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Two CD95 (APO-1/Fas) signaling pathways

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We have identified two cell types, each using almost exclusively one of two different CD95 (APO-1/Fas) signaling pathways. In type I cells, caspase-8 was activated within seconds and caspase-3 within 30 min of receptor engagement, whereas in type II cells cleavage of both caspases was delayed for ~60 min. However, both type I and type II cells showed similar kinetics of CD95-mediated apoptosis and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Upon CD95 triggering, all mitochondrial apoptogenic activities were blocked by Bcl-2 or Bcl-x_L overexpression in both cell types. However, in type II but not type I cells, overexpression of Bcl-2 or Bcl-x_L blocked caspase-8 and caspase-3 activation as well as apoptosis. In type I cells, induction of apoptosis was accompanied by activation of large amounts of caspase-8 by the death-inducing signaling complex (DISC), whereas in type II cells DISC formation was strongly reduced and activation of caspase-8 and caspase-3 occurred following the loss of $\Delta\Psi_m$. Overexpression of caspase-3 in the caspase-3-negative cell line MCF7-Fas, normally resistant to CD95-mediated apoptosis by overexpression of Bcl-x_L, converted these cells into true type I cells in which apoptosis was no longer inhibited by Bcl-x_L. In summary, in the presence of caspase-3 the amount of active caspase-8 generated at the DISC determines whether a mitochondria-independent apoptosis pathway is used (type I cells) or not (type II cells).

Keywords: apoptosis/Bcl-2/caspases/cytochrome *c*/mitochondria

Introduction

Recently, a new subfamily of the tumor necrosis factor receptor superfamily, the death receptors, has emerged (Peter *et al.*, 1998). Death receptors such as TNF-R1, DR3 (APO-3/TRAMP/Wsl-1/LARD), DR4 (TRAIL-R1), DR5 (TRAIL-R2) and CD95 (APO-1/Fas) are characterized by the presence of a death domain (DD) within the cytoplasmic region and have been shown to trigger apoptosis upon binding of their cognate ligands or specific agonistic antibodies. The receptor-proximal events have been best characterized for CD95. Stimulation of CD95 results in aggregation of its intracellular death domains, leading to the recruitment of two key signaling proteins that together with the receptor form the death-inducing signaling complex (DISC) (Kischkel *et al.*, 1995). FADD (MORT-1) (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995, 1996a) couples through its C-terminal DD to cross-linked CD95 receptors and recruits caspase-8 (FLICE/MACHα1/Mch5) (Boldin *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996; Muzio *et al.*, 1996) through its N-terminal death effector domain (DED) to the DISC.

Caspase-8 is part of a growing family of cysteine proteases that were shown to be involved in many forms of apoptosis. All caspases are synthesized as inactive proenzymes that have to be activated by proteolytic cleavage after specific aspartate residues (Nicholson and Thornberry, 1997). Recently, we have shown that caspase-8 can be activated by association with the CD95 DISC, leading to the release of the active subunits p18 and p10 into the cytosol (Medema *et al.*, 1997). Downstream of the DISC, the CD95 signaling pathway has been shown to involve activation of additional caspases such as caspase-1 (Enari *et al.*, 1995; Los *et al.*, 1995), caspase-3 (Enari *et al.*, 1996; Hasegawa *et al.*, 1996; Schlegel *et al.*, 1996), caspase-4 (Kamada *et al.*, 1997), caspase-6 (Takahashi *et al.*, 1997) and caspase-7 (Fernandes-Alnemri *et al.*, 1995). Caspase-3, which is functionally similar to the *Caenorhabditis elegans* cell-death protein CED-3 (Xue *et al.*, 1996), appears to play an important role in the effector pathway in apoptosis.

Recently, involvement of mitochondria in apoptotic processes has been demonstrated (Shimizu *et al.*, 1996a; Kroemer *et al.*, 1997). This was also shown for CD95-mediated apoptosis. Early after induction of apoptosis, a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) can be measured together with the formation of permeability transition (PT) pores. At the same time, cytochrome *c* is inactivated (Krippner *et al.*, 1996; Adachi *et al.*, 1997) and then released into the cytoplasm where it results in activation of caspase-3, presumably with the help of Apaf-1, a homolog of the *C.elegans* cell-death protein CED-4 (Zou *et al.*, 1997) following activation of Apaf-3/caspase-9 (Li *et al.*, 1997a). All mitochondrial activities in apoptosis can be blocked by overexpression of Bcl-2 or Bcl-x_L, which are homologous to the anti-apoptotic *C.elegans* protein CED-9 (Vaux *et al.*, 1992; Shimizu *et al.*, 1996a; Kluck *et al.*, 1997a; Vander Heiden *et al.*, 1997; Yang *et al.*, 1997a). As in *C.elegans*, the molecular order of the cell death proteins seems to be Bcl-2 acting upstream of Apaf-1 and Apaf-1 acting upstream of caspase-3. Although in most cells caspase-3 is activated

during CD95-mediated apoptosis, the ability of Bcl-2 or Bcl-x_L to inhibit this kind of apoptosis has been controversially discussed (reviewed in Peter *et al.*, 1998).

We now have identified two different cell types that use distinct CD95 apoptosis signaling pathways. Type I cells require activation of caspase-8 at the DISC closely followed by activation of caspase-3. Blocking mitochondrial function by heterologous expression of Bcl-2 has no effect on caspase-8 or caspase-3 cleavage, or on the CD95 sensitivity of these cells. In type II cells, caspase-8 and caspase-3 are primarily activated downstream of mitochondria, and their activation and apoptosis is blocked by overexpression of Bcl-2 or Bcl-x_L. In these cells, DISC formation is strongly reduced despite normal expression levels of the DISC components CD95, FADD and caspase-8. Furthermore, in MCF7-Fas cells DISC formation and caspase-8 activation occur as in type I cells (Medema *et al.*, 1998; Srinivasan *et al.*, 1998). However, apoptosis is blocked by Bcl-x_L overexpression as in type II cells. This mixed phenotype is probably due to a caspase-3 deficiency since heterologous expression of caspase-3 in MCF7 cells overcomes the ability of Bcl-x_L to block apoptosis, converting the cells into true type I cells.

Results

Type I and type II cells differ in their kinetics of caspase-8 and caspase-3 activation

We have recently shown that in many cell lines the first detectable event during CD95-mediated apoptosis is the appearance of caspase-8 cleavage products that could be detected as early as 5 s after receptor cross-linking (Medema *et al.*, 1997; Scaffidi *et al.*, 1997). Activation of caspase-8 in these cells only occurred at the DISC level (Medema *et al.*, 1997). This mechanism of caspase-8 activation is reflected in the early appearance of caspase-8 active subunits in the cytoplasm (Figure 1A, lanes 1–12). The early caspase-8 activation (shown after 10 min stimulation with anti-APO-1) was followed by activation of caspase-3 (Figure 1B, lanes 1–12). In the B lymphoblastoid cell line SKW6.4, caspase-3 cleavage was first detectable after 10 min of stimulation (Figure 1B, lanes 1–6). The active subunit p17 was first detected after 30 min and began to degrade further at 60 min. The T lymphoma cell line H9 was somewhat slower in cleaving both caspases (Figure 1A and B, lanes 7–12).

We have now identified cells in which caspase-8 and caspase-3 cleavage was significantly delayed despite high CD95 expression. Cells with a rapid cleavage of caspase-8, such as SKW6.4 and H9, we designated type I cells and those with the delayed cleavage (Jurkat and CEM) we called type II cells. In these cells, the first cleavage products of caspase-8 and caspase-3 did not appear before 60 min of stimulation (Figure 1A and B, lanes 13–24). As in type I cells, cleavage of caspase-8 in type II cells preceded cleavage of caspase-3 (Figure 1A and B, lanes 13–24), which was also delayed for ~60 min. In general, all caspase-3-like protease activation was delayed for at least 30 min in type II cells, as seen by the cleavage of the typical caspase-3 substrate poly (ADP-ribose) polymerase (PARP) (Figure 1C).

Looking closely at the way caspase-8 and caspase-3 were cleaved in the two cell types, more specific differences can be seen. In type I cells, caspase-8 cleavage occurred through the active subunit p18 which was then slowly processed to a smaller p16 fragment (Figure 1A, lanes 1–12). In both type II cells tested the smaller p16 subunit was formed directly but in a delayed manner, without any detectable p18 formation (Figure 1A, lanes 13–24). Moreover, when comparing type I and type II cells, differences can be seen with respect to the way the active p17 subunit of caspase-3 is generated. In both type I cells, initial cleavage of caspase-3 generated a p20 subunit that has been reported to represent the p17 subunit plus the short caspase-3 prodomain (Fernandes-Alnemri *et al.*, 1996), suggesting that the first cleavage occurred between the two active subunits p17 and p10. Such an activation is consistent with the activation by another caspase (Fernandes-Alnemri *et al.*, 1996). The p20 fragment was then slowly converted into the active p17 subunit in agreement with the general activation scheme for caspases (Nicholson and Thornberry, 1997) (Figure 1B, lanes 1–12). Type II cells differed in that caspase-3 activation proceeded directly to formation of the active subunit p17. The p20 intermediate could not be detected (Figure 1B, lanes 13–24). The data suggest that type I and II cells differ in the mechanism of activation of both caspase-8 and caspase-3, indicating that they may use different apoptosis signaling pathways.

Type I and type II cells are equally sensitive to CD95-mediated apoptosis

Assuming that delayed caspase activation would influence CD95 apoptosis sensitivity, the four cell lines were tested for both dose dependence and kinetics of CD95-mediated apoptosis (Figure 2). Surprisingly, no significant difference could be seen. In the dose response shown in Figure 2A, the cell line with the slowest rate of caspase-8 and caspase-3 cleavage (Jurkat) was the most sensitive with respect to apoptosis induction by the anti-APO-1 antibody. The kinetics of apoptosis seemed to be independent of the apoptosis cell type (Figure 2B). In summary, since all four cell lines tested expressed high levels of surface CD95 (data not shown) and died with similar kinetics, differences between the two cell types must lie in the signaling pathway.

Both type I and type II cells activate mitochondria following CD95 triggering

Recently, a number of reports have demonstrated that induction of apoptosis is often accompanied by a drop in $\Delta\Psi_m$ (Shimizu *et al.*, 1996a), the opening of PT pores and the release of cytochrome *c* from mitochondria (Kluck *et al.*, 1997a; Yang *et al.*, 1997a). Apoptogenic activity of cytochrome *c* was shown to require a cytoplasmic factor to activate caspase-3 (Liu *et al.*, 1996). In addition, 'activated' mitochondria released another apoptosis-inducing factor (AIF) that was shown to activate caspase-3 and to induce DNA fragmentation on isolated nuclei (Susin *et al.*, 1996). To test whether type I and type II cells differ with respect to their activation of mitochondria during CD95-mediated apoptosis, a number of experiments were performed. Figure 3A shows the kinetics of the loss of $\Delta\Psi_m$ in the four cell lines tested. In all cases, loss of $\Delta\Psi_m$ followed similar kinetics with no significant difference

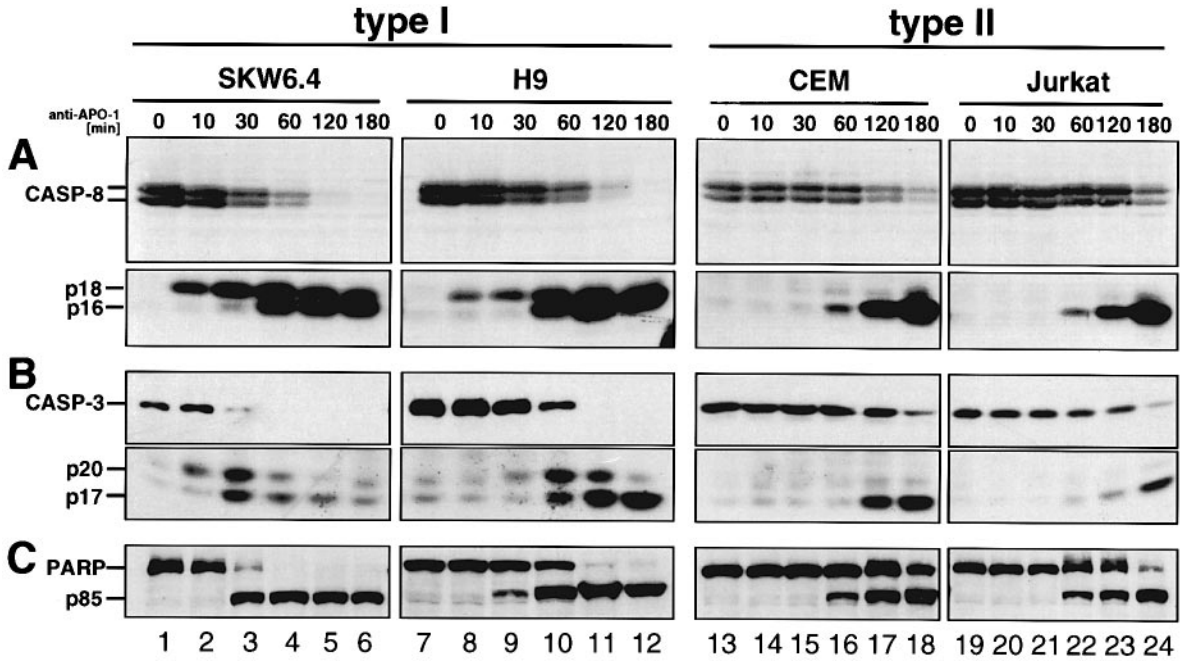

Fig. 1. Caspase-8, caspase-3 and PARP are cleaved with different kinetics in type I and type II cells. Time course of caspase-8 (**A**), caspase-3 (**B**) and PARP (**C**) cleavage. Type I cells SKW6.4 and H9 and type II cells CEM and Jurkat were treated with anti-APO-1 (1 μg/ml) for indicated periods of time and subsequently lysed as described in Materials and methods. Lysates equivalent to 10^6 cells were subjected to 12% SDS–PAGE and immunoblotted with the respective mAb. The migration position of full length caspase-8 (CASP-8) which exists as two isoforms (Scaffidi *et al.*, 1997), the active subunit p18, its further processing product p16, full-length caspase-3 (CASP-3), the cleavage intermediate p20, the active subunit p17, full-length PARP and the cleavage product p85 are indicated.

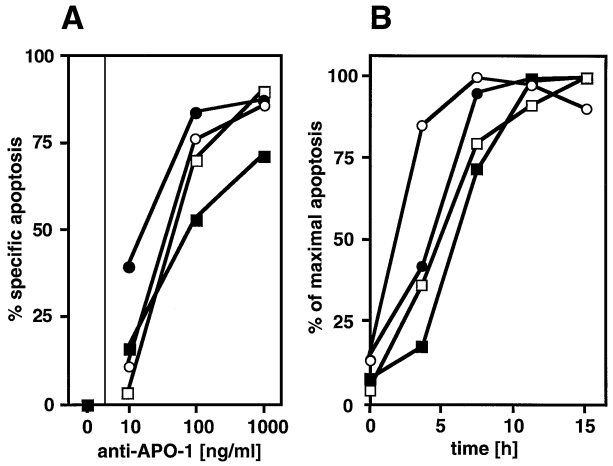

Fig. 2. Similar sensitivity to CD95-mediated apoptosis in type I and type II cells. (**A**) Dose dependence of CD95-mediated apoptosis. Type I cells SKW6.4 (○) and H9 (□) and type II cells CEM (■) and Jurkat (●) were incubated for 16 h with the indicated concentrations of anti-APO-1. After this incubation cells were harvested and analyzed by flow cytometry for DNA fragmentation using nuclear staining with propidium iodide. The percentage of specific apoptosis was calculated as follows: [(% experimental apoptosis – % spontaneous apoptosis)/(100 – % spontaneous apoptosis)]×100. (**B**) Kinetics of CD95-mediated apoptosis. Type I cells SKW6.4 (○) and H9 (□) and type II cells CEM (■) and Jurkat (●) were treated with anti-APO-1 (1 μg/ml) for different periods of time and analyzed as described in (A). For comparison, the percentage of maximum apoptosis for each cell line was calculated. Maximal apoptosis determined was: SKW6.4, 76.0%; H9, 91.8%; CEM, 77.7%; Jurkat, 94.4%. The data shown are representative of three independent experiments.

between type I and type II cells (Figure 3A). Next, the activity of isolated mitochondria from anti-APO-1 treated cells was tested (Figure 3B). Isolated mitochondria of all tested cells were able to induce DNA fragmentation on isolated SKW6.4 nuclei in a dose-dependent way, again with no difference between mitochondria from type I and type II cells. When isolated from untreated non-apoptotic cells, none of the mitochondrial preparations was active in this assay (Figure 3B). Isolated mitochondria from anti-APO-1-activated type I and type II cells were similarly active in inducing caspase-8, caspase-3 and PARP cleavage when added to cytosolic extracts of unstimulated cells (data not shown). Finally, in all cells almost all cytochrome *c* was released from mitochondria after CD95 triggering for 2 h (Figure 3C). These data demonstrate that both type I and type II cells similarly activated mitochondria during their CD95-specific apoptotic program. Therefore, the differences between type I and type II cells in the kinetics of activation of caspase-8 and caspase-3 cannot be attributed to a difference in activation of mitochondria. However, type I and type II cells could differ in their dependence on the mitochondrial contribution to apoptosis.

In type II cells CD95-mediated apoptosis is blocked by Bcl-2

A growing body of data indicates that overexpression of Bcl-2 can block all apoptosis-specific mitochondrial activities. Bcl-2 has been shown to inhibit PT formation (Shimizu *et al.*, 1996a), release of AIF (Susin *et al.*, 1996) and of cytochrome *c* (Kluck *et al.*, 1997a; Yang *et al.*, 1997a) in a number of experimental systems. We therefore used Bcl-2-overexpressing cell lines to test the dependence of type I and type II cells on the mitochondrial contribution to apoptosis. As type I cells we chose SKW6 cells (Strasser *et al.*, 1995) and as type II cells, Jurkat cells (Armstrong *et al.*, 1996). Consistent with published data, the SKW6

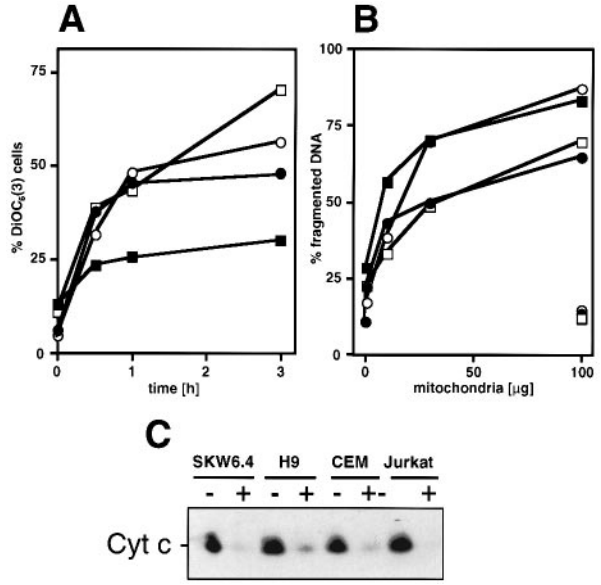

Fig. 3. Mitochondria are similarly activated in type I and type II cells. (**A**) Loss of mitochondrial transmembrane potential. SKW6.4 (○), H9 (□), CEM (■) and Jurkat (●) cells were incubated with anti-APO-1 (1 µg/ml) for indicated periods of time and analyzed by flow cytometry for $\Delta\Psi_m$ using the fluorochrome $DiOC_6(3)$. The percentage of cells with low $\Delta\Psi_m$ is shown. (**B**) Activated mitochondria induce nuclear fragmentation. SKW6.4 (○), H9 (□), CEM (■) and Jurkat (●) cells were treated with anti-APO-1 (1 µg/ml) for 2 h and mitochondria were isolated. Indicated amount of mitochondria (expressed as µg mitochondrial protein) was incubated with isolated nuclei of SKW6.4 cells. As control 100 µg of mitochondrial protein from isolated mitochondria of untreated cells was incubated with nuclei of SKW6.4 cells (separate symbols). DNA fragmentation was assessed by propidium iodide staining and flow cytometry. (**C**) Loss of mitochondrial cytochrome *c*. Mitochondria of untreated cells (–) or cells treated with anti-APO-1 (1 µg/ml) for 2 h (+) were isolated. 10 µg of mitochondrial proteins were separated by 15% SDS–PAGE. Western blot analysis of mitochondrial cytochrome *c* was done as described in Materials and methods.

cells were not affected in their CD95-sensitivity by overexpression of Bcl-2 (Figure 4A) despite very high Bcl-2 expression (Figure 4G). In contrast, Jurkat-bcl-2 cells expressing much lower amounts of Bcl-2 than SKW6-bcl-2 cells were significantly resistant to apoptosis after CD95 triggering (Figure 4D). For both cells different transfectants were tested to exclude clonal effects. Next we tested whether Bcl-2 affected mitochondrial functions during CD95-mediated apoptosis in both cell types. In SKW6-bcl-2 and Jurkat-bcl-2 cells, Bcl-2 was present in the isolated mitochondrial fraction (Figure 4G) and blocked loss of $\Delta\Psi_m$ in mitochondria (Figure 4B and E). In addition, in both Bcl-2-overexpressing cell types the ability of isolated mitochondria after CD95 stimulation to induce DNA cleavage on isolated nuclei (Figure 4C and F), or to induce caspase cleavage in cytosolic extracts (data not shown), was blocked as well as cytochrome *c* release from these mitochondria (Figure 4H). These data support the finding that both type I and type II cells activate mitochondria, resulting in the release of cytochrome *c*. However, only type II cells depend on the apoptogenic activity of mitochondria since blocking of this activity by Bcl-2 overexpression reduced CD95-mediated apoptosis sensitivity in the type II cell line Jurkat, but not in the type I cell line SKW6.

