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Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8

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The involvement of the death adaptor protein FADD and the apoptosis-initiating caspase-8 in CD95 and TRAIL death signalling has recently been demonstrated by the analysis of the native death-inducing signalling complex (DISC) that forms upon ligand-induced receptor cross-linking. However, the role of caspase-10, the other death-effector-domain-containing caspase besides caspase-8, in death receptor signalling has been controversial. Here we show that caspase-10 is recruited not only to the native TRAIL DISC but also to the native CD95 DISC, and that FADD is necessary for its recruitment to and activation at these two protein complexes. With respect to the function of caspase-10, we show that it is not required for apoptosis induction. In addition, caspase-10 can not substitute for caspase-8, as the defect in apoptosis induction observed in caspase-8-deficient cells could not be rescued by overexpression of caspase-10. Finally, we demonstrate that caspase-10 is cleaved during CD95-induced apoptosis of activated T cells. These results show that caspase-10 activation occurs in primary cells, but that its function differs from that of caspase-8.

Keywords: caspase/CD95 (APO-1/Fas)/death-inducing signalling complex/death receptor/TRAIL

Introduction

Apoptosis is an essential process during the development of the immune system and for the maintenance of T- and B-cell homeostasis. Besides tumour necrosis factor (TNF) itself, two other apoptosis-inducing members of the TNF family, CD95 (APO-1/Fas) ligand (CD95L) and TNF-related apoptosis-inducing ligand (TRAIL/APO-2L), have been shown to be involved in various immunological processes. The involvement of the CD95 receptor–ligand system in inflammation, activation-induced death of peripheral T cells, immune privilege, tumour evasion from the immune system, autoimmunity and AIDS is well established (Nagata, 1997; Krammer, 2000). The functional analysis of the TRAIL receptor–ligand system has been complicated by the fact that a total of five different receptors for this cytokine have been identified (Griffith and Lynch, 1998; Locksley *et al*., 2001). Recently, TRAIL has been shown to be functional in various processes of the immune system and tumour surveillance (Walczak and Krammer, 2000). Of particular interest was the recent finding that TRAIL was necessary for natural killer cell-mediated suppression of liver metastasis in a mouse tumour model (Smyth *et al*., 2001; Takeda *et al*., 2001).

The early biochemical events that result in apoptosis induction by TRAIL and CD95L have been studied by analysis of the so-called death-inducing signalling complex (DISC) (Kischkel *et al*., 1995; Walczak and Sprick, 2001). Cross-linking of CD95 or the two apoptosis-inducing TRAIL receptors results in the recruitment of FADD and caspase-8 (Boldin *et al*., 1995; Chinnaiyan *et al*., 1995) to the respective DISC (Boldin *et al*., 1996; Muzio *et al*., 1996; Bodmer *et al*., 2000; Kischkel *et al*., 2000; Sprick *et al*., 2000). In a homotypic interaction, the death domain (DD) of FADD binds to the DD of CD95. The death effector domain (DED) of FADD in turn interacts with the DED of pro-caspase-8 and thereby recruits this pro-enzyme to the CD95 DISC (Medema *et al*., 1997). Pro-caspase-8 is proteolytically cleaved and thereby activated at the DISC. Activated caspase-8 then initiates the apoptosis-executing caspase cascade (Peter *et al*., 1999).

Studies using cells from mice deficient in either FADD (Yeh *et al*., 1998) or caspase-8 (Varfolomeev *et al*., 1998) showed that these two proteins are essential for CD95L-induced apoptosis. However, Holler *et al*. (2000) and Matsumura *et al*. (2000) reported that human Jurkat cells deficient in caspase-8 underwent caspase-independent necrotic cell death after stimulation with highly active CD95L. The molecular mechanisms responsible for this type of cell death remain largely unclear, although the molecule RIP has been proposed to be essential for CD95L-induced necrosis (Holler *et al*., 2000).

