Loss of Caspase-9 Provides Genetic Evidence for the Type I/II Concept of CD95-mediated Apoptosis*

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The death receptor CD95 triggers apoptosis upon formation of a death-inducing signaling complex and the activation of caspase-8. Two types of CD95-mediated apoptosis have been distinguished that differ in their efficiency of death-inducing signaling complex formation and the requirement of mitochondria for caspase activation. The validity of the type I/II model, however, has been challenged, as Bcl-2 expression or the use of various CD95 agonists resulted in different apoptosis effects. By identifying a caspase-9-deficient T cell line, we now provide genetic evidence for the two-pathway model of CD95-mediated apoptosis and demonstrate that type II cells strongly depend on caspase-9. Caspase-9-deficient cells revealed strongly impaired apoptosis, caspase activation, and mitochondrial membrane depolarization upon CD95 triggering, whereas, surprisingly, activation of Bak and cytochrome c release were not inhibited. Furthermore, caspase-9-deficient cells did not switch to necrosis, and reconstitution of caspase-9 expression restored CD95 sensitivity. Finally, we also show that different death receptors have a distinct requirement for caspase-9.

CD95 (APO-1/Fas) is the prototype member of the death receptor family (1). Stimulation of CD95 with its cognate ligand or agonistic antibodies results in receptor oligomerization and recruitment of signaling proteins in a death-inducing signaling complex (DISC) (2, 3). Essential for apoptosis signaling through death receptors are the DISC components Fas-associated death domain and caspase-8. Recruitment of caspase-8 into the DISC leads to its dimerization-induced activation and the autoproteolytic release of its active subunits into the cytosol. The active caspase-8 activates downstream caspases, such as caspase-3, -6 and -7, that are responsible for most of the morphological manifestations of cell death (1). This death receptor-mediated pathway is also called the extrinsic pathway and is distinguished from the intrinsic mitochondrial death pathway. Apoptosis induction via mitochondria is regulated by the Bcl-2 family and involves early loss of mitochondrial membrane potential (ΔΨM), release of cytochrome c, and other apoptogenic factors (4). In the cytosol, cytochrome c binds to Apaf-1 leading to caspase-9 recruitment. At this apoptosome complex, caspase-9 is activated and initiates the caspase cascade and subsequent apoptosis.

An involvement of mitochondria has also been demonstrated in CD95 signaling, suggesting a functional role of this organelle in death receptor-mediated apoptosis of certain cell types (5, 6). Two types of CD95 signaling pathways have been proposed (7). CD95 type I cells show efficient DISC formation and strong activation of caspase-8 at the receptor level, which directly triggers the caspase cascade. Type II cells, in contrast, form a weak DISC and produce very little active caspase-8 at the receptor level, which is insufficient to initiate the apoptotic process. The low amounts of active caspase-8 can cleave Bid, a proapoptotic Bcl-2 protein that translocates to mitochondria and induces apoptosome formation (5, 6). Thus, type II cells strongly rely on the mitochondrial amplification loop. The two-pathway model is mostly based on the criterion that expression of antiapoptotic Bcl-2 proteins inhibits apoptosis in type II but not type I cells (3, 7, 8). However, overexpression of Bcl-2 or Bcl-xL only delays or attenuates (but does mostly not abolish) CD95-mediated apoptosis, making an unambiguous classification of CD95 signaling difficult. Moreover, differences in the effects of CD95-stimulating agents, such as agonistic antibodies and recombinant CD95L, have challenged the existence of the two cell types and validity of the type I/II model (9, 10).

We report here a direct genetic proof of the type I/II model using a T cell line with caspase-9 deficiency. Upon CD95 stimulation, caspase-9-deficient cells revealed highly impaired activation of effector caspase-3 and DNA fragmentation, which was restored by reconstitution of caspase-9 expression. Loss of caspase-9 did also not lead to a switch of cell death toward necrosis. Interestingly, caspase-9-deficient cells showed no loss of the mitochondrial transmembrane potential (ΔΨM), although cytochrome c was released. Thus, these findings suggest that both events can be uncoupled and that caspase-9 is required for loss of ΔΨM but not for cytochrome c release. Finally, we demonstrate that different death receptors differentially depend on a caspase-9-triggered mitochondrial amplification loop.
EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, penicillin, and streptomycin (PAA Laboratories, Linz, Austria). The following antibodies were used: anti-CD95 (2R2) and anti-caspase-8 (12F5; both from BioCheck, Münster, Germany), anti-caspase-3 (catalog number AF-605-NA; R&D Systems, Wiesbaden, Germany), anti-caspase-9 (catalog number 9502; Cell Signaling Technology, Danvers, MA), anti-cytochrome c (7H8.2C12 and 6H2.B4) and anti-Bcl-x (catalog number 610212; BD Biosciences), conformation-specific anti-Bak (Ab-2; Oncogene), anti-Bcl-2 (C2; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (AC-74) and anti-tubulin (DM1A; both from Sigma). For detection of murine proteins the following antibodies were used: anti-Bid (catalog number AF860; R&D Systems), anti-caspase-3 (clone 46; BD Biosciences), anti-caspase-8 (1G12; Alexis, Lausen, Switzerland), and anti-caspase-9 (catalog number 9504) and anti-Erk (catalog number 9102; both from Cell Signaling Technology). The horseradish peroxidase-conjugated goat antimouse IgG, IgG2a, and IgG2b were from Southern Biotechnology Associates (Birmingham, AL), the horseradish peroxidase-conjugated goat anti-rabbit IgG was from Santa Cruz Biotechnology. Recombinant human and mouse TRAIL were purchased from R&D Systems (TEC-375) and Alexis (ALX-201-130), respectively. The human cytokines TNF and human and mouse TRAIL were a kind gift from R. U. Jänicke and H. Wajant, respectively. The caspase inhibitor N-(2-quinoilyl)valeryl-aspartyl-(2,6-difluoro-phenoxy)methyl ketone (Q-VD-OPh) was purchased from MP Biomedicals (Irvine, CA).

Fluorescence-activated Cell Sorter Assays—Surface stainings, DNA fragmentation, and ΔΨm assays were performed as described (11). Flow cytometric analysis of cytochrome c release was carried out as described previously (12). Uptake of propidium iodide (2 μg/ml) or 7-aminoactinomycin D (7AAD) (1 μg/ml) into nonfixed cells was evaluated by flow cytometric analyses of the FL2 or FL3 profile, respectively (13). For flow cytometric analysis of Bak conformational change, cells were fixed in phosphate-buffered saline, 0.5% paraformaldehyde on ice for 30 min and subsequently washed three times in phosphate-buffered saline, 1% fetal calf serum. Staining with conformation-specific antibody against Bak and isotype matched control antibody was performed with a 1:50 dilution of the respective antibody in 50 μl of staining buffer (phosphate-buffered saline, 1% fetal calf serum, 50 μg/ml digitonin). Then, cells were washed and resuspended in 50 μl of staining buffer containing 0.1 μg of Alexafluor 488-labeled chicken anti-mouse for 30 min in the dark. Cells were washed again and analyzed on a FACSCalibur machine (BD Biosciences).

Immunoprecipitation, Western Blot Analysis, and Caspase Activity Assays—Immunoprecipitation of the CD95 DISC and Western blot analyses were performed as described (11). Caspase-3-like activity was measured in triplicates using Asp-Glu-Val-Asp-aminomethylcoumarin (Biomol, Hamburg, Germany) as a substrate (14).

Transfection and Reverse Transcriptase-PCR Analysis—Caspase-9-deficient Jurkat cells were electroporated at 250 V and 950 μF with an N-terminally FLAG-tagged pro-caspase-9 construct and selected for hygromycin resistance. RNA was isolated and PCR amplified for caspase-9 and glyceraldehyde-3-phosphate dehydrogenase using standard protocols. The 3′ and 5′ primers used for amplification were 5′-ATG GAC GAA GCG GAT CGG and 5′-CCC TGG CCT TAT GAT GTT-3′ for caspase-9 and 5′-GTG GAA GGA GGA CTC ATG ACC ACA G-3′ and 5′-CTG GTG CTC AGT GTA GCC CAG-3′ for glyceraldehyde-3-phosphate dehydrogenase.

siRNA Transfection—siRNAs targeting CASP9 and control siRNAs directed against luciferase were obtained from Dharmacon RNA Technologies, Inc. (Lafayette, CO). Transfection into HT1080 and SKW6.4 cells was performed with Dharmafect 4 according to manufacturer’s procedures (Dharmacon). At 48 h after transfection, cells were stimulated with CD95L or left untreated for 16 h. Subsequently, cell death was assessed by staining with 7AAD and flow cytometry. Specific cell death was calculated as follows: specific apoptosis % = (experimental apoptosis – spontaneous apoptosis)/(100 – spontaneous apoptosis) × 100.

RESULTS

We screened different Jurkat subclones for their sensitivity toward CD95-induced cell death. To this end, Jurkat cells were incubated with different concentrations of anti-CD95 antibody for 18 h, and apoptosis was measured by quantification of sub-diploid DNA in a flow cytometer. To ensure efficient cross-linking of the CD95 death receptor, we added 10 ng/ml protein A to the samples. We observed that one Jurkat clone, designated JMR, exhibited a profound resistance toward CD95-mediated apoptosis when compared with J16 or other Jurkat clones (Fig. 1A). At a concentration of 100 ng/ml of anti-CD95, J16 cells showed 41.8% ± 7.3% apoptosis compared with 11.6% ± 3.6% in JMR cells. When very high concentrations of antibody were used, i.e. 1 μg/ml anti-CD95, a certain number of JMR cells showed DNA fragmentation (25.8% ± 0.8%). However, the extent of apoptosis was still considerably less than in J16 cells (72.0% ± 1.5%).

To ensure that resistance of JMR cells was not simply caused by down-regulation of CD95, we analyzed CD95 cell surface expression by flow cytometry. As shown in Fig. 1B, both Jurkat clones had comparable levels of CD95 on the cell surface, suggesting a defect in the intracellular signaling cascade. Importantly and in line with this observation, JMR cells showed no expression of caspase-9 protein or mRNA as analyzed by Western blotting and reverse transcriptase-PCR analysis, whereas protein expression of Bcl-2 and Bcl-xL was similar in J16 and JMR cells (Fig. 1C). Expression levels of other caspases, Bcl-2 family proteins, and inhibitor of apoptosis proteins were comparable between the two Jurkat clones (data not shown).

CD95 is able to induce necrotic cell death under certain circumstances (15, 16). To test whether JMR cells might switch to necrosis upon CD95 stimulation, we analyzed cell death by the propidium iodide dye exclusion assay. When J16 cells were stimulated with anti-CD95, ~50% of the cells became propidium iodide-positive within 12 h, which further increased up to 80% after 36 and 48 h of CD95 triggering (Fig. 1D). In contrast, JMR cells showed only a basal cell death rate of 10–20% at

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the time points analyzed. Strikingly, unlike overexpression of Bcl-2 that conferred only a transient protection, caspase-9 deficiency of JMR cells provided a substantial and sustained resistance against anti-CD95 (Fig. 1D). We made similar findings when J16 and JMR cells were stimulated for 20 h with anti-CD95 and subsequently analyzed by light microscopy.

Although most of the J16 cells showed an apoptotic appearance upon CD95 stimulation, JMR cells remained morphologically normal (Fig. 1E). Thus, JMR cells show highly impaired apoptosis upon CD95 triggering and do not switch to necrotic cell death.

To address whether caspase-9 deficiency affected activation of other caspases, we treated J16 and JMR cells with recombinant cross-linked CD95L for various time points. As assessed by Western blot analysis, the proteolytic processing of caspase-8 was significantly delayed in caspase-9-deficient JMR cells (Fig. 2A). Although the active p18 fragment of caspase-8 was detected in J16 cells already after 1 h of CD95 stimulation, it appeared in JMR cells only after 4 h. Similarly, caspase-3 activation was evident in J16 cells at 2 h and paralleled that of caspase-9. In contrast, caspase-3 processing was hardly detectable at the 8-h time point in the caspase-9 deficient Jurkat clone. Because caspase-8 processing in the cytosol might not necessarily reflect its gain of proteolytic activity (14), we further investigated its recruitment to CD95 by performing DISC analyses. Both in J16 and JMR cells, Fas-associated death domain and caspase-8 were recruited to the DISC at similar levels (Fig. 2B). Thus, DISC formation is not affected by the loss of caspase-9. However, caspase-9 activity is required for efficient activation of the effector caspase-3 during CD95-mediated apoptosis in Jurkat cells.

To analyze whether or not caspase-9 deficiency affects the CD95 pathway only in Jurkat cells, we employed an RNA interference approach in type I and type II cells. HT1080 (type II) and SKW6.4 (type I) cells were transfected with caspase-9-specific siRNAs or control siRNAs. 48 h after transfection, caspase-9 expression was significantly reduced in both cell lines transfected with caspase-9-specific siRNAs compared with control siRNA-transfected cells (Fig. 3A). When HT1080 type II cells were treated for additional 16 h with CD95L, knockdown of caspase-9 resulted in reduced CD95-mediated apoptosis (Fig. 3B). In SKW6.4 type I cells, however, no difference in
apoptosis induction was observed between control and caspase-9 siRNA-treated cells (Fig. 3B), even though the knockdown of caspase-9 was more complete in these cells than in HT1080 cells (Fig. 3A). Thus, caspase-9 expression is required for efficient induction via CD95 in type II (HT1080) but not in type I (SKW6.4) cells.

To prove that the changes detected in JMR cells were specifically caused by the absence of caspase-9, we stably transfected JMR cells with caspase-9. Several clones were selected that expressed procaspase-9 at levels comparable with J16 cells (Fig. 4A). These clones showed comparable sensitivity toward anti-CD95 and CD95L treatment as J16 cells suggesting that caspase-9 deficiency is the only defect within the CD95 pathway in JMR cells (Fig. 4B). Furthermore, as shown for two representative clones (Fig. 4C), CD95 expression was not altered in the transfected cells. One of these clones, termed F9, was chosen for further experiments. JMR and F9 cells were treated with recombinant CD95L for various time points, and caspase processing was analyzed by Western blotting. Fig. 4D shows that the caspase-9 transfected F9 cells processed caspase-8 and -9 as well as caspase-3 after 2 h of stimulation with CD95L. At this time point J16 cells revealed similar activation of caspase-3, whereas in the caspase-9 deficient JMR cells no caspase-3 processing was observed (see Fig. 2A).

To further analyze the effect of caspase-9 reconstitution, we
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First, we measured the conformational status of Bak, a gatekeeper of mitochondrial permeabilization (18), using a conformation-specific antibody and flow cytometry. Interestingly, within 2 h of CD95 stimulation Bak was activated and underwent a conformational change in J16, JMR, and F9 cells (Fig. 6A). In line, Bid cleavage upon CD95 activation, as assessed by Western blotting, was comparable in the three cell lines (data not shown). Flow cytometric measurement of cytochrome c release further revealed that within 2 h of anti-CD95 treatment apoptogenic factors were released from the mitochondria of JMR, J16, and F9 cells (Fig. 6B). Surprisingly, however, loss of $\Delta \Psi_\text{M}$ was only observed in J16 and F9 cells but not in caspase-9-deficient JMR cells (Fig. 6C). These data, therefore, show that caspase-9 deficiency can uncouple cytochrome c release and loss of $\Delta \Psi_\text{M}$ as two separate events in apoptosis.

Next, we investigated whether only CD95-mediated apoptosis or also TNFα- and TRAIL-mediated cell death depends on caspase-9 in Jurkat cells. To this end, J16, JMR, and F9 cells were incubated with different concentrations of CD95L, TNFα/cycloheximide, or TRAIL for 18 h and, subsequently, were analyzed for cell death at the level of DNA fragmentation and membrane permeability. As expected, JMR cells were resistant to CD95L, whereas J16 and F9 cells readily underwent apoptosis (Fig. 7, A and B). Thus, the resistance of JMR cells is observed against agonistic antibodies as well as against recombinant ligand. Similarly, JMR cells were protected against TRAIL-induced apoptosis (Fig. 7, C and D). Interestingly, J16 and F9 cells showed slightly different sensitivities depending on the readout used. Nevertheless, both cell lines were more susceptible toward TRAIL-induced cell death when compared with JMR cells (Fig. 7, C and D). In contrast to CD95L and TRAIL, caspase-9-deficient and -proficient cells died to a similar extent when TNFα/cycloheximide was used as a death stimulus (Fig. 7, E and F). Thus, death receptor-induced apoptosis differentially depended on caspase-9 in a given cell type.

To investigate whether absence of caspase-9 affects death receptor signaling also in non-transformed cells, we analyzed wild-type and caspase-9-deficient mouse embryonic fibroblasts (MEFs) for their sensitivity toward CD95-mediated apoptosis. Caspase-9 deficiency has been shown not to affect CD95 sensitivity in thymocytes or activated splenocytes (19, 20), but no experiments were performed in type II cells such as MEFs. Therefore, we treated wild-type and caspase-9-deficient MEFs with varying concentrations of CD95L or TNFα. Although wild-type MEFs died in a dose-dependent manner upon stimulation of CD95, cell death in caspase-9-deficient MEFs was substantially reduced (Fig. 8A). In contrast, both cell types died readily upon addition of TNFα (Fig. 8B). Thus, similar as in the Jurkat model, caspase-9 deficiency affects CD95L- but not TNFα-induced apoptosis.

We investigated also the effect of caspase inhibitors. Upon treatment with CD95L for 24 h, cell death was only marginally induced in caspase-9-deficient MEFs, whereas about 50% of wild-type cells died (Fig. 8C). Addition of the pan-caspase inhibitor Q-VD-OPh reduced cell death to background levels in caspase-9-deficient MEFs. Though also strongly impaired, there was still some cell death detectable in wild-type cells in the presence of the caspase inhibitor. Finally, we analyzed caspase and Bid processing in CD95L-treated wild-type and caspase-9-deficient MEFs by Western blotting (Fig. 8D). The caspase-8 zymogen was cleaved in both cell types, although less processing was observed in caspase-9-deficient MEFs. As expected, procaspase-9 was processed in wild-type cells, whereas expression in the knock-out MEFs was absent. Bid and caspase-3 were rapidly cleaved in wild-type cells. However, caspase-9-deficient MEFs showed hardly detectable amounts of the fully processed fragments of Bid (p15) and caspase-3

FIGURE 5. Reconstitution of caspase-9 expression restores CD95 sensitivity. Wild-type J16, JMR, and caspase-9-reconstituted JMR Jurkat cells (clone F9) were treated with 500 ng/ml of anti-CD95 for 8 h and then analyzed in triplicates for (A) DNA fragmentation, (B) propidium iodide uptake, and (C) caspase-3-like activity using Asp-Glu-Val-Asp-aminomethylcoumarin cleavage activities (Fig. 5B) and showed comparable Asp-Glu-Val-Asp-aminomethylcoumarin cleavage activities (Fig. 5C) as J16 cells, whereas JMR cells remained negative in both assays. Thus, reconstitution of caspase-9 expression in JMR cells restored sensitivity toward CD95-mediated apoptosis.

treated J16, JMR, and F9 cells with anti-CD95 for 24 h and analyzed several apoptotic events. As measured by DNA fragmentation, F9 cells showed similar levels of apoptosis as J16 cells, whereas the parental JMR cells were almost resistant toward anti-CD95 treatment (Fig. 5A). Caspase-9-reconstituted F9 cells became also positive for propidium iodide (Fig. 5B) and showed comparable Asp-Glu-Val-Asp-aminomethylcoumarin cleavage activities (Fig. 5C) as J16 cells, whereas JMR cells remained negative in both assays. Thus, reconstitution of caspase-9 expression in JMR cells restored sensitivity toward CD95-mediated apoptosis.

Reproduction of proapoptotic factors from mitochondria, such as cytochrome c, is thought to be associated with loss of $\Delta \Psi_\text{M}$ (17). However, the exact connection between these two events is unclear and discussed controversially. Therefore, we wanted to investigate apoptotic events at the mitochondria in more detail. First, we measured the conformational status of Bak, a gatekeeper of mitochondrial permeabilization (18), using a conformation-specific antibody and flow cytometry. Interestingly, within 2 h of CD95 stimulation Bak was activated and underwent a conformational change in J16, JMR, and F9 cells (Fig. 6A). In line, Bid cleavage upon CD95 activation, as assessed by Western blotting, was comparable in the three cell lines (data not shown). Flow cytometric measurement of cytochrome c release further revealed that within 2 h of anti-CD95 treatment
Therefore, also in non-transformed type II cells the caspase-9-mediated amplification loop seems to be required for full caspase activation and efficient cell death.

**DISCUSSION**

In this study, we employed a caspase-9-deficient tumor cell line to delineate the contribution of the mitochondrial pathway in death receptor-mediated apoptosis. By using caspase-9-deficient Jurkat cells and caspase-9-specific siRNAs we provide direct genetic evidence that type II cells depend on the mitochondrial amplification loop to rapidly execute CD95-mediated apoptosis. In contrast, caspase-9 is dispensable in type I cells like SKW6.4. Cells from caspase-9-deficient mice have been shown to have various defects (19, 20). However, these earlier studies analyzed only type I cells, such as thymocytes and activated splenocytes, but not type II cells from caspase-9 deficient mice for their CD95 sensitivity. Thus, the two pathway model of apoptosis has never been directly tested in a situation when components of the apoptosome are missing. Here, we show that mouse embryonic fibroblasts from caspase-9-deficient mice are more resistant toward CD95-mediated apoptosis than their wild-type counter parts. Furthermore, in cellular systems mainly the overexpression of Bcl-2 has been employed to study the impact of mitochondria, which, however, has provided controversial results. This is presumably caused by the fact that despite high overexpression, Bcl-2 only delays or attenuates but cannot completely block CD95-mediated apoptosis. In line, also against TRAIL-mediated apoptosis Bcl-2 protects only transiently or at low doses of the death ligand (21). On the other hand, cells deficient for Bid or Apaf-1 show highly reduced sensitivity toward CD95-mediated apoptosis (22, 23). In the current study, we addressed the role of the mitochondrial pathway in a loss-of-function approach. Our finding that Jurkat cells depend on caspase-9 for efficient killing via CD95 provides genetic evidence for the validity of the two pathway model in CD95-mediated apoptosis.

It has been argued that differences between type I and type II cells are only evident when using agonistic antibodies but not CD95L (9, 10), suggesting that these two cell types might not exist at a physiological level. In contrast, we report here that CD95-mediated apoptosis in type II cells is dependent on mitochondria also when recombinant CD95L is used.

We have shown previously that type I and type II cells reveal differences also in DISC formation upon stimulation with CD95L (3). Moreover, other data indicate that this distinction might be also valid in primary cell types. For instance, thymocytes and long term activated peripheral T cells behave as type I cells, whereas fibroblasts and hepatocytes resemble type II cells (5). It was also shown that short term activated primary T cells are type II cells, which switch to a type I phenotype upon prolonged activation (24).

In DISC analyses we found that the initial recruitment of caspase-8 to CD95 was not affected by the presence or absence of caspase-9. However, when the processing of caspase-8 was analyzed in cell lysates, caspase-8 cleavage was strongly impaired in caspase-9-deficient cells. This suggests that processing of total cellular caspase-8 does presumably not correlate with the receptor-mediated activation of caspase-8 and its gain of proteolytic activity. Rather it might be argued that the detection of caspase-8 processing in cell lysates merely reflects a substrate cleavage event, resulting in a processed but inactive caspase-8 monomer.

**FIGURE 6.** Intact mitochondrial transmembrane potential in the presence of active Bak and cytochrome c release. J16, caspase-9-deficient JMR, and caspase-9 reconstituted F9 cells were stimulated with 1 μg/ml anti-CD95 and 10 ng/ml protein A (gray line) or left untreated (bold black line). After 2 h cells were stained by flow cytometry for Bak conformational change (A) or cytochrome c release (B). Mitochondrial transmembrane potential (ΔΨm) was analyzed by JC-1 staining and flow cytometry after 24 h (C).
During mitochondrial dysfunction, a number of apoptogenic factors are released from mitochondria including cytochrome c, apoptosis-inducing factor, HtrA2/Omi, and Smac/Diablo (4). Cytochrome c release leads to formation of the apoptosome and activation of caspase-9, whereas Smac/Diablo releases caspases from inhibition by inhibitor of apoptosis proteins. Although it was shown that CD95-mediated apoptosis in Jurkat cells could be inhibited by overexpression of Bcl-2 or Bcl-xL, it remains unclear whether all apoptogenic factors have to be released from mitochondria or whether one single pathway is sufficient. Our results show that CD95L- and TRAIL-mediated apoptosis are highly dependent on caspase-9, suggesting that at least in the cell types analyzed here apoptogenic factors from the mitochondrial intramembrane space other than cytochrome c only play a minor role in cell death induced by these death ligands. Only at very high concentrations of CD95L some apoptosis could be observed in JMR cells suggesting that at high doses of the triggering death ligand sufficient amounts of caspases are activated independently of the mitochondrial amplification loop. Interestingly, caspase-9 deficiency did not interfere with TNFα-induced apoptosis, suggesting that distinct signaling components might be important in this pathway. The current model for TNFα-induced cell death rather suggests that inhibition of NF-κB and activation of JNK are required for cell death to occur. NF-κB promotes survival by inducing a number of anti-apoptotic genes including c-FLIP (25) and by inhibiting prolonged JNK activation (26, 27). In the absence of NF-κB activation, JNK signaling prevails and leads to degradation of c-FLIP via the ubiquitin ligase Itch, resulting in caspase activation at the receptor level (28). Thus, cell death by TNFα might be initiated independently of mitochondria. This is in line with a recent report showing that TNFα-mediated hepatocyte apoptosis and liver failure is not prevented by a Bcl-2 transgene in RelA-deficient mice (29). It is also conceivable that a given cell type could be of type I for one death receptor yet type II for another. Interestingly, a caspase-9 inhibitor could impair TRAIL-mediated apoptosis in some cells lines, whereas others were not affected (30). One might also imagine that the dependence of caspase-9 is controlled by different receptor internalization or formation of distinct receptor signaling complexes, as it was proposed for TNFα- and recently also for TRAIL-mediated apoptosis (31–33).

Our data also show that cytochrome c release and loss of ΔΨM, which are often thought to occur in parallel, can be separated as two independent events. We observed that loss of ΔΨM occurred only in the presence of active caspases and not in JMR cells in which caspase activation is highly impaired upon death receptor triggering. This caspase requirement for the loss of ΔΨM is consistent with findings that cytochrome c release is upstream of caspase activation and independent of mitochondrial depolarization (34). Interestingly, cytochrome c is able to maintain ΔΨM after its release into the cytosol (35), which may explain why JMR cells survive prolonged stimulation of the CD95 death receptor. Moreover, loss of ΔΨM might be caused by the cleavage of proteins within complex I of the respiratory chain (36, 37). Although all of these studies were done in cell-free systems or permeabilized cells, our study extends these findings to intact cells and shows for the first time that loss of ΔΨM can be uncoupled from cytochrome c release during death receptor-mediated apoptosis. The

FIGURE 7. Death receptors differentially depend on caspase-9. J16, JMR, and F9 cells were treated with indicated concentrations of cross-linked FLAG-CD95L (A and B), TRAIL (C and D), or TNFα and 1 μg/ml cycloheximide (E and F). Apoptosis was assessed 18 h after the addition of the respective death ligand by measuring DNA fragmentation (A, C, and E) or 7AAD dye exclusion (B, D, and F).
caspase-9-deficient Jurkat line JMR will prove a very useful tool for further analysis of cell death mechanisms in the absence of apoptosis

function.

Acknowledgments—We thank C. Meyer and D. Scholtyssek for expert technical assistance and Drs. R. U. Jänicke, G. Salvesen, A. Strasser, and H. Wajant for reagents.

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Caspase-9 Dependence of CD95 Type II Cells

FIGURE 8. Caspase-9 is required for CD95-mediated apoptosis in MEFs. A and B, wild-type and caspase-9-deficient MEFs were treated for 24 h with the indicated concentrations of CD95L (A) or TNFα (B) before cell death was analyzed by 7AAD staining. C, wild-type and caspase-9-deficient MEFs were treated for 24 h with 4 ng/ml CD95L in the presence or absence of 20 μM of the caspase inhibitor Q-VD-OPh (QVD) or a DMSO (DMSO) vehicle control. Subsequently, cell death was analyzed by the 7AAD assay. The results in A–C show the mean ± S.D. of a representative experiment of three independent experiments performed in duplicate. D, wild-type and caspase-9-deficient MEFs were treated with 20 ng/ml CD95L. After the indicated times, cellular lysates were prepared and analyzed by Western blotting with antibodies specific for caspase-3, -8, -9, and Bid. Staining for Erk served as a loading control.