In type I cells caspase-8 activation occurs upstream, and in type II cells downstream, of mitochondria

To define the role of mitochondria in the activation of caspases in type I and type II cells, we tested how blocking of mitochondrial activation by Bcl-2 overexpression would affect cleavage of caspase-8, caspase-3 and PARP (Figure 5). In SKW6 cells, Bcl-2 did not block caspase activities (Figure 5A, B and C, lanes 1–10), consistent with the fast cleavage kinetics of caspase-8, caspase-3 and PARP in type I cells. In contrast, in the type II cell line Jurkat, cleavage of both caspase-8 and caspase-3 was completely blocked by overexpression of Bcl-2 (Figure 5A and B, lanes 11–20), consistent with the slow activation kinetics of both caspases in these cells. In addition, cleavage of PARP was significantly delayed indicating that almost all caspase-3-like protease activity in these cells was blocked by Bcl-2. Bcl-x_L has been shown to be functionally equivalent to Bcl-2 (Huang *et al.*, 1997) and to test whether Bcl-x_L could also block apoptosis in type II cells, we generated CEM cells which stably overexpressed Bcl-x_L. In these cells, overexpression of Bcl-x_L inhibited CD95-mediated apoptosis as well as caspase-8 activation and PARP cleavage (Figure 6). This underlines again the dependence of caspase activation and apoptosis on mitochondrial function in type II cells. Taken together, these data suggest that in type I cells caspase-8 is activated directly at the DISC level. Active caspase-8 subunits may directly cause cleavage of caspase-3 and this activation is independent of mitochondrial activation. In contrast, in type II cells strong activation of caspase-8 and caspase-3 occurs at a level downstream of mitochondria. It is inhibited by Bcl-2 or Bcl-x_L and may depend on mitochondrial release of cytochrome *c*.

DISC formation is strongly reduced in Jurkat and CEM T cells

Cross-linking of the CD95 receptor on most cells expressing high levels of CD95 results in instant recruitment of FADD and caspase-8 to the receptor (Kischkel *et al.*, 1995), leading to the activation of caspase-8 at the DISC (Medema *et al.*, 1997). Since activation of caspase-8 in the type II cells occurred downstream of mitochondria, we compared formation of the DISC between type I and type II cells. As previously reported (Medema *et al.*, 1997), the DISC of the type I cells SKW6.4 and H9 contained high quantities of both FADD and caspase-8 (Figure 7A, lanes 2 and 4) which were not detected in unstimulated cells (Figure 7A, lanes 1 and 3). In contrast, recruitment of both FADD and caspase-8 was substantially reduced in the type II cells CEM and Jurkat (Figure 7A, lanes 6 and 8). Upon overexposure, very little FADD or caspase-8 was detected in the DISC in either type II cell line (data not shown). Testing 11 different CD95-positive cell lines of different origin [SKW6.4, HuT78, H9, BJAB, Raji, CEM, Jurkat, HepG2, HT29 and human CD95-expressing BL60 (K50) and L929] (Kischkel *et al.*, 1995; Medema *et al.*, 1997; C.Scaffidi, F.Kischkel and M.Peter, unpublished), CEM and Jurkat T cells were the only cells with that phenotype. The lack of significant DISC formation in CEM and Jurkat cells was not due to the absence of either FADD or caspase-8 since both proteins were expressed at similar levels in the four cell

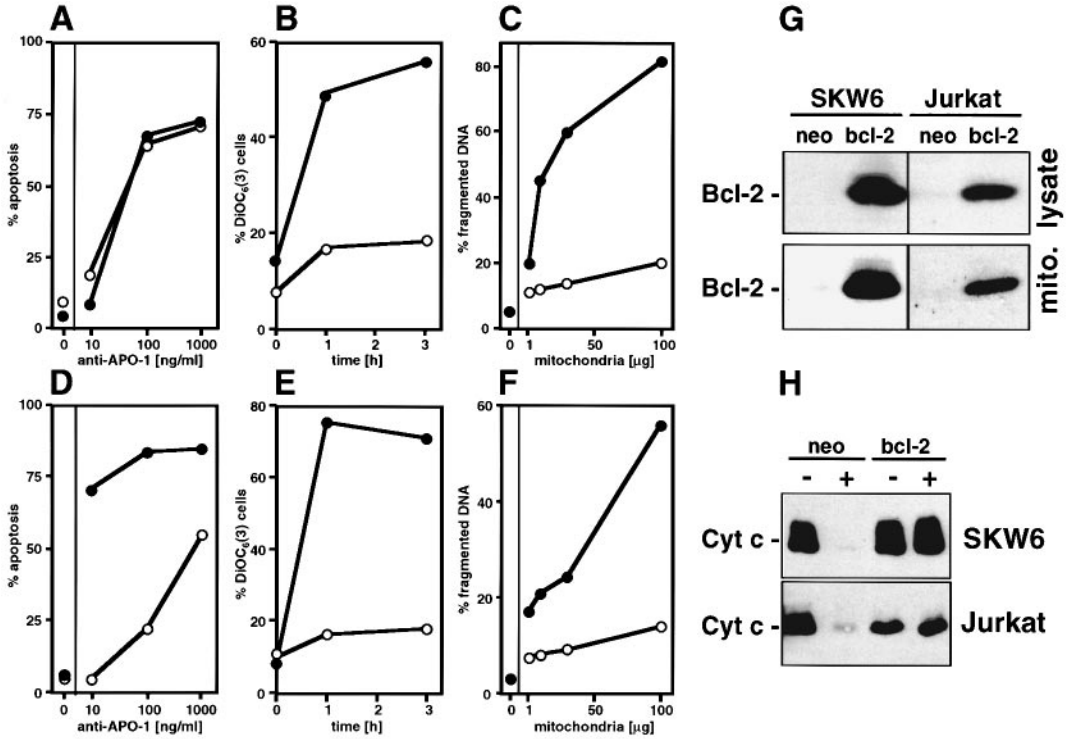

Fig. 4. Block of mitochondrial activation by Bcl-2. Overexpression of Bcl-2 inhibits CD95-mediated apoptosis only in type II cells. (A) and (D) Bcl-2 blocks CD95-mediated apoptosis in type II, but not in type I cells. (**A**) SKW6 or (**D**) Jurkat cells stably transfected with neomycin vector only (●) or Bcl-2 expression vector (○) were treated with indicated concentrations of anti-APO-1 for 16 h. Apoptosis was assessed by propidium iodide staining and flow cytometry. (B) and (E) Loss of $\Delta\Psi_m$. (**B**) SKW6 or (**E**) Jurkat cells transfected with vector control (●) or Bcl-2 expression vector (○) were incubated with anti-APO-1 (1 µg/ml) for indicated periods of time and analyzed by flow cytometry for $\Delta\Psi_m$ using the fluorochrome $DiOC_6(3)$. The percentage of cells with low $\Delta\Psi_m$ is shown. (C) and (F) Bcl-2 prevents nuclear fragmentation induced by mitochondria isolated from type I and type II cells. Isolated mitochondria of anti-APO-1 (1 µg/ml, 2 h) treated (**C**) SKW6 or (**F**) Jurkat cells transfected with vector control (●) or Bcl-2 expression vector (○) were incubated at indicated concentrations (expressed as µg mitochondrial protein) with isolated nuclei of SKW6.4 cells. DNA fragmentation was assessed by propidium iodide staining and flow cytometry. (**G**) Expression of Bcl-2 in transfected cell lines. Cellular or mitochondrial proteins of SKW6 or Jurkat cells stably transfected with neomycin vector only or Bcl-2 expression vector were separated by 12% SDS–PAGE. Western blot analysis of Bcl-2 was performed using anti-Bcl-2 antibody and ECL. Western blots shown for Jurkat cells were exposed five times longer than the ones shown for SKW6 cells. (**H**) Bcl-2 prevents cytochrome *c* release from mitochondria of type I and type II cells. Mitochondria were isolated from either untreated (–) or anti-APO-1 stimulated (+) (1 µg/ml, 2 h) SKW6 or Jurkat cells stably transfected with neomycin vector control or Bcl-2 expression vector. Mitochondrial proteins (10 µg) were separated by 15% SDS–PAGE and subjected to Western blot analysis to determine their cytochrome *c* content.

lines as determined by Western blot analysis of cellular lysates (Figure 7B). The data suggest that the amount of active caspase-8 generated by recruitment to the DISC determines the apoptosis cell type.

The reduced FADD and caspase-8 recruitment to the DISC in type II cells could be caused by a protein bound to CD95, blocking DISC formation. We therefore analyzed the receptor complex of both cell types for CD95-specific association of a protein found only in type II cells using 2D IEF/SDS–PAGE analysis as previously described (Kischkel *et al.*, 1995). We identified a 120 kDa protein specifically associated with CD95 in CEM and Jurkat cells only (Figure 7C), independent of the activation status. This protein was not detected in immunoprecipitates using an isotype-matched control mAb (Figure 7C, FII23) or an anti-transferrin receptor mAb (Figure 7C, anti-TfR). The protein was not found to be associated with CD95 in SKW6.4 cells (Figure 7C) or other type I cells (Medema *et al.*, 1997).

Conversion of MCF7-Fas into a type I cell line by overexpression of pro-caspase-3

We have recently shown that in MCF7 cells stably expressing CD95 (MCF7-Fas), caspase-8 is recruited to the DISC after triggering of CD95 (Medema *et al.*, 1998). Overexpression of Bcl-x_L did not interfere with activation of caspase-8, however, it blocked CD95-mediated apoptosis (Jäättelä *et al.*, 1995; Medema *et al.*, 1998; Srinivasan *et al.*, 1998). Thus, MCF7-Fas cells behave like type I cells in terms of DISC formation and caspase-8 activation, but like type II cells in terms of the ability of Bcl-x_L to block CD95-mediated apoptosis. Recently, we have shown that MCF7-Fas cells are deficient in caspase-3 expression and this accounts for the inability of micro-injected cytochrome *c* to induce apoptosis in these cells (Li *et al.*, 1997b; Srinivasan *et al.*, 1998). These observations raised the possibility that in the absence of caspase-3 activated caspase-8 signals through a Bcl-x_L-inhibitable mitochondrial step. To test this possibility, the ability of Bcl-x_L to inhibit CD95-mediated apoptosis in MCF7-Fas cells was examined in pro-caspase-3 transiently transfected cells. When MCF7-Fas cells were transfected with vector control (Figure 8A, middle column), CD95-mediated apoptosis was inhibited when Bcl-x_L was present in the cells as well (Figure 8A, right hand column). In contrast, when transiently transfected with pro-caspase-3, the MCF7-Fas-bcl-x_L cells were no longer protected from

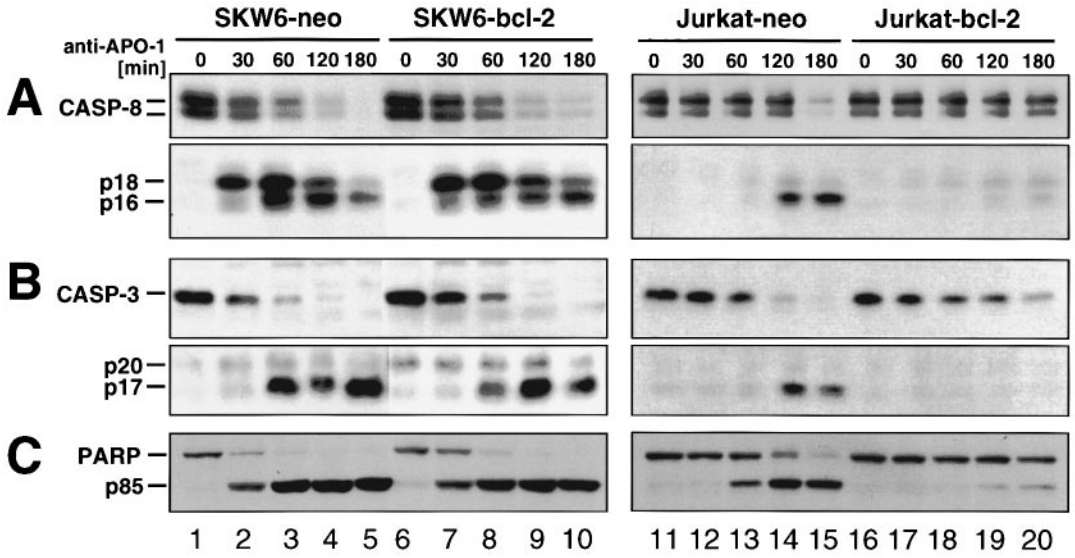

Fig. 5. In the type II cell Jurkat, caspase-8 and caspase-3 cleavage is blocked by Bcl-2 overexpression. Time course of (**A**) caspase-8, (**B**) caspase-3, and (**C**) PARP processing. SKW6 or Jurkat cells transfected with vector control (neo) or Bcl-2 expression vector were treated with anti-APO-1 (1 μg/ml) for indicated periods of time and subsequently lysed as described in Materials and methods. Lysates of 10^6 cells were subjected to 12% SDS–PAGE and immunoblotted with the respective mAb. The migration position of full length caspase-8 (CASP-8), the active subunit p18, its further processing product p16, full-length caspase-3 (CASP-3), the cleavage intermediate p20, the active subunit p17, full-length PARP and the cleavage product p85 are indicated.

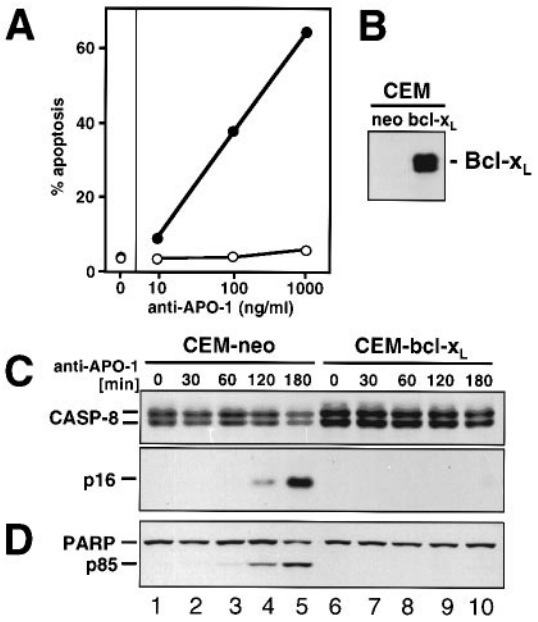

Fig. 6. Bcl-x_L overexpression in the type II cells CEM blocks CD95-mediated apoptosis as well as cleavage of caspase-8 and PARP. (**A**) CEM cells stably transfected with neomycin vector only (●) or Bcl-x_L expression vector (○) were treated with indicated concentrations of anti-APO-1 for 16 h. Apoptosis was assessed by propidium iodide staining and flow cytometry. Shown are the results of one of three different clones tested. (**B**) Bcl-x_L expression of transfected CEM cells. Cellular lysates of CEM cells transfected with vector control (neo) or Bcl-x_L expression vector were analyzed by Western blotting. (**C**, **D**) Time course of caspase-8 (C) and PARP (D) processing. CEM cells transfected with vector control (neo) or Bcl-x_L expression vector were treated as described in Figure 6. The migration position of full-length caspase-8 (CASP-8), the cleavage product p16, full-length PARP and the cleavage product p85 are indicated.

CD95-mediated apoptosis and became independent of the mitochondrial pathway (Figure 8B, right column). In summary, heterologous expression of caspase-3 converted MCF7-Fas cells to true type I cells with respect to the inability of Bcl-x_L to block CD95-mediated apoptosis.

Discussion

Caspases have been recognized as playing a major role in execution of the death signal during apoptosis induced by various stimuli (Nicholson and Thornberry, 1997). During CD95-mediated apoptosis they were shown to be activated shortly after receptor triggering (Enari *et al.*, 1996; Hasegawa *et al.*, 1996; Medema *et al.*, 1997; Scaffidi *et al.*, 1997). Caspase-3-like activities seem to be most important (Hasegawa *et al.*, 1996; Schlegel *et al.*, 1996) although caspase-3 alone does not seem to be absolutely required in most cases as caspase-$3^{-/-}$ mice did not have a defect in the CD95 pathway in most tissues (Kuida *et al.*, 1996). Recently, using different systems involving cell-free extracts, it became clear that mitochondrial components are essential for many forms of apoptosis in mammalian cells. Consistent with our data, ~30 min after apoptosis induction a drop in $\Delta\Psi_m$ can be detected (Zamzami *et al.*, 1996). In parallel, cytochrome *c* is first inactivated (Krippner *et al.*, 1996; Adachi *et al.*, 1997) and later released from mitochondria (Liu *et al.*, 1996; Kluck *et al.*, 1997a; Perry *et al.*, 1997; Yang *et al.*, 1997a). Irrespective of its redox state, cytochrome *c* seems to be important for activation of caspase-3 (Kluck *et al.*, 1997b; Yang *et al.*, 1997a). This activation step requires the cellular factors Apaf-1, a mammalian cytochrome *c* binding homologous to the *C.elegans* CED-4 protein (Zou *et al.*, 1997), and Apaf-3/caspase-9 (Li *et al.*, 1997a). Recent work has suggested that in mammalian cells at least two levels exist at which caspase-3-like proteases act during CD95-mediated apoptosis. First level caspases act upstream and second level caspases downstream of the mitochondrial check-point (Peter *et al.*, 1997a).

Both Bcl-2 and Bcl-x_L, shown to be functionally equiva-

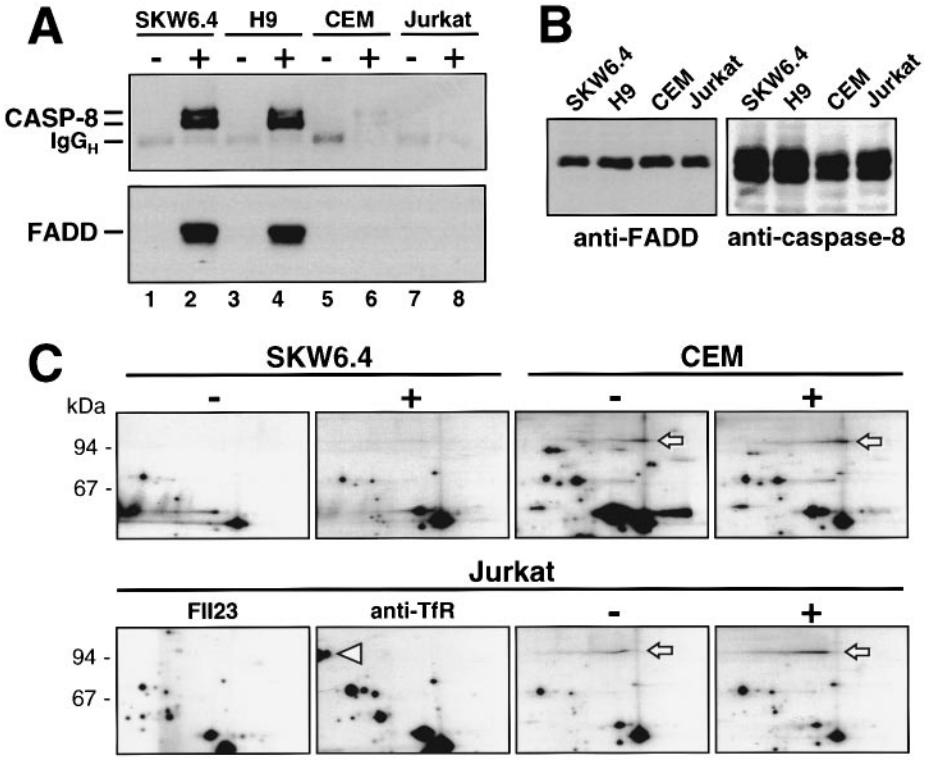

Fig. 7. DISC formation is strongly reduced in CEM and Jurkat cells. (**A**) Different recruitment of caspase-8 and FADD to activated CD95 receptors in CEM and Jurkat cells. CD95 was immunoprecipitated from either 10^7 untreated (–) or anti-APO-1-treated (5 min) (+) SKW6.4, H9, CEM or Jurkat cells. Immunoprecipitates were washed, subjected to 12% SDS–PAGE and immunoblotted with anti-caspase-8 mAb C15 or anti-FADD mAb. Migration positions of caspase-8 and FADD are indicated. The heavy chain of the anti-APO-1 antibody used in immunoprecipitation is detected due to cross-reactivity of the secondary antibody used in Western blot analysis. Strongly reduced DISC formation in the type II cells was also found when equal amounts of immunoprecipitated CD95 were loaded as determined by surface biotinylation (data not shown). (**B**) Similar expression of FADD and caspase-8 in SKW6.4, H9, CEM and Jurkat cells. Cellular lysates equivalent to 20 μg protein were subjected to 12% SDS–PAGE and immunoblotted using anti-FADD or anti-caspase-8 (C15) mAbs. (**C**) First row: CD95 from unstimulated (–) or anti-APO-1 treated (+) metabolically labeled SKW6.4 or CEM cells was immunoprecipitated and analyzed by 2D IEF–PAGE as described in Materials and methods. Second row: metabolically labeled Jurkat cells were lysed and progressively immunoprecipitated with (left to right) FII23, anti-TfR mAb coupled to anti-IgG1 agarose and anti-APO-1 coupled to protein A–Sepharose (–). CD95 from stimulated (+) Jurkat cells was immunoprecipitated as described above. Only the upper acidic halves of all 2D gels are shown. Migration position of a type II-cell specific CD95 associated protein of 120 kDa is labeled with an arrow. A small fraction of the immunoprecipitated TfR present on the area of the gels shown is labeled with an arrow head.

lent (Huang *et al.*, 1997), are in part expressed in the outer mitochondrial membrane. Recently, a large body of evidence has supported the view that Bcl-2 or Bcl-x_L can block all activities of mitochondria shown to be involved in apoptosis signaling (Newmeyer *et al.*, 1994; Shimizu *et al.*, 1996a,b; Adachi *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997a; Vander Heiden *et al.*, 1997; Yang *et al.*, 1997a). Consistent with this ability, others have shown that Bcl-2 and Bcl-x_L act upstream of a caspase-3-like activity (Armstrong *et al.*, 1996; Chinnaiyan *et al.*, 1996b; Cosulich *et al.*, 1996; Monney *et al.*, 1996; Shimizu *et al.*, 1996b; Estoppey *et al.*, 1997; Perry *et al.*, 1997), establishing caspase-3 as a second level caspase acting downstream of mitochondria.

Involvement of mitochondria defines two different CD95 signaling pathways

Despite the growing number of key discoveries in apoptosis signaling, a number of unsolved questions have remained, two of which are currently being controversially discussed for CD95 signaling: (i) do mitochondria play a central role in CD95-mediated apoptosis; and (ii) can Bcl-2 or Bcl-x_L inhibit CD95-mediated apoptosis? We can now address these questions. (i) After recognition of the importance of mitochondria in apoptosis signaling, it was suggested that all forms of apoptosis may depend on activation of mitochondria (Marchetti *et al.*, 1996; Kroemer *et al.*, 1997). We have now demonstrated that both type I and type II cells activate mitochondria similarly with respect to PT formation, release of cytochrome *c* and their ability to induce DNA fragmentation of isolated nuclei or caspase activation in cytosolic extracts. However, type I cells have developed a way to bypass mitochondrial functions, as they activate caspase-8 at the DISC followed by caspase-3 activation independent of mitochondrial activity. Only type II cells seem to depend on mitochondria during induction of apoptosis, and activation of both caspase-8 and caspase-3 can be prevented by blocking mitochondrial activity. (ii) The role of Bcl-2 and Bcl-x_L in CD95 signaling is unclear. A large number of publications have addressed the question of whether mammalian CED-9 homologs can inhibit CD95-mediated apoptosis. Reports range from no effect (Chiu *et al.*, 1995; Memon *et al.*, 1995; Strasser *et al.*, 1995; Chinnaiyan *et al.*, 1996b; Moreno *et al.*, 1996; Huang *et al.*, 1997; Susin *et al.*, 1997), through partial effect (Itoh *et al.*, 1993; Memon *et al.*, 1995; Boise and Thompson, 1997), to substantial inhibition (Jäättelä *et al.*, 1995; Takayama *et al.*, 1995;

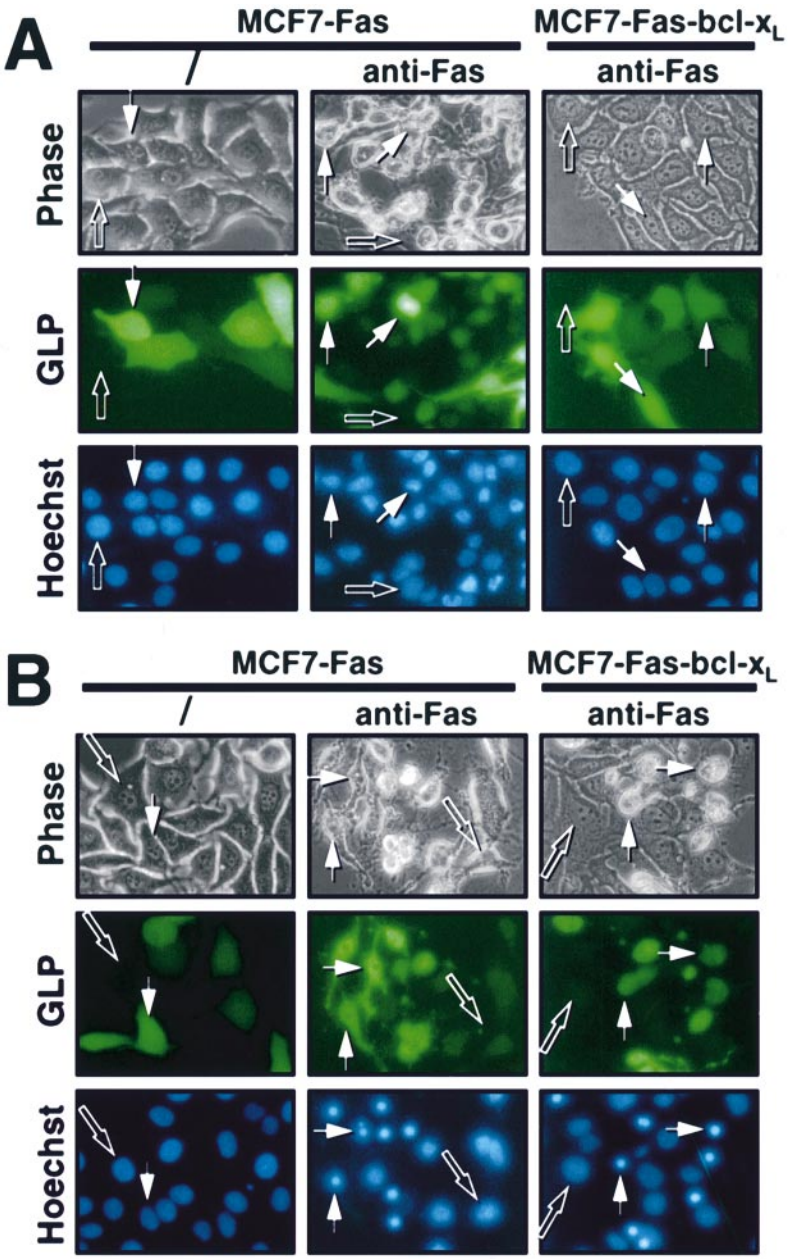

Fig. 8. Expression of caspase-3 in MCF7-Fas cells renders these cells independent of mitochondria. (**A**) Bcl-x_L inhibits Fas-induced apoptosis in vector transfected MCF7-Fas cells. Phase contrast and corresponding fluorescence micrographs of MCF7-Fas and MCF7-Fas-bcl-x_L cells transfected with pcDNA3 DNA and pCMV green lantern protein (GLP). The left hand column shows untreated, transfected MCF7-Fas cells; the middle and right columns are MCF7-Fas and MCF7-Fas-bcl-x_L cells, respectively, treated with 500 ng/ml anti-Fas and 1 μg/ml cycloheximide for 8 h. The top row shows phase contrast images of the cells (Phase), the middle row shows cells positive for the co-transfected marker GLP and the bottom row shows the nuclear morphology (Hoechst). Untransfected cells are indicated with open arrows; transfected cells are indicated with closed arrows. (**B**) Bcl-x_L does not inhibit Fas-mediated apoptosis in pro-caspase-3-transfected cells. Phase contrast and corresponding fluorescence micrographs of MCF7-Fas and MCF7-Fas-bcl-x_L cells transfected with pcDNA3-pro-caspase-3 DNA and pCMV GLP. The left hand column shows untreated, transfected MCF7-Fas cells; the middle column and the right column are MCF7-Fas and MCF7-Fas-bcl-x_L cells, respectively, treated with 500 ng/ml anti-Fas and 1 μg/ml cycloheximide for 8 h. Note: the nuclei in the anti-Fas treated MCF7-Fas cells exhibit a shrunken, misshapen, slightly-marginated morphology (A, bottom row, middle panel). The nuclei of both the transfected, untreated MCF7-Fas cells (A, bottom row, left panel) and the transfected, anti-Fas treated MCF7-Fas-bcl-x_L cells (A, bottom row, right panel) exhibit a round, normal morphology showing that Bcl-x_L inhibits Fas-induced nuclear changes. The nuclei of pro-caspase-3 transfected, untreated MCF7-Fas cells exhibit a round, normal morphology (B, bottom row, left panel) indicating that expression of pro-caspase-3 alone is insufficient to induce apoptosis in MCF7 cells. Untransfected cells in the anti-Fas treated MCF7-Fas cells exhibit the previously described shrunken, misshapen morphology (compare with A, bottom row, middle panel). However, following anti-Fas treatment, the nuclei of pro-caspase-3 transfected cells in both MCF7-Fas cells (B, bottom row, middle panel) and MCF7-Fas-bcl-x_L cells (B, bottom row, right panel) exhibit an intensely condensed, occasionally fragmented morphology. In (A), 78.3% of the GLP-positive MCF7-Fas cells and 16.5% of the GLP-positive MCF7-Fas-bcl-x_L cells showed marginated nuclei after anti-Fas treatment. In (B), 67.2% of the GLP-positive MCF7-Fas and 57.1% of the GLP-positive MCF7-Fas-bcl-x_L cells had a condensed or fragmented nucleus. No nuclear changes were observed without anti-Fas treatment (A and B). This experiment was repeated five times and data from a representative experiment are shown.

Armstrong *et al.*, 1996; Lee *et al.*, 1996; Mandal *et al.*, 1996; Vander Heiden *et al.*, 1997). We have now demonstrated that the ability of human CED-9 homologs to inhibit CD95-mediated apoptosis is dependent on the apoptosis cell type tested. Expression of Bcl-2 in the type I SKW6 cells did inhibit mitochondrial functions, however, it failed to inhibit apoptosis and activation of caspase-8 and caspase-3 *in vivo*, indicating that type I cells use a system parallel to mitochondria. In contrast, in the type II cells Jurkat and CEM, when DISC formation was strongly impaired and most caspase-8 and caspase-3 was primarily activated downstream of mitochondria, activation of these caspases and apoptosis were blocked by Bcl-2 or Bcl-x_L, respectively. Most studies reporting inhibition of CD95-mediated apoptosis by Bcl-2 or Bcl-x_L have used Jurkat T cells, a typical type II cell line (Memon *et al.*, 1995; Takayama *et al.*, 1995; Armstrong *et al.*, 1996; Boise and Thompson, 1997; Vander Heiden *et al.*, 1997). Only a few reports exist where no inhibition of CD95-mediated apoptosis was found by overexpression of Bcl-2 or Bcl-x_L in type II cells such as Jurkat or CEM (Chinnaiyan *et al.*, 1996b; Susin *et al.*, 1997). These contradictory results may be due to the different cell lines used in various studies. More detailed analysis would be necessary to clarify whether these specific clones really are type II cells, and whether Bcl-2 or Bcl-x_L inhibited all mitochondrial activity in these cells. Studies that did not find any inhibition by Bcl-2 or Bcl-x_L used type I cells such as SKW6 (Strasser *et al.*, 1995; Huang *et al.*, 1997).

What are the characteristics of type I and type II cells?

Type I cells such as SKW6.4 and H9 form a DISC and activate caspase-8 at the receptor level within seconds of CD95 triggering (Figure 9A). In these cells, caspase-8 acts as a first level caspase. Active caspase-8 subunits lead to the activation of mitochondria, similar to that in type II cells. However, the large quantities of active caspase-8 generated may start a mitochondria-independent caspase cascade, or in direct processing of caspase-3. The latter assumption is supported by *in vitro* data (Srinivasula *et al.*, 1996; Muzio *et al.*, 1997). As caspase activation in these cells is independent of mitochondrial function it cannot be blocked by overexpression of Bcl-2.

Type II cells show only reduced DISC formation (Figure 9B). Mitochondria in these cells may function as an amplifier, activating both caspase-8 and caspase-3. Only in these cells can activation of these caspases be blocked by Bcl-2 or Bcl-x_L and apoptosis sensitivity is strongly reduced. We found that caspase-3 was activated by addition of cytochrome *c* to cytosolic extracts whereas caspase-8 was not activated (S.Fulda and M.Peter, unpublished). It is therefore unclear what activates caspase-8 downstream of mitochondria. This question is even more intriguing considering that caspase-8 activation apparently preceded activation of caspase-3 in our experiments.

Do type II cells form an alternative DISC?

Since type I and type II cells activate mitochondria similarly, it is unlikely that type II cells use an alternative receptor-proximal signaling pathway and form an alternative DISC. However, two proteins other than FADD have been suggested to couple with CD95. First, Daxx, a protein interacting with the CD95 death domain and activating Jun kinases (Yang *et al.*, 1997b). Apoptosis induced by Daxx was suggested to be inhibitable by Bcl-2 whereas activation of cell death by FADD was suggested to be independent of Bcl-2. Daxx therefore would fit the criteria of a protein activating the mitochondrial pathway. However, when previously testing the kinetics of Jun kinase activation in the type II cell Jurkat, we did not find faster activation of Jun kinases compared with the type I cell SKW6.4 in which Jun kinase activation occurs downstream of caspases (Cahill *et al.*, 1996). Therefore, binding of Daxx to CD95 is unlikely to be responsible for the distinct phenotype of type II cells.

Another candidate for binding to CD95 in type II cells is caspase-10 (Fernandes-Alnemri *et al.*, 1996; Vincenz *et al.*, 1997). Involvement of caspase-10 would be consistent with data demonstrating that caspases act upstream of mitochondria (Susin *et al.*, 1997). However, using a rabbit serum specific for the p18 subunit of caspase-10, we found processing of caspase-10 in both the type I cell SKW6.4 and the type II cell Jurkat after activation of mitochondria at 30 min (unpublished). In addition, caspase-10 processing in the type II cell Jurkat was blocked by Bcl-2 (C.Scaffidi and M.Peter, unpublished). It is therefore unlikely that caspase-10 is part of the CD95 DISC. It may, however, be activated by other death receptors. In summary, caspase-10 cannot be the first level caspase that activates mitochondria in type II cells upon triggering of CD95.

The amount of active caspase-8 generated by the CD95 DISC can determine whether a cell reacts like a type I or a type II cell

Comparable activation of mitochondria in type I and type II cells makes it unlikely that an unknown signaling protein would specifically bind to CD95 in type II cells instead of FADD. FADD and caspase-8 seem to initiate the death signal at the receptor level in both apoptosis cell types. This is supported by data demonstrating that a dominant negative form of FADD could inhibit CD95-mediated apoptosis in the type I cell line BJAB (Chinnaiyan *et al.*, 1995) as well as in the type II cell line MCF7 (Chinnaiyan *et al.*, 1996a).

An alternative explanation of the difference in the receptor-proximal events between type I and type II cells comes from experiments using the previously described *in vitro* caspase-8 cleavage assay (Medema *et al.*, 1997). With this assay, a low ability of the DISC from Jurkat cells to process caspase-8 was detected (data not shown). Although very low, this ability may be enough to activate mitochondria in type II cells. In these cells, mitochondria may serve as a signal amplifier for low caspase activity generated at the DISC. This caspase activity, however, may be insufficient to start a caspase cascade such as the one in type I cells (Figure 9A).

Experiments using MCF7-Fas cells provided strong evidence that caspase-8 generated at the DISC is also responsible for the activation of mitochondria in type II cells. Unlike other type II cells, MCF7-Fas cells formed large quantities of active caspase-8 at the DISC (Medema *et al.*, 1998). This may have been due to the fact that these cells stably overexpress CD95. Using micro-injection experiments we found that active caspase-8 was able to

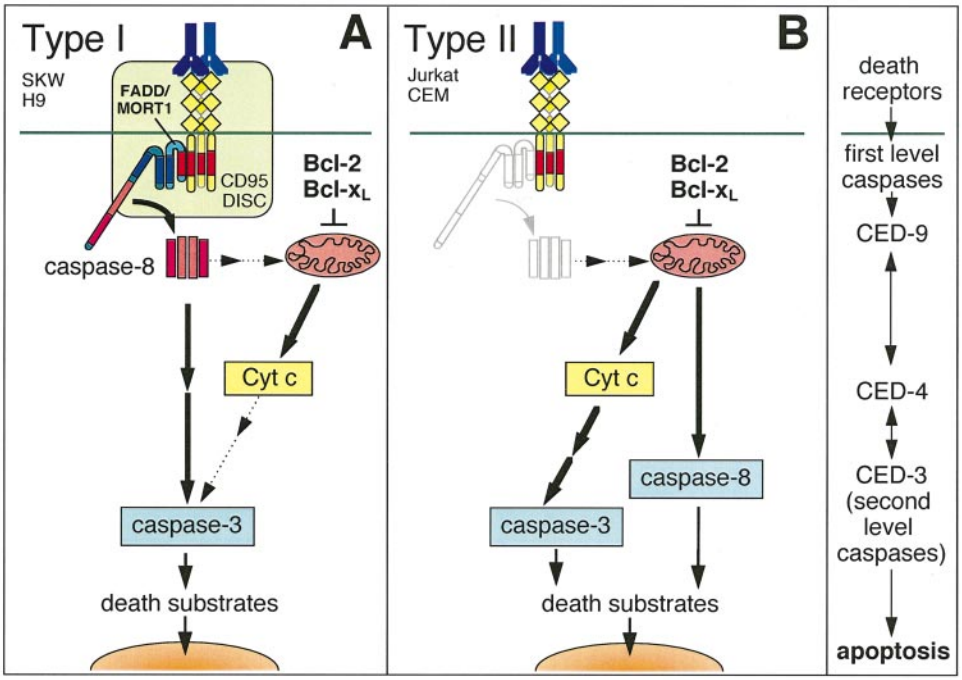

Fig. 9. Model of the two CD95 signaling pathways. (**A**) In type I cells CD95 triggering leads to strong caspase-8 activation at the DISC which bypasses mitochondria directly leading to activation of other caspases such as caspase-3 and subsequently to apoptosis. (**B**) In type II cells only a little DISC is formed leading to the activation of mitochondria, in turn resulting in cleavage of caspase-8 and caspase-3 downstream of mitochondria. Therefore, blocking activation of mitochondria by Bcl-2 or Bcl-x_L inhibits apoptosis only in type II cells. Red box, death domain; blue box, death effector domain.

start a mitochondria-dependent apoptotic pathway, and that even high caspase-8 concentrations in these cells did not result in the induction of mitochondria-independent apoptosis (Srinivasan *et al.*, 1998). This was probably due to a lack of caspase-3 expression in MCF7 cells. Expression of caspase-3 enabled these cells to bypass mitochondria by directly activating caspase-3. These cells were then no longer resistant to CD95-mediated apoptosis, despite overexpressing Bcl-x_L. These data provide strong evidence for a model in which the amount of active caspase-8 generated at the DISC determines whether a cell uses mitochondria when caspase-3 is present. The regulation of development into one or the other cell type must therefore start at the inhibition of the recruitment of FADD to CD95. What mechanism prevents FADD from associating with activated CD95 in type II cells is currently unknown. A possible candidate for such a blocking molecule could be the 120 kDa protein found to be associated only with CD95 in type II cells (Figure 7C). This protein qualifies as such an inhibitor since it is also associated with the non-cross-linked receptor. It may therefore prevent formation of the DISC.

Evolution of the two CD95 apoptosis pathways

Apoptosis in the nematode *C.elegans* has served as a paradigm for mammalian apoptosis pathways. In *C.elegans*, apoptosis is executed by a caspase-3-like protease, CED-3, that requires another death promoting protein, CED-4, for its activity (Yuan and Horvitz, 1990). A third protein, CED-9, acts as a negative regulator, presumably by direct physical association with CED-4 (Chinnaiyan *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997). The mammalian mitochondrial apoptosis pathway found in type II cells is most similar to the apoptosis pathway in *C.elegans* (Figure 9B). Consistent with this model, apoptosis in type II cells is inhibited by the CED-9 homologs Bcl-2 and Bcl-x_L. Therefore, the apoptosis pathway of type II cells may be evolutionary older than that of type I cells. In contrast to the nematode, mammals have developed an additional apoptotic pathway bypassing mitochondria by direct activation of caspases at the level of the death receptors. Therefore in type I cells, in which this mitochondria independent pathway is operative, apoptosis cannot be inhibited by Bcl-2 or Bcl-x_L. To overcome this lack of regulation, mammals may have developed a new kind of inhibitor, FLICE inhibitory protein (cFLIP) (for review see Peter *et al.*, 1998), which inhibits caspase-8 activation directly at the receptor level without affecting recruitment of FADD (C.Scaffidi and M.Peter, unpublished). cFLIP was reported to inhibit CD95-mediated apoptosis in both type I (Raji) and type II cells (Jurkat) (Irmler *et al.*, 1997), again supporting a model in which caspase-8 activation at the DISC is essential for both apoptosis pathways.

The physiological role of the two CD95 signaling pathways

Most cell lines tested during this study were of type I; only two type II cells were found but this may not reflect the relative importance of the two pathways in the body. However, data have led us to speculate about the physiological roles of type I and type II CD95-mediated apoptosis in the body. At least two tissues can be categorized: peripheral T cells and thymocytes seem to be type I cells. We have recently shown that human peripheral T cells form a DISC and that inhibition of CD95 signaling in apoptosis-resistant T cells resulted from a failure to recruit pro-caspase-8 to the DISC rather than from upstream

regulation of Bcl-x_L that was also detected (Peter *et al.*, 1997b). This assessment is consistent with data from studies on the direct effects of Bcl-2 and Bcl-x_L on normal peripheral T cells. Bcl-2 and Bcl-x_L overexpression in normal mouse and human T-cell blasts did not inhibit Fas-mediated apoptosis but glucocorticoid- and etoposide-induced apoptosis were blocked (Moreno *et al.*, 1996). In addition, thymocytes and activated T cells were not protected by a Bcl-2 transgene (Strasser *et al.*, 1995). These data support the notion that peripheral T cells and thymocytes have a type I phenotype and may therefore be independent of mitochondrial functions following CD95 signaling. A typical type II tissue seems to be the liver: a Bcl-2 transgene protected mice from anti-CD95 antibody-induced apoptosis in liver tissue (Lacronique *et al.*, 1996; Rodriguez *et al.*, 1996). Future work will determine what role both described CD95 signaling pathways play *in vivo*, and whether these two pathways are used by other members of the death receptor family. In addition, a growing number of diseases have been correlated with a dysregulated CD95 apoptosis system (Peter *et al.*, 1997a). Therapeutic strategies that involve modulation of CD95-mediated apoptosis will first target the tissue to be determined as type I or type II, and will require an evaluation of the contribution of the two pathways in each case.

Materials and methods

Cell lines

The B lymphoblastoid cell line SKW6.4, and the T cell lines H9 and CEM were maintained in RPMI 1640 (Gibco-BRL), 10 mM HEPES (Gibco-BRL), 2 mg/ml Gentamycin (Gibco-BRL) and 10% fetal calf serum (Gibco-BRL) in 5% CO_2. The T cell line Jurkat (clone J16) was maintained in Iscove's modified Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented as described above. Jurkat cells transfected with empty vector or Bcl-2 were cultured as described elsewhere (Armstrong *et al.*, 1996). SKW6 cells transfected with vector control or Bcl-2 were kindly provided by Dr A.Strasser (WEHI, Melbourne, Australia) and cultured as described elsewhere (Strasser *et al.*, 1995).

Antibodies and reagents

Monoclonal antibodies against FADD and caspase-3, and the rabbit polyclonal serum against Bcl-x_L were purchased from Transduction Laboratories (Lexington, Kentucky). The mouse monoclonal antibody against PARP (C-II-10) was a kind gift of Dr A.Bürkle (German Cancer Research Center, Heidelberg, Germany). The C15 mAb recognizes the p18 subunit of FLICE (Scaffidi *et al.*, 1997), and anti-APO-1 is an agonistic monoclonal antibody (IgG3, κ) recognizing an epitope on the extracellular part of APO-1 (CD95/Fas) (Trauth *et al.*, 1989). The TfR-specific mAb PA-1 (IgG1) was a generous gift from Dr G.Moldenhauer (German Cancer Research Center, Heidelberg, Germany). FII23c is a non-binding murine mAb (IgG3). The anti-cytochrome *c* antibody (7H8.2C12) was from PharMingen, and the anti-Bcl-2 antibody (N-19) and the HRPO-conjugated goat anti-rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). The HRPO-conjugated goat anti-mouse IgG1 and IgG2b were from Southern Biotechnology Associates (Birmingham, AL). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma (St Louis, MO).

DISC analysis by Western blotting

The amount of DISC-associated FLICE and FADD was determined as follows: 10^7 cells were either first treated with 2 μg/ml anti-APO-1 for 5 min at 37°C and then lysed in lysis buffer [30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and small peptide inhibitors (Kischkel *et al.*, 1995), 1% Triton X-100 (Serva) and 10% glycerol] (stimulated condition), or first lysed and then supplemented with anti-APO-1 (unstimulated condition). The CD95 DISC was then precipitated for 2 h at 4°C with protein A–Sepharose (Sigma). After immunoprecipitation the beads were washed five times with 20 volumes of lysis buffer. For Western blotting, immunoprecipitates or cytosolic proteins equivalent to 10^6 cells or 20 μg of protein were separated by 12% SDS–PAGE and transferred to Hybond nitrocellulose membrane (Amersham), blocked with 2% BSA in PBS/Tween (PBS + 0.05% Tween-20) for at least 1 h, washed with PBS/Tween and incubated with the primary antibody in PBS/Tween for 16 h at 4°C. Blots were developed with HRPO-conjugated secondary antibody diluted 1:20 000 in PBS/Tween. After washing with PBS/Tween the blots were developed with the chemiluminescence method (ECL) following the manufacturer's protocol (Amersham). Protein concentrations of cellular lysates were determined by the BCA method (Pierce).

Metabolic labeling and immunoprecipitation

Metabolic labeling and immunoprecipitation of CD95 was done essentially as described previously (Kischkel *et al.*, 1995). In short, 5×10^7 cells were washed twice with PBS and labeled in 15 ml RPMI without cysteine and methionine (Gibco-BRL) for 20 h with 1 mCi ^{35}S ProMix (Amersham). Untreated or anti-APO-1 (2 μg/ml) treated cells were washed with PBS and lysed in lysis buffer. After preclearing with FII23 mAb covalently coupled to CNBr-activated Sepharose beads (Pharmacia), and subsequent immunoprecipitation with an anti-transferrin receptor mAb coupled to anti-IgG1 Agarose (Sigma), the CD95 was immunoprecipitated for 1 h with either protein A–Sepharose (Sigma) (stimulated condition) or anti-APO-1 coupled to protein A–Sepharose (unstimulated condition). All immune complexes were subjected to IEF/SDS–PAGE analysis as described (Kischkel *et al.*, 1995).

Preparation of mitochondria and nuclei, and Western blot analysis for cytochrome c

Cells (3×10^8 per sample) were washed twice with ice-cold PBS and resuspended in five volumes of buffer A (50 mM Tris, 1 mM EGTA, 5 mM 2-mercaptoethanol, 0.2% BSA, 10 mM KH_2PO_4, pH 7.6, 0.4 M sucrose) and allowed to swell on ice for 20 min. Cells were homogenized with 30 strokes of a teflon homogenizer and centrifuged at 4000 *g* for 1 min at 4°C. The supernatants were further centrifuged at 10 000 *g* for 10 min at 4°C and the resulting pellets were resuspended in buffer B (10 mM KH_2PO_4, pH 7.2, 0.3 mM mannitol, 0.1% BSA). Mitochondria were separated by sucrose gradient (lower layer: 1.6 M sucrose, 10 mM KH_2PO_4, pH 7.5, 0.1% BSA; upper layer: 1.2 M sucrose, 10 mM KH_2PO_4, pH 7.5, 0.1% BSA). Interphases containing mitochondria were washed with buffer B centrifuged at 18 000 *g* for 10 min at 4°C and the resulting mitochondrial pellets were resuspended in buffer B. Protein concentrations of mitochondria were determined by Bradford method (Bio-Rad).

For isolation of nuclei, SKW6.4 cells were washed twice in ice-cold PBS, resuspended in 10 volumes of buffer C (10 mM PIPES, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DDT, 1 mM PMSF, 10 μM cytochalasin B), allowed to swell on ice for 20 min and homogenized using a Teflon homogenizer. Homogenisates were layered over 30% sucrose in buffer C and centrifuged at 800 *g* for 10 min. The resulting nuclear pellets were resuspended in buffer C and washed three times. Nuclei were stored at –80°C in aliquots at 2×10^8 nuclei/ml until required. For determination of nuclear fragmentation, mitochondria were incubated with nuclei in buffer D (10 mM HEPES, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DDT, 2 mM ATP, 10 mM phosphocreatine, 50 μg/ml creatine kinase, 10 μM cytochalasin B) for 2 h at 37°C. Nuclei were stained with propidium iodide and analyzed by flow cytometry. For determination of mitochondrial cytochrome *c*, isolated mitochondria were lysed using 1% NP-40 containing lysis buffer, and 10 μg of solubilized mitochondrial proteins were subjected to 15% SDS–PAGE and Western blot analysis using anti-cytochrome *c* mAb and ECL. To confirm equal loading of mitochondrial proteins, all Western blots were also developed with an antibody directed against a 60 kDa mitochondrial antigen (data not shown).

Determination of mitochondrial membrane potential and reactive oxygen species production

To measure $\Delta\Psi_m$ and reactive oxygen species (ROS) generation, anti-APO-1 (1 μg/ml) treated or untreated cells (5×10^5/ml) were incubated with 3,3-dihexyloxacarbocyanine iodide [$DiOC_6(3)$, 460 ng/ml; FL-1] (Moleculare Probes, Inc., Eugene, OR) for $\Delta\Psi_m$ and dihydroethidine (HE, 126 ng/ml, FL-3) (Moleculare Probes, Inc.) for ROS generation for 12 min at 37°C in the dark followed by analysis on a flow cytometer (FACScan).

Cytotoxicity assay

5×10^5 cells were incubated in 24-well plates (Costar, Cambridge, MA) with anti-APO-1 in 1 ml of medium at 37°C. Quantification of DNA

fragmentation as a specific measure of apoptosis was carried out by nuclear staining with propidium iodide, essentially as described elsewhere (Peter *et al.*, 1995).

Cell transfection

CEM cells were transfected by electroporation using a Gene-pulser (Bio-Rad) with control vector (pcDNA3) or Bcl-x_L expression vector (pcDNA3-bcl-x_L). Transfectants were selected by growth in G418 (1 mg/ml). Clones expressing high levels of Bcl-x_L were identified by Western blotting. MCF7 cells stably transfected to express CD95 alone (denoted MCF7-Fas) or both CD95 and Bcl-x_L (denoted MCF7-Fas-bcl-x_L) (gifts of Dr V.Dixit, University of Michigan) were plated on glass cellocate coverslips (Eppendorf) at a density of 60 000 per coverslip and placed in single wells of a 24-well plate. 24 h after plating, using Lipofectin (Life Technologies) cells were transfected with 0.5 μg of either pcDNA3 vector DNA (Invitrogen) or pcDNA3-pro-caspase-3; in both instances 0.2 μg of pCMV-green lantern protein was co-transfected as a transfection marker (Gibco-BRL). 24 h after transfection, cells were treated with 500 ng/ml anti-Fas (clone CH–11, PanVera Labs, Madison, WI) plus 1 μg/ml cycloheximide for 8 h. Hoechst dye 33342 (Sigma) was added to the medium to a final concentration of 4 μg/ml. Cells were incubated with Hoechst dye at 37°C for 30 min and photographed under UV illumination.

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References

Adachi,S., Cross,A.R., Babior,B.M. and Gottlieb,R.A. (1997) Bcl-2 and the outer mitochondrial membrane in the inactivation of cytochrome *c* during Fas-mediated apoptosis. *J. Biol. Chem.*, **272**, 21878–21882.

Armstrong,R.C. *et al.* (1996) Fas-induced activation of the cell death-related protease CPP32 is inhibited by Bcl-2 and by ICE family protease inhibitors. *J. Biol. Chem.*, **271**, 16850–16855.

Boldin,M.P., Varfolomeev,E.E., Pancer,Z., Mett,I.L., Camonis,J.H. and Wallach,D. (1995) A novel protein that interacts with the death domain of Fas/APO-1 contains a sequence motif related to the death domain. *J. Biol. Chem.*, **270**, 7795–7798.

Boldin,M.P., Goncharov,T.M., Goltsev,Y.V. and Wallach,D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell*, **85**, 803–815.

Boise,L.H. and Thompson,C.B. (1997) Bcl-x_L can inhibit apoptosis in cells that have undergone Fas-induced protease activation. *Proc. Natl Acad. Sci. USA*, **94**, 3759–3764.

Cahill,M.A., Peter,M.E., Kischkel,F.C., Chinnaiyan,A.M., Dixit,V.M., Krammer,P.H. and Nordheim,A. (1996) CD95 (APO-1/Fas) induces activation of SAP kinases downstream of ICE-like proteases. *Oncogene*, **13**, 2087–2096.

Chinnaiyan,A.M., O'Rourke,K., Tewari,M. and Dixit,V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 505–512.

Chinnaiyan,A.M., Tepper,C.G., Seldin,M.F., O'Rourke,K., Kischkel,F.C., Hellbardt,S., Krammer,P.H., Peter,M.E. and Dixit,V.M. (1996a) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.*, **271**, 4961–4965.

Chinnaiyan,A.M., Orth,K., O'Rourke,K., Duan,H., Poirier,G.G. and Dixit,V.M. (1996b) Molecular ordering of the cell death pathway. Bcl-2 and Bcl-x_L function upstream of the CED-3-like apoptotic proteases. *J. Biol. Chem.*, **271**, 4573–4576.

Chinnaiyan,A.M., O'Rourke,K., Lane,B.R. and Dixit,V.M. (1997) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science*, **275**, 1122–1126.

Chiu,V.K., Walsh,C.M., Liu,C.C., Reed,J.C. and Clark,W.R. (1995) Bcl-2 blocks degranulation but not Fas-based cell-mediated cytotoxicity. *J. Immunol.*, **154**, 2023–2032.

Cosulich,S.C., Green,S. and Clarke,P.R. (1996) Bcl-2 regulates activation of apoptotic proteases in a cell-free system. *Curr. Biol.*, **6**, 997–1005.

Enari,M., Hug,H. and Nagata,S. (1995) Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature*, **375**, 78–81.

Enari,M., Talanian,R.V., Wong,W.W. and Nagata,S. (1996) Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature*, **380**, 723–726.

Estoppey,S., Rodriguez,I., Sadoul,R. and Martinou,J.-C. (1997) Bcl-2 prevents activation of CPP32 cysteine protease and cleavage of poly (ADP-ribose) polymerase and U1-70 kDa proteins in staurosporine-mediated apoptosis. *Cell Death Differ.*, **4**, 34–38.

Fernandes-Alnemri,T. *et al.* (1995) Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.*, **55**, 6045–6052.

Fernandes-Alnemri,T. *et al.* (1996) *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl Acad. Sci. USA*, **93**, 7464–7469.

Hasegawa,J., Kamada,S., Kamiike,W., Shimizu,S., Imazu,T., Matsuda,H. and Tsujimoto,Y. (1996) Involvement of CPP32/Yama(-like) proteases in Fas-mediated apoptosis. *Cancer Res.*, **56**, 1713–1718.

Huang,D.C., Cory,S. and Strasser,A. (1997) Bcl-2, Bcl-x_L and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene*, **14**, 405–414.

Itoh,N., Tsujimoto,Y. and Nagata,S. (1993) Effect of Bcl-2 on Fas antigen-mediated cell death. *J. Immunol.*, **151**, 621–627.

Irmler,M. *et al.* (1997) Inhibition of death receptor signals by cellular FLIP. *Nature*, **388**, 190–195.

Jäättelä,M., Benedict,M., Tewari,M., Shayman,J.A. and Dixit,V.M. (1995) Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene*, **10**, 2297–2305.

Kamada,S., Washida,M., Hasegawa,J., Kusano,H., Funahashi,Y. and Tsujimoto,Y. (1997) Involvement of caspase-4(-like) protease in Fas-mediated apoptotic pathway. *Oncogene*, **15**, 285–290.

Kim,N.C., Wang,X., Huang,Y., Ibrado,A.M., Liu,L., Fang,G. and Bhalla,K. (1997) Overexpression of Bcl-x_L inhibits Ara-C-induced mitochondrial loss of cytochrome *c* and other perturbations that activate the molecular cascade of apoptosis. *Cancer Res.*, **57**, 3115–3120.

Kischkel,F.C., Hellbardt,S., Behrmann,I., Germer,M., Pawlita,M., Krammer,P.H. and Peter,M.E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.*, **14**, 5579–5588.

Kluck,R.M., Bossy-Wetzel,E., Green,D.R. and Newmeyer,D.D. (1997a) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275**, 1132–1136.

Kluck,R.M., Martin,S.J., Hoffman,B.M., Zhou,J.S., Green,D.R. and Newmeyer,D.D. (1997b) Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. *EMBO J.*, **16**, 4639–4649.

Kuida,K., Zheng,T.S., Na,S., Kuan,C., Yang,D., Karasuyama,H., Rakic,P. and Flavell,R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature*, **384**, 368–372.

Krippner,A., Matsuno-Yagi,A., Gottlieb,R.A. and Babior,B.M. (1996) Loss of function of cytochrome *c* in Jurkat cells undergoing Fas-mediated apoptosis. *J. Biol. Chem.*, **271**, 21629–21636.

Kroemer,G., Zamzami,N. and Susin,S.A. (1997) Mitochondrial control of apoptosis. *Immunol. Today*, **18**, 44–51.

Lacronique,V. *et al.* (1996) Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nature Med.*, **2**, 80–86.

Lee,R.K., Spielman,J. and Podack,E.R. (1996) Bcl-2 protects against Fas-based but not perforin-based T cell-mediated cytolysis. *Int. Immunol.*, **8**, 991–1000.

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

Li,F., Srinivasan,A., Wang,Y., Armstrong,R.C., Tomaselli,K.J and Fritz,L.C. (1997b) Cell-specific induction of apoptosis by microinjection of cytochrome *c*. *J. Biol. Chem.*, **272**, 30299–30305.

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

Los,M. *et al.* (1995) Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. *Nature*, **375**, 81–83.

Mandal,M., Maggirwar,S.B., Sharma,N., Kaufmann,S.H., Sun,S.C. and Kumar.R. (1996) Bcl-2 prevents CD95 (Fas/APO-1)-induced degradation of lamin B and poly(ADP-ribose) polymerase and restores the NF-κB signaling pathway. *J. Biol. Chem.*, **271**, 30354–30359.

Marchetti,P. *et al.* (1996) Mitochondrial permeability transition is a central coordinating event of apoptosis. *J. Exp. Med.*, **184**, 1155–1160.

Medema,J.P., Scaffidi,C., Kischkel,F.C., Shevchenko,A., Mann,M., Krammer,P.H. and Peter,M.E. (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J.*, **16**, 2794–2804.

Medema,J.P., Scaffidi,C., Krammer,P.H. and Peter,M.E. (1998) Bcl-x_L acts downstream of caspase-8 activation by the death-inducing signaling complex. *J. Biol. Chem.*, **273**, 3388–3393.

Memon,S.A., Moreno,M.B., Petrak,D. and Zacharchuk,C.M. (1995) Bcl-2 blocks glucocorticoid- but not Fas- or activation-induced apoptosis in a T cell hybridoma. *J. Immunol.*, **155**, 4644–4652.

Monney,L., Otter,I., Olivier,R., Ravn,U., Mirzasaleh,H., Fellay,I., Poirier,G.G. and Borner,C. (1996) Bcl-2 overexpression blocks activation of the death protease CPP32/Yama/apopain. *Biochem. Biophys. Res. Commun.*, **221**, 340–345.

Moreno,M.B., Memon,S.A. and Zacharchuk,C.M. (1996) Apoptosis signaling pathways in normal T cells: differential activity of Bcl-2 and IL-β-converting enzyme family protease inhibitors on glucocorticoid- and Fas-mediated cytotoxicity. *J. Immunol.*, **157**, 3845–3849.

Muzio,M. *et al.* (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex (DISC). *Cell*, **85**, 817–827.

Muzio,M., Salvesen,G.S. and Dixit,V.M. (1997) FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. *J. Biol. Chem.*, **272**, 2952–2956.

Newmeyer,D.D., Farschon,D.M. and Reed,J.C. (1994) Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell*, **79**, 353–364.

Nicholson,D.W. and Thornberry,N.A. (1997) Caspases: killer proteases. *Trends Biochem. Sci.*, **22**, 299–306.

Perry,D.K., Smyth,M.J., Wang,H.-G., Reed,J.C., Duriez,P., Poirier,G.G., Obeid,L.M. and Hannun,Y. (1997) Bcl-2 acts upstream of the PARP protease and prevents its activation. *Cell Death Differ.*, **4**, 29–33.

Peter,M.E., Hellbardt,S., Schwartz-Albiez,R., Westendorp,M.O., Moldenhauer,G., Grell,M. and Krammer,P.H. (1995) Cell surface sialylation plays a role in modulating sensitivity towards APO-1-mediated apoptotic cell death. *Cell Death Differ.*, **2**, 163–171.

Peter,M.E., Heufelder,A. and Hengartner,M.O. (1997a) Advances in apoptosis research. *Proc. Natl Acad. Sci. USA*, **94**, 12736–12737.

Peter,M.E., Kischkel,F.C., Scheuerpflug,C., Medema,J.P., Debatin,K.-M. and Krammer,P.H. (1997b) Resistance of cultured peripheral T cells towards activation induced cell death involves a lack of recuitment of FLICE to the death-inducing signaling complex (DISC). *Eur. J. Immunol.*, **27**, 1207–1212.

Peter,M.E., Scaffidi,C., Medema,J.P., Kischkel,F. and Krammer,P.H. (1998) The death receptors. In Kumar,S., (ed.), *Apoptosis, Problems and Diseases*. Springer, Heidelberg, in press.

Rodriguez,I., Matsuura,K., Khatib,K., Reed,J.C., Nagata,S. and Vassalli,P. (1996) A Bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection. *J. Exp. Med.*, **183**, 1031–1036.

Scaffidi,C., Medema,J.P., Krammer,P.H. and Peter,M.E. (1997) Two FLICE isoforms, caspase-8/a and caspase-8/b, are recruited and activated by the CD95 death-inducing signaling complex (DISC). *J. Biol. Chem.*, **272**, 26953–26958.

Schlegel,J., Peters,I., Orrenius,S., Miller,D.K., Thornberry,N.A., Yamin,T.T. and Nicholson,D.W. (1996) CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in Fas-mediated apoptosis. *J. Biol. Chem.*, **271**, 1841–1844.

Shimizu,S., Eguchi,Y., Kamiike,W., Waguri,S., Uchiyama,Y., Matsuda,H. and Tsujimoto,Y. (1996a) Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene*, **13**, 21–29.

Shimizu,S., Eguchi,Y., Kamiike,W., Matsuda,H. and Tsujimoto,Y. (1996b) Bcl-2 expression prevents activation of the ICE protease cascade. *Oncogene*, **12**, 2251–2257.

Spector,M.S., Desnoyers,S., Hoeppner,D.J. and Hengartner,M.O. (1997) Interaction between the *C.elegans* cell-death regulators CED-9 and CED-4. *Nature*, **385**, 653–656.

Srinivasan,A., Li,F., Wong,A., Kodandapani,L., Smidt,R., Krebs,J.F., Fritz,L.C., Wu,J.C. and Thomaselli,K.J. (1998) Bcl-x_L functions downstream of caspase-8 to inhibit Fas- and TNFR1-induced apoptosis in MCF7 breast carcinoma cells. *J. Biol. Chem.*, **273**, 4523–4529.

Srinivasula,S.M., Ahmad,M., Fernandes-Alnemri,T., Litwack,G. and Alnemri,E.S. (1996) Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple CED-3/ICE-like cysteine proteases. *Proc. Natl Acad. Sci. USA*, **93**, 14486–14491.

Strasser,A., Harris,A.W., Huang,D.C., Krammer,P.H. and Cory,S. (1995) Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J.*, **14**, 6136–6147.

Susin,S.A., Zamzami,N., Castedo,M., Hirsch,T., Marchetti,P., Macho,A., Daugas,E., Geuskens,M. and Kroemer,G. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J. Exp. Med.*, **184**, 1331–1341.

Susin,S.A., Zamzami,N., Castedo,M., Daugas,E., Wang,H.G., Geley,S., Fassy,F., Reed,J.C. and Kroemer,G. (1997) The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. *J. Exp. Med.*, **186**, 25–37.

Takahashi,A. *et al.* (1997) Affinity labeling displays the stepwise activation of ICE-related proteases by Fas, staurosporine, and CrmA-sensitive caspase-8. *Oncogene*, **14**, 2741–2752.

Takayama,S., Sato,T., Krajewski,S., Kochel,K., Irie,S., Millan,J.A. and Reed,J.C. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell*, **80**, 279–284.

Trauth,B.C., Klas,C., Peters,A.M., Matzku,S., Möller,P., Falk,W., Debatin,K.-M. and Krammer,P.H. (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science*, **245**, 301–305.

Vander Heiden,M.G., Chandel,N.S., Williamson,E.K., Schumacker,P.T. and Thompson,C.B. (1997) Bcl-x_L regulates the membrane potential and volume homeostasis of mitochondria. *Cell*, **91**, 627–637.

Vaux,D.L., Weissman,I.L. and Kim,S.K. (1992) Prevention of programmed cell death in *Caenorhabditis elegans* by human Bcl-2. *Science*, **258**, 1955–1957.

Vincenz,C. and Dixit,V.M. (1997) Fas-associated death domain protein interleukin-1β-converting enzyme 2 (FLICE2), an ICE/CED-3 homolog, is proximally involved in CD95- and p55-mediated death signaling. *J. Biol. Chem.*, **272**, 6578–6583.

Wu,D., Wallen,H.D. and Nuñez,G. (1997) Interaction and regulation of subcellular localization of CED-4 by CED-9. *Science*, **275**, 1126–1129.

Xue,D., Shaham,S. and Horvitz,H.R. (1996) The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes Dev.*, **10**, 1073–1083.

Yang,J., Liu,X., Bhalla,K., Kim,C.N., Ibrado,A.M., Cai,J., Peng,T.I., Jones,D.P. and Wang,X. (1997a) Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science*, **275**, 1129–1132.

Yang,X., Koshravi-Far,R., Chang,H.Y. and Baltimore,D. (1997b) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell*, **89**, 1067–1076.

Yuan,J.Y. and Horvitz,H.R. (1990) The *Caenorhabditis elegans* genes CED-3 and CED-4 act cell autonomously to cause programmed cell death. *Dev. Biol.*, **138**, 33–41.

Zamzami,N., Susin,S.A., Marchetti,P., Hirsch,T., Gomez-Monterrey,I., Castedo,M. and Kroemer,G. (1996) Mitochondrial control of nuclear apoptosis. *J. Exp. Med.*, **183**, 1533–1544.

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

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