Until recently, the molecular explanation for a rare immunological disorder in children called autoimmune lymphoproliferative syndrome (ALPS) had been restricted to the CD95 receptor–ligand system (Straus *et al*., 1999; Fischer *et al*., 2000). However, two patients with severe ALPS have recently been identified who did not carry mutations in either the CD95 or the CD95L gene. Comprehensive analysis of apoptosis-associated genes in these patients revealed mutations in the caspase-10 gene. These mutations were shown to result in a disease termed ALPS II (Wang *et al*., 1999). In this study, it was proposed that TRAIL resistance of mature DC and activated peripheral T cells from ALPS II patients was due to the mutated non-functional caspase-10 and causative for the disease. Later, one of the mutations identified in this study was found to be a common polymorphism in the Danish population (Gronbaek *et al*., 2000), although to date, besides the ALPS II patient identified by Wang *et al*. (1999), no other individual homozygous for this

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caspase-10 mutation has been described. This finding raised the question whether this mutation in caspase-10 alone is causative for the disease.

Caspase-10 (Mch4, FLICE-2) is the second known DED-containing caspase besides caspase-8, and was identified by homology cloning (Fernandes-Alnemri *et al*., 1996; Vincenz and Dixit, 1997). The FLICE-2 and Mch4 isoforms represent different splice variants derived from the same gene. Later, two additional isoforms were identified (Ng *et al*., 1999). Mch4 is now known as caspase-10a, FLICE-2 as caspase-10b, and the two recently identified variants as caspase-10c and caspase-10d. While the caspase-10a, -10b and -10d isoforms represent proteins that contain both the large and small catalytic subunits, the caspase-10c variant represents a truncated form yielding a catalytically inactive molecule. While previous studies on the function of caspase-10 relied on overexpression of the different isoforms or catalytically inactive variants, no detailed study under native conditions with the endogenously expressed proteins has been conducted so far. Protein overexpression experiments are prone to artefacts, yielding a cautionary note to the results obtained in these systems. Furthermore, the expression and distribution of the different caspase-10 isoforms have been unclear, as only mRNA levels have been investigated.

We therefore set out to study (i) the endogenous expression levels of caspase-10, (ii) whether it forms part of the TRAIL and the CD95 DISCs under native conditions, and (iii) whether caspase-8 and caspase-10 are functionally redundant or might fulfil different functions.

Results

Caspase-10 is expressed in three isoforms

The role of caspase-10 in CD95- and TRAIL-mediated apoptosis has been controversial. At least four splice variants of caspase-10 have been reported (Fernandes-Alnemri *et al*., 1996; Vincenz and Dixit, 1997; Ng *et al*., 1999). Three of the reported caspase-10 isoforms contain the large and the small subunits of caspase-10 and yield potentially active enzymes. The fourth variant, caspase-10c, codes for a truncated form that lacks the catalytic subunits and, thus, represents a catalytically inactive form of caspase-10 reminiscent of the CAP3 form of caspase-8 (Kischkel *et al*., 1995; Muzio *et al*., 1996). To investigate the potential role of caspase-10 during TRAIL- and CD95L-mediated apoptosis, we first set out to identify a caspase-10-specific antibody. Amongst the panel of commercially available antibodies that we tested, only one specifically recognized bands at 55 and 59 kDa in Chinese hamster ovary (CHO) cells transfected with expression plasmids encoding recombinant caspase-10a and -10d, respectively (Figure 1A). The epitope recognized by this antibody is localized in the DED-containing prodomain as the antibody also detects the recombinantly expressed His-tagged prodomain (Figure 1A). All splice variants of caspase-10 reported thus far contain this prodomain. Therefore, this antibody should recognize all known isoforms of caspase-10, while all other antibodies we tested did not react with either the recombinant prodomain or with overexpressed caspase-10a or -10d in CHO cells (data not shown).

Having identified a specific antibody for caspase-10, we investigated which isoforms of caspase-10 are expressed in the human Burkitt's lymphoma cell line BJAB and the human T-cell line Jurkat. Two large isoforms of caspase-10 (p55 and p59) were detected in the lysates derived from both cell lines (Figure 1B), reminiscent of the two large isoforms of caspase-8. At ~28 kDa, an additional isoform of caspase-10 was detected. To determine to which of the published isoforms the individual bands correspond, we performed two-dimensional (2D) gel electrophoretic analysis (Figure 1B). The calculated molecular weights and isoelectric points (pI) for the different isoforms of caspase-10 are shown in Figure 1C. As a control, the same blot was calibrated by detection of caspase-8, showing that the 2D-determined pI of the p55 and p53 caspase-8 isoforms exactly matched their predicted pI of 4.9 and 5.0, respectively (Figure 1D). Determination of the pI of the two expressed large isoforms of caspase-10 revealed that they represent caspase-10d (p59) and caspase-10a (p55). This analysis further excluded the possibility that one of them could represent caspase-10b (calculated pI 7.3). As the pI of the 28 kDa band matched that of caspase-10c, this protein spot most likely corresponded to this isoform of caspase-10.

Caspase-10 is cleaved early during CD95- and TRAIL-induced apoptosis

Caspase-8 has been shown to be the apoptosis-initiating caspase in the CD95 and the TRAIL DISCs and, thus far, caspase-8 has been thought to be the earliest caspase to become cleaved during death-receptor-mediated apoptosis. Knowing that caspase-8 is cleaved during TRAIL- and CD95L-induced apoptosis, we next analysed whether caspase-10 might also be processed during death-receptor-mediated apoptosis and whether this cleavage occurs prior to, concomitantly with, or following caspase-8 cleavage (Figure 2). During TRAIL- and CD95L-induced apoptosis, the two large isoforms of caspase-10 were cleaved, resulting in two intermediate cleavage products of caspase-10a and -10d, p43 and p47, respectively (Figure 2). Further cleavage of these two intermediates resulted in the appearance of a 25 kDa band representing the prodomain of caspase-10. Also, the 28 kDa caspase-10c was cleaved during TRAIL- and CD95-induced apoptosis. Thus, caspase-10 is present in both BJAB and Jurkat cells in three different isoforms, and all of these isoforms are cleaved during TRAIL- and CD95-mediated apoptosis. Depending on cell type and stimulus, this cleavage was apparent between 15 and 30 min after onset of the incubation with TRAIL or CD95L, concomitantly with the appearance of caspase-8 cleavage in the cytoplasm.

Caspase-10 is recruited to the CD95 and the TRAIL DISCS in BJAB and Jurkat cells

Like caspase-8, caspase-10 contains the tandem DEDs necessary for homotypic interaction with the adapter protein FADD, making recruitment of caspase-10 to death receptor-bound FADD-containing complexes possible. Caspase-8 is recruited to the CD95, the TRAIL-R1 and the TRAIL-R2 signalling complexes after ligand-induced

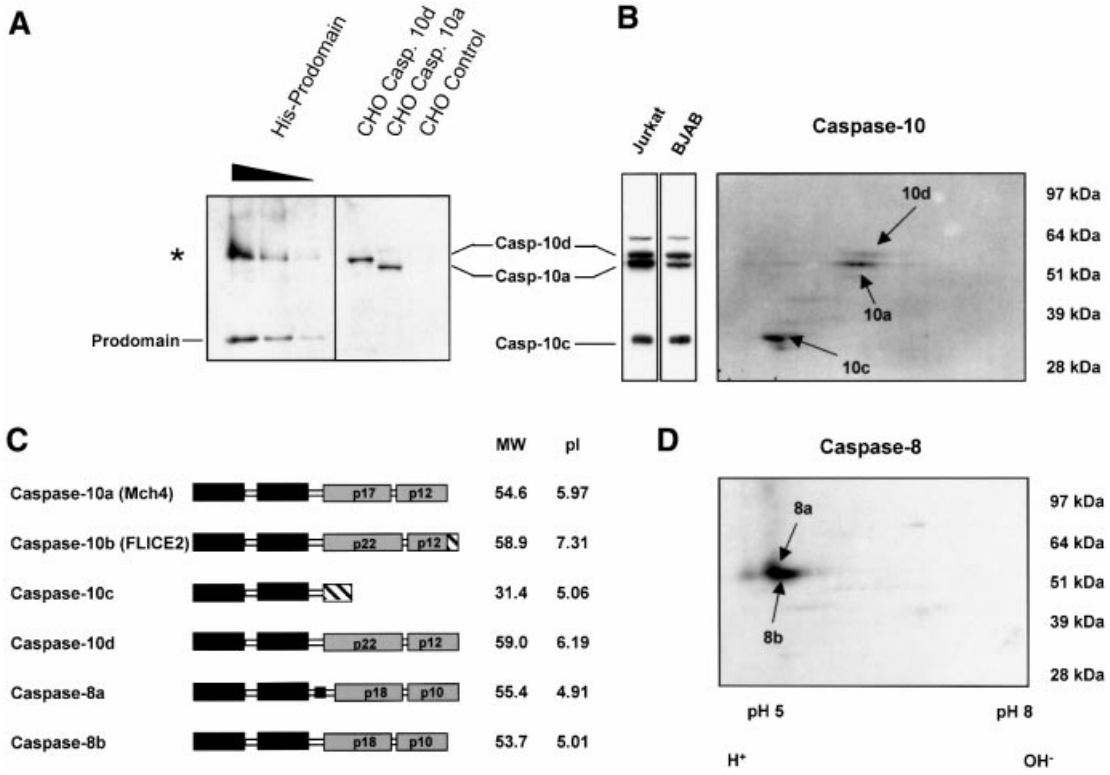

Fig. 1. Three isoforms of caspase-10 are expressed in BJAB and Jurkat cell lines. (**A**) Specificity of the caspase-10 antibody was determined by blotting decreasing amounts of bacterially expressed His-tagged caspase-10 prodomain or lysates from CHO cells transfected with expression plasmids for human caspase-10d, -10a or a control plasmid. The asterisk denotes a co-purified protein resulting from inefficient termination of translation. (**B**) Three endogenous isoforms of caspase-10 can be detected in lysates from BJAB and Jurkat cells. Proteins (10 μg) from the indicated cell lines were resolved by SDS–PAGE, blotted and detected with the caspase-10 antibody. Lysates from BJAB cells were resolved on 2D gels spanning pH 5–8, blotted and subsequently detected with caspase-10 antibody. The pI and molecular weight (MW) of the large caspase-10 isoforms correspond to caspase-10d and caspase-10a, and the small protein to the pI and MW of caspase-10c. (**C**) Overview of the calculated MW and pI of the reported caspase-10 and caspase-8 isoforms. (**D**) As a control for the pI determination, the 2D-gel blot was reprobed with an antibody to caspase-8, showing the caspase-8 isoforms at the expected positions.

stimulation of the respective receptors in a FADD-dependent fashion. As results concerning protein–protein interactions that rely solely on transient protein expression are prone to artefacts, we analysed the receptor complexes under native conditions. We compared the DISCs that form after stimulation with CD95L and TRAIL in both Jurkat and BJAB cells, and tested whether caspase-10 is recruited to these complexes (Figure 3). To control for efficiency of DISC formation, we analysed recruitment of caspase-8 and FADD, two already known components of the TRAIL and the CD95 DISCs (Figure 3A). Whereas none of the proteins analysed was bound to the non-stimulated receptors, we observed a stimulation-dependent recruitment of FADD and caspase-8 to CD95 and the TRAIL death receptors, as described before (Bodmer *et al*., 2000; Kischkel *et al*., 2000; Sprick *et al*., 2000). For caspase-10 the analysis revealed ligand-induced recruitment of all three expressed caspase-10 isoforms to the native TRAIL DISC and the native CD95 DISC in both cell types (Figure 3B). As with caspase-8, the products of the first activation step of caspase-10a and -10d (p43 and p47) were also associated with the TRAIL and the CD95 DISCs. These two forms represent the prodomain with the p17 subunit of caspase-10a and -10d, respectively, after separation of the p12 subunit by proteolytic cleavage. In addition, the prodomain is also apparent in both DISCs (Figure 3B, lower panel).

Caspase-10 is recruited to the TRAIL-R1 and the TRAIL-R2 DISCs

So far, no biochemical differences in recruitment of adaptor and effector proteins between TRAIL-R1 and TRAIL-R2 have been identified. To investigate whether caspase-10 is recruited selectively to one of the apoptosis-inducing TRAIL receptors, we performed differential DISC analysis for TRAIL-R1 and TRAIL-R2 on BJAB cells (Figure 3C). Either one of the two receptors was blocked by incubation with a monoclonal antibody (mAb) specific for TRAIL-R1 or TRAIL-R2 before stimulation of the other receptor by the ligand. Efficiency of inhibition of the receptors was demonstrated by complete inhibition of FADD and caspase-8 recruitment upon simultaneous TRAIL-R1 and TRAIL-R2 blockage. The analysis of the resulting complexes shows that both caspase-10a and -10d were recruited to both receptors independently of the other receptor.

FADD is necessary for the recruitment of caspase-10 to the CD95, the TRAIL-R1 and the TRAIL-R2 DISCs

FADD has been shown to be required for the recruitment and subsequent activation of caspase-8 to the TRAIL-R2 and the CD95 DISCs. To test whether FADD is also required for recruitment of caspase-10 to the CD95, TRAIL-R1 and TRAIL-R2 DISCs, we investigated the

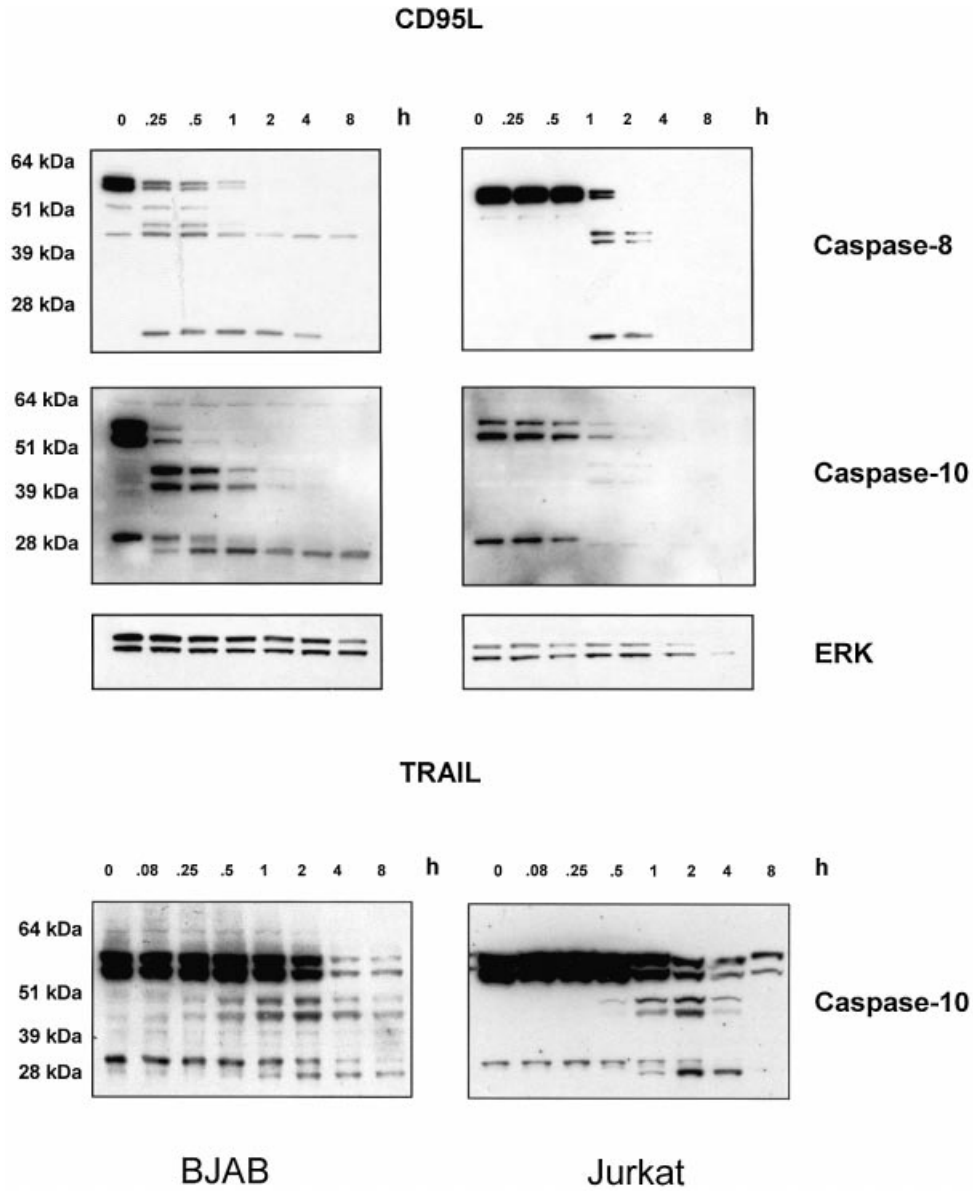

Fig. 2. Caspase-10 is processed in TRAIL- and CD95L-sensitive cell lines after stimulation with the respective ligands. Cells were stimulated for the indicated time periods with TRAIL (1 μg/ml) or CD95L (500 ng/ml). Lysates were prepared, and analysed by western blotting with antibodies to caspase-8 and caspase-10. As a control, the blot containing lysates from cells stimulated with CD95L was reprobed with an antibody to ERK. Cleavage of all caspase-10 isoforms can be observed as early as 15–30 min for stimulation with CD95L (upper panel) or TRAIL (lower panel), respectively. Cleavage of caspase-10 leads to the appearance of two intermediate products, p47 and p43, and a smaller band corresponding to the prodomain at 25 kDa. The cleavage pattern is similar to that observed for caspase-8, which is included as a control. The lower amount of ERK control observed after 8 h of stimulation is the result of almost complete cell death observed at this time point.

respective protein complexes in cells that lack FADD but express all three receptors (Figure 4). As the original FADD-deficient Jurkat cells only expressed TRAIL-R2 (Bodmer *et al*., 2000; Sprick *et al*., 2000), we established a stable subclone of this cell line transfected with a TRAIL-R1 expression plasmid. This clone, which we named Jurkat FADD^{def}-TR1/2, expressed TRAIL-R1 and TRAIL-R2 on its surface (Figure 4A). The protein complexes that formed upon stimulation with CD95L and TRAIL are shown in Figure 4B. While the CD95 and the TRAIL DISCs were efficiently formed in the parental Jurkat cells by incubation of these cells with CD95L and TRAIL, respectively, the absence of FADD resulted in failure of the cross-linked receptors to recruit caspase-8 and caspase-10. Thus, FADD is not only necessary for recruitment and activation of caspase-8, but also for recruitment and activation of caspase-10 at the TRAIL and the CD95 DISCs.

Caspase-10 is not necessary for death-receptor-mediated apoptosis and caspase-8 activation at the DISC

As both caspase-8 and caspase-10 are present in the DISC complexes, it could be possible that both are needed for apoptosis to be initiated at the CD95 death receptors and the TRAIL receptors. We noted that several cell lines, including the Burkitt's lymphoma cell line BL60 and the CD95-transfected BL60 subclone K50, do not express

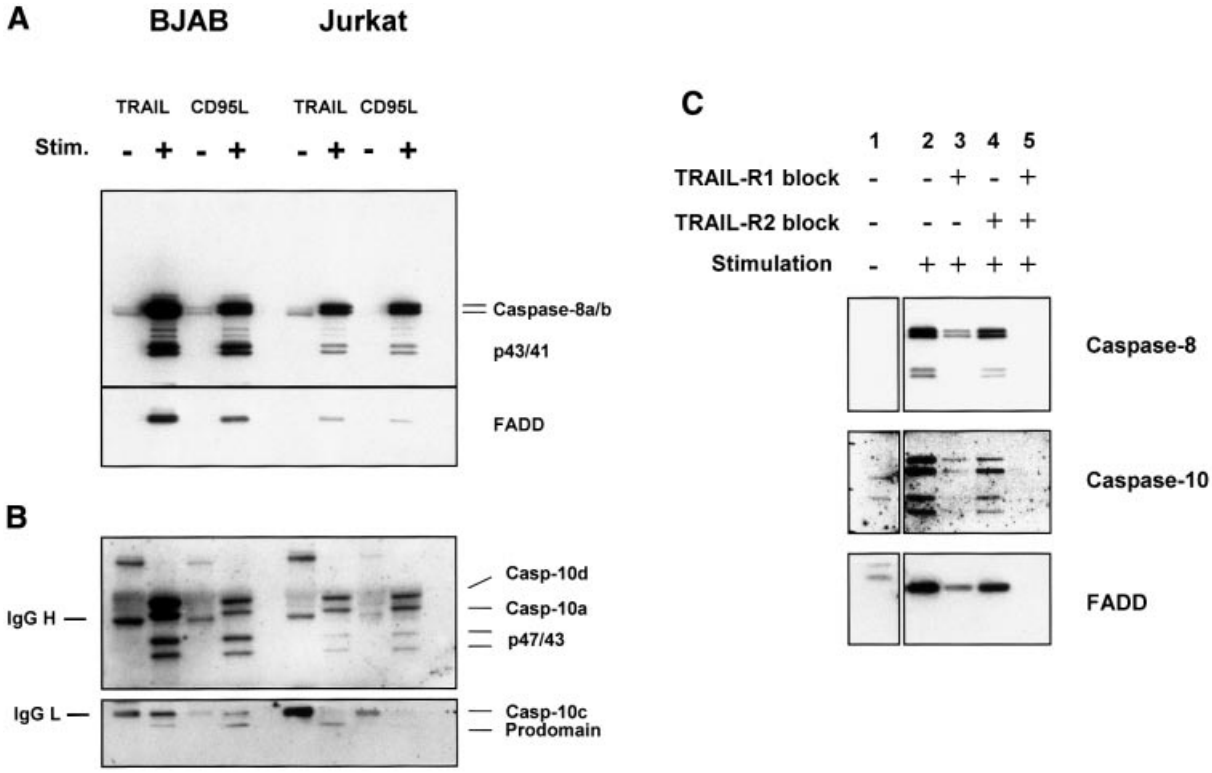

Fig. 3. Caspase-10 is recruited to the native TRAIL and CD95 DISCs. Precipitation of the DISC complexes, which form upon stimulation with TRAIL and CD95L, was performed in BJAB and Jurkat cells. The resulting precipitated protein complexes were separated by SDS–PAGE, and analysed by western blotting. (**A**) To control for DISC formation, the western blots were probed for the known DISC components caspase-8 and FADD, showing recruitment of these proteins to the TRAIL and the CD95 DISC in BJAB and Jurkat cells. (**B**) The DISC complexes were also analysed for the presence of caspase-10, showing that caspase-10 is recruited to the TRAIL DISC and the CD95 DISC in both cell lines tested. All three caspase-10 isoforms associate with both complexes. In addition, the intermediate products p47 and p43 and the fragment corresponding to the prodomain p25 are also associated with the CD95 and the TRAIL DISCs. The light chain of the M2 anti-FLAG antibody migrates at the same MW as the caspase-10 isoform. Correlation of heavy and light chain levels reveals caspase-10c association with cross-linked receptors. (**C**) Caspase-10 associates with homomeric TRAIL-R1 and TRAIL-R2 complexes. Differential TRAIL DISC analysis was carried out by blocking either TRAIