior to our report, however, binding of active caspase-8 binding to BAR had not been tested. We now demonstrate that BAR binds active caspase-8, pro-caspase-8, and the caspase-8 prodomain (containing the two tandem death effector domains) in vitro. The binding characteristics of BAR are similar to those of
HM fractions of MCF7-Fas-Bcl-xL cells, implicating BAR as a candidate for binding of active caspase-8 by Bcl-xL-expressing mitochondria. It should be noted, however, that recent data suggest, that depending on the cell type, BAR together with Bcl-2/Bcl-xL can also be found in the ER. To test BARs role in the CD95 apoptosis resistance of MCF7-Fas-Bcl-xL cells we recently downregulated BAR in these cells using specific antisense oligonucleotides. Reduction of BAR expression resulted in a significant sensitization of MCF7-Fas-Bcl-xL cells to anti-CD95 induced apoptosis.

Our data also support a model in which binding of active caspase-8 to mitochondrial BAR in the presence of Bcl-xL results in its inactivation. MCF7 cells express large amounts of the mitochondrial protein BAR and lack expression of caspase-3. They therefore have a high capacity to buffer active caspase-8. We observed that after an initial detection of the active p18 subunits of caspase-8 which peaks between 2 and 4 hrs after CD95 stimulation in both the vector-transfected and the Bcl-xL-expressing cells, all caspase-8 fragments are apparently degraded as they are no longer detectable by immunoblotting. Degradation of active caspase subunits is a commonly observed phenomenon, though it has never adequately been explained. Late during apoptosis, when cells undergo secondary necrosis, many cellular substrates are degraded due to the destruction of the cells. However, secondary necrosis cannot explain our results because active caspase-8 was also rapidly degraded in MCF7-Fas-Bcl-xL cells and these cells did not die. Given that the BAR protein binds caspase-8 and possesses a RING domain with probable E3-like activity, we speculate that active caspase-8 might be targeted for ubiquitination and proteasome dependent degradation. However, we failed to prevent the degradation of caspase-8 by pre-treating cells with proteasome inhibitors or by adding zVAD-fmk to the cells. This finding suggests that the apparent degradation of caspase-8 as seen by immune blotting may reflect an intrinsic instability of the active caspase-8.
enzyme molecule. Consistent with that idea, degradation of caspase-8 in the surviving MCF7-Fas-Bcl-x<sub>L</sub> cells occurred with the same kinetics as in cells undergoing apoptosis, despite the complete lack of PARP cleavage.

Recently, we have demonstrated that shortly after binding of anti-CD95 antibody or CD95 ligand, the CD95 receptor forms clusters on the cell surface followed by receptor internalization, a process dependent on polymerization of actin and which requires caspase-8 activity (19). Interestingly, we now show that even non-apoptosing MCF7-Fas-Bcl-x<sub>L</sub> cells cluster and internalize activated CD95, and this process can be inhibited by a caspase-8-selective inhibitor. Our data suggest that initially MCF7-Fas-Bcl-x<sub>L</sub> cells activate caspase-8 at the DISC, resulting in generation of small amounts of active caspase-8. This activity is then so effectively sequestered as a result of BAR and Bcl-x<sub>L</sub> co-expression that even Bid and plectin are not cleaved. Our data therefore provide an explanation for the striking finding that even microinjection of recombinant active caspase-8 did not induce apoptosis in these cells (36). Late after the triggering of CD95, caspase-8 activity is specifically lost in the Bcl-x<sub>L</sub>-expressing MCF7-Fas cells at time-points when we detect caspase-8 active subunits at mitochondria. At this time, the bound caspase-8 subunits are not active, since we detected no caspase activity in these cells in vivo. We therefore conclude that binding of caspase-8 to mitochondria results in its inactivation or at least its sequestration in a manner that effectively neutralizes the protease. This mechanism may also be important in other Type II cells in which less active caspase-8 is produced at the DISC. In these cells, a much lower expression of BAR may be sufficient to neutralize the smaller amounts of active caspase-8 generated at the DISC. However, this hypothesis could be very difficult to test, due to the detection limits of the assays currently available. Details of the mechanism of sequestration and inactivation of caspase-8 at mitochondria of Bcl-x<sub>L</sub>-expressing Type II cells await further investigation.
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Footnotes

3 Both authors contributed equally to this work.

4 Abbreviations: DISC, death inducing signaling complex; FADD, Fas associated death domain; c-FLIP, cellular FLICE-inhibitory protein; PARP, poly (ADP-ribose) polymerase; HM, heavy membranes.

5 Unpublished data.

6 W. Roth and J. Reed, unpublished data.
Figure legends

FIG. 1  MCF7-Fas-Bcl-xL cells tolerate large amounts of active caspase-8 without signs of 
apoptosis.  
A, MCF7-Fas cells were left untreated (-) or treated with anti-CD95 for 16 h (+). HM 
fractions of treated and untreated MCF7-Fas cells were prepared and both the cytochrome c (Cyt c) 
content of mitochondria as well as the ∆Ψm of these cells was measured.  
B, The time-course of 
cleavage of PARP and caspase-8 was examined in MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells. The 
migration positions of caspase-8/a, -8/b (CASP-8) the processed intermediates of caspase-8 
(p43/41) and the caspase-8 active subunit p18 are indicated.  
C, Cleavage of Bid and plectin in cells 
treated as described in A was examined by immunoblotting. Probing blots with antibodies to 
cytokeratin 8 (K8) served as a noncleavable loading control.

FIG. 2  Pro-caspase-8 in MCF7 cells is localized on mitochondria and is accessible to activation 
by caspase-8.  
A, Left panel, staining for caspase-8 in MCF7 cells reconstituted with pro-caspase-3 
using FITC-labeled C5 antibody specific for the caspase-8 active subunit p10 using confocal laser 
scanning microscopy.  
Center panel, MitoTracker.  
Right panel, overlay of the two stainings.  
B, 
Active caspase-8 processes mitochondria-associated pro-caspase-8 of MCF7-Fas-Bcl-xL cells. 
Equivalent specific activities of recombinant caspases were incubated with isolated mitochondria of 
MCF7-Fas-Bcl-xL cells for 1 hr at 37ºC. The amount of cleaved pro-caspase-8 was determined by 
immunoblotting using the anti-caspase-8 antibody N2, which is specific for the caspase-8 
prodomain (5) and therefore does not recognize the added exogenous recombinant caspase-8. The 
experiment is representative of four independent experiments.  
C, DISC activity assay of MCF7-
Fas-Vec and MCF7-Fas-Bcl-xL cells. Immunoprecipitation of CD95 following stimulation of cells
was performed as described in the method section. Activity of caspase-8 associated with the DISC was determined using the caspase-8 selective peptide substrate zIETD-AFC in a fluorometric assay. Data are expressed as relative fluorescence units normalized for total protein content.

FIG. 3 **Active caspase-8 subunits localize to a membrane fraction in Bcl-xL-expressing MCF7-Fas cells.** 

**A,** Caspase-8 is activated at the DISC followed by activation of mitochondrial caspase-8. Anti-CD95 immunoprecipitations (DISC), isolation of heavy membranes, and cytosolic extracts were performed on cells stimulated with anti-CD95 for the indicated times. Note that cytochrome c is detected only in the cytosolic extract of MCF7-Fas-Vec cells. Appearance of cyt c in the cytosol was always paralleled by a complete loss of cyt c from the HM fraction$^5$ (see Fig. 1A). Lysates derived from cells were pretreated with zIETD-fmk for 45 min before stimulation as indicated. The caspase-8/a and -8/b forms (CASP-8), the processed intermediates of caspase-8 (p43, p41), c-FLIP long (c-FLIPL) and its cleavage product (p43), and cytochrome c (Cyt c) are indicated. Note that the amount of caspase-8 detected in the DISC cannot directly be compared to the amount in HM fractions due to the difference in the methods used to generate these lysates/extracts (see Method section). 

**B,** Cells were treated with anti-CD95 for the indicated times, and treated with lysophosphatidylcholine at 4°C until cells were >99% permeabilized. Supernatant was separated from pellet by centrifugation. These fractions were incubated with the caspase-8-selective peptide substrate zIETD-AFC (bottom), or subjected to immunoblot analysis using antibodies recognizing caspase-8 or Tim23 (top). Shown is mean of the activity assays plus standard deviation of three independent experiments. The Western blot above represents one of these experiments.
FIG. 4 Early in vivo caspase-8 activity detected in both vector and Bcl-xL expressing transfectants. A, Flow cytometric analysis of MCF7-Fas-Vec (vector) or MCF7-Fas-Bcl-xL (Bcl-xL) cells stimulated with anti-CD95 followed by incubation with fluorogenic inhibitor substrates. Cells were stimulated for indicated times followed by addition of CaspaTag 8 before harvest. Fluorescence was detected by flow cytometry. Three independent experiments are shown. Due to the low activity of caspase-8 in the MCF7-Fas-Bcl-xL cells results from vector transfected and Bcl-xL-expressing cells are shown in different scales. Due to the low fluorescence of CaspaTag8 percent positive cells do not directly correlate to percent of the cells that have fully processed caspase-8. B, Cells were stained with anti-CD95 antibody, followed by FITC conjugated anti-mouse antibodies for indicated times, fixed, and analyzed by immunofluorescence microscopy. Isotype matched mouse antibody, against an irrelevant protein was used as a control (mIgG). Cells in the far right panels were preincubated with zIETD-fmk (+zIETD) for 45 minutes prior to stimulation. C, Cells with redistributed CD95 were counted in untreated and anti-CD95 treated (2 hrs) MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells. Shown is the mean percentage (+/- SD) of cells which have internalized the receptor.

FIG. 5 Active caspase-8 subunits in anti-CD95-treated MCF7-Fas-Bcl-xL cells colocalize with Bcl-xL on mitochondria. A, B, Apoptosis sensitive MCF7-Fas-Vec cells (A) and apoptosis resistant MCF7-Fas-Bcl-xL cells (B) were left untreated (0 hrs) or treated with anti-CD95 for 16 hrs and stained for caspase-8 (anti-caspase-8 C5, green panel). MCF7-Fas-Bcl-xL cells were additionally stained for Bcl-xL (red panels in B). The far right column represents the overlay of the two stainings. The identity of the rod shaped structures as mitochondria was confirmed by costaining with MitoTracker®. C, Immunoblot analysis of caspase-8 was performed using 10^6 cells scraped
from the same glass slides that were used to do the staining shown in A and B. D, A mitochondria containing heavy membrane fraction (HM) was separated from cytoplasm (C) from MCF7-Fas-Vec (V) or MCF7-Fas-Bcl-xL cells (B) following stimulation with anti-CD95. The amount of bound active caspase-8 was determined using a fluorometric assay with the caspase-8 selective peptide substrate zIETD-AFC. The specificity of this substrate for caspase-8 as used here has been confirmed (16). Values of unstimulated cells were subtracted from values of stimulated cells. The experiments shown are representative of four independent experiments.

FIG. 6 BAR colocalizes with Bcl-xL and caspase-8 on the outer mitochondrial membrane and binds active caspase-8 in vitro. A, Heavy membranes were isolated from MCF7-Fas-Bcl-xL cells and subjected to a trypsination assay as described in method section. The heavy membranes were subjected to SDS-PAGE and probed with the indicated antibodies. Tim23 is an integral transmembrane protein of the inner mitochondrial membrane. Its general susceptibility to trypsin was established by treating Triton X-100 detergent lysates with trypsin. Tim23 was readily degraded upon this treatment (+ TX-100). B, Heavy membrane fractions (HM) of MCF7-Fas-Bcl-xL cells were treated with 80 µg of trypsin as described in (A) and were tested for in vitro binding of 35S-labeled caspase-8 (CASP-8) (lanes 1-5) and for protein expression in an immunoblot analysis (lanes 6 and 7) using the indicated antibodies. I, input; B, bound; U, unbound. Note that the reduced amount of unbound [35S]caspase-8 in this experiment with added trypsin is due to the extreme sensitivity of caspase-8 to cleavage by trypsin resulting from residual activity even after addition of excess trypsin inhibitor. C, 35S-labeled pro-caspase-8 (CASP-8), pro-caspase-3 (CASP-3), N-CASP-8 (AA 1-216, containing the prodomain), C-CASP-8 (AA 224-479, containing the catalytic domain), or processed C-CASP-8 (caspase-8 active subunits) were used in a binding assay with
isolated heavy membranes. Note that a larger p18 subunit (p18*) can also be seen in the gels. The p18* product is derived from cleavage of CASP-8*, which represents an N-terminally extended aberrant \textit{in vitro} translated C-CASP-8 caused by an upstream start site. The identity of these fragments was confirmed by immunoprecipitation using subunit-specific anti-caspase-8 mAbs. I, input; B, bound; U, unbound. The same amount of \textit{in vitro} translated material used for the binding assay was loaded as input. D, GST-BAR-DED-L fusion protein was incubated with the indicated 35S-labeled \textit{in vitro} translated caspase-8 constructs, and recovered with GSH-agarose beads (B). Beads with GSH alone were used as a control (G). Proteins adsorbed to beads were subjected to SDS-PAGE and autoradiography. The same amount of \textit{in vitro} translated material used for the binding assay was loaded as input.

\textbf{FIG. 7} \textbf{BAR in conjunction with Bcl-x\textsubscript{L} inhibits caspase-8 activity.} \textit{A}, 293T cells were transfected with vector control or plasmids encoding BAR, Bcl-x\textsubscript{L}, or BAR and Bcl-x\textsubscript{L} and cotransfected with either a plasmid encoding a CD8-caspase-8 (which generates large amounts of active caspase-8) fusion protein or control vector. Cells were harvested and DNA fragmentation was determined as described. Shown is the mean (-/+- SD) of three experiments. \textit{B}, 293T cells were transfected with either myc-tagged wild-type BAR or myc-tagged BAR lacking the N-terminal RING domain (BAR\textDelta R). Heavy membrane (HM) and cytosolic (C) fractions were separated and subjected to SDS-PAGE and probed with anti-myc, anti-Erk and anti-Tim-23 as cytosolic or heavy membrane fractionation controls, respectively. \textit{C}, Heavy membranes of 293T cells transfected with myc-BAR\textDelta R, Bcl-x\textsubscript{L}, or both were isolated and incubated with extracts of unstimulated or stimulated MCF7-Fas vector cells. After incubation, the extracts were incubated with the caspase-8 selective peptide substrate zIETD-AFC and activity determined by fluorometry (bottom). The
percentage of depletion of caspase-8 activity is shown. Expression of the transfected proteins was controlled by immunoblotting using a myc-tag and Bcl-xL specific antibodies (top). Tim-23 is shown as a fractionation control.
Figure 1

A

| anti-CD95 | Vec | Bcl-xL |
|-----------|-----|--------|
| -         | -   | -      |
| +         | +   | +      |

Cyt c

\[ \Delta \Psi_m \text{ (% loss)} \]

B

| anti-CD95 | Vec | Bcl-xL |
|-----------|-----|--------|
| -         | 0   | 0      |
| +         | 2   | 2      |
|           | 4   | 4      |
|           | 6   | 6      |
|           | 8   | 8      |
|           | 12  | 12     |
|           | 16  | 16     |

| anti-CD95 | Vec | Bcl-xL |
|-----------|-----|--------|
| -         | 0   | 0      |
| +         | 2   | 2      |
|           | 4   | 4      |
|           | 6   | 6      |
|           | 8   | 8      |
|           | 12  | 12     |
|           | 16  | 16     |

| anti-CD95 | Vec | Bcl-xL |
|-----------|-----|--------|
| -         | 0   | 0      |
| +         | 2   | 2      |
|           | 4   | 4      |
|           | 6   | 6      |
|           | 8   | 8      |
|           | 12  | 12     |
|           | 16  | 16     |

K8

Bid

Plectin

PARP

p85

CASP-8

p43/41

p18

Vec Bcl-xL

anti-CD95

0    2    4     6   8  12  16   0    2    4     6     8  12  16  (h)
Figure 3

A

|       | Vec | DISC | Bcl-x<sub>L</sub> |
|-------|-----|------|------------------|
|       | 0'  | 5'   | 30'  | 2'  | 4'  | 16'  | 0'  | 5'   | 30'  | 2'  | 4'  | 16'  |
| CASP-8|     |      |      |     |     |      |     |      |      |     |     |      |
| p43   |     |      |      |     |     |      |     |      |      |     |     |      |
| p41   |     |      |      |     |     |      |     |      |      |     |     |      |
| c-FLIP<sub>L</sub> |     |      |      |     |     |      |     |      |      |     |     |      |
| p43   |     |      |      |     |     |      |     |      |      |     |     |      |

B

|       | Vec | Bcl-x<sub>L</sub> |
|-------|-----|------------------|
|       | 0   | 0               |
|       | 3   | 3               |
|       | 0   | 0               |
|       | 3   | 0               |

anti-CD95 (h)

|       | Vec | Bcl-x<sub>L</sub> |
|-------|-----|------------------|
|       | 0   | 3               |
|       | 0   | 3               |

Bcl-x<sub>L</sub>

CASP-8

p43/p41

cytosol

heavy membranes

Casp-8

p43/p41

Cyt c

rel. fluor.

0 1 2 3 4 5 6 7 8
Figure 4

A

Exp. #1 | Exp. #2 | Exp. #3

| vector | vector | vector |

% positive cells

| 0 | 4 | 24 | 0 | 4 | 24 | 0 | 4 | 24 |

B

mlG | 0 | 2h | 2h (+zIETD)

Vec

mlG | 0 | 2h | 2h (+zIETD)

Bcl-xL

Council-

C

% CD95 internalized

| vec | Bcl-xL |

0 | 2 |

0 | 2 |
Figure 5

A
MCF7-Fas-Vec

CASP-8

0 hrs

16 hrs

B
MCF7-Fas-Bcl-xL

CASP-8

0 hrs

Bcl-xL

0 hrs

CASP-8 + Bcl-xL

0 hrs

C

anti-CD95

- +

CASP-8:

Vec

FU (x10^{-2})

D

V B

V B

C

HM
Figure 7

A

B

C

% apoptosis

Vec
BAR
Bcl-xL
BAR + Bcl-xL

- +

CD8:CASP-8

% depletion of active caspase-8

293T-

vec BAR BARΔR HM C HM C HM C

BAR - BARΔR -

Erk -

Tim23 -
**Supplementary figure legends**

SUPPLEMENTARY FIG. 1. **Caspase-8 binds to purified mitochondria.** A, Mitochondria were purified from CEM-Bcl-xL cells using a sucrose gradient as described previously (9). 35S-labeled N-CASP-8 (AA 1-216, containing the prodomain), C-CASP-8 (AA 224-479, containing the catalytic domain), or processed C-CASP-8 (caspase-8 active subunits) were used in an *in vitro* binding assay (see Materials and Methods and legend to Fig. 6). The migration positions of the proteins are indicated. I, input; B, bound; U, unbound. B, Purified mitochondria from CEM-Vec or CEM-Bcl-xL cells were subjected to SDS-PAGE and immunoblot analysis. The migration positions of caspase-8 and Bcl-xL are indicated. Tim23 is shown as a loading control. Note, there is a increase of bound procaspase-8 to mitochondria purified from Bcl-xL expressing cells when compared to mitochondria isolated from vector transfected cells.

SUPPLEMENTARY FIG. 2. **Fluorogenic caspase substrates are selective.** MCF7-Fas or Jurkat cells were treated with an agonistic anti-CD95 antibody or with the kinase inhibitor staurosporine for the indicated periods of time. Cells were harvested, lysed in a Triton X-100 containing lysis buffer and the caspase activation profile was determined using fluorogenic peptide substrates selective for caspase-2 (zVDVAF-AFC), caspase-3+7 (zDEVD-AFC), caspase-6 (zVEID-AFC), caspase-8 (zIETD-AFC) and caspase-9 (AcLEHD-AFC).

SUPPLEMENTARY FIG. 3. **The cytoplasmic and mitochondrial pool of caspase-8 are processed to similar amounts and with similar kinetics in MCF7-Fas-Vec and MCF7-Fas-Bcl-xL cells.** Caspase-8 is activated at the DISC followed by activation of mitochondrial caspase-8.
Anti-CD95 immunoprecipitates (DISC), isolated heavy membranes, and cytosolic extracts were prepared on cells stimulated with anti-CD95 for the indicated times. Lysates derived from cells were pretreated with zIETD-fmk for 45 min before stimulation as indicated. The caspase-8/a and -8/b forms (CASP-8), the processed intermediates of caspase-8 (p43, p41), c-FLIP long (c-FLIP_L) and its cleavage product (p43), and cytochrome c (Cyt c) are indicated.
supplementary figure 1

purified Mitochondria (CEM-Bcl-x<sub>L</sub>)

|            | N-CASP-8 | C-CASP-8 | C-CASP-8 processed |
|------------|----------|----------|-------------------|
| kDa        | I        | B        | U                  |
| 67         |          |          |                   |
| 46         |          |          |                   |
| 30         |          |          |                   |
| 21         |          |          |                   |
| 14.4       |          |          |                   |

Binding assay of 35S-labeled CASP-8

CEM
vec  Bcl-x<sub>L</sub>
= CASP-8
- Bcl-x<sub>L</sub>
- Tim23

Western of mitos
Selectivity of the fluorogenic caspase substrates

**MCF7 cells**

- **anti-CD95 [h]**
  - 0
  - 2
  - 6
  - 16

**Jurkat**

- **staurosporine [h]**
  - 0
  - 6
  - 12
  - 16

- **rel. fluorescence**
  - 0
  - 5000
  - 10000
  - 15000
  - 20000
  - 25000

- **caspase-2**
- **caspase-3+7**
- **caspase-6**
- **caspase-8**
- **caspase-9**
| DISC | Vec | Bcl-x_L | zIETD |
|------|-----|---------|-------|
|      |     |         |       |
| anti-CD95 (h) | 0 | 5' | 30' | 2 | 4 | 16 |
|       | 0 | 5' | 30' | 2 | 4 | 16 |
|       | 5' | 5' |     |   |   |   |
|       | CASP-8 |     |     |   |   |   |
|       | p43 |     |     |   |   |   |
|       | p41 |     |     |   |   |   |
|       | c-FLIP \_ p43 |     |     |   |   |   |
|       |       | heavy membranes |     |   |   |   |
|       | CASP-8 |     |     |   |   |   |
|       | p43 |     |     |   |   |   |
|       | p41 |     |     |   |   |   |
|       | cyt c- |     |     |   |   |   |
|       |       | cytosol |     |   |   |   |
|       | cyt c- |     |     |   |   |   |
Inactivation of caspase-8 on mitochondria of Bcl-xL expressing MCF7-Fas cells: Role for the BAR protein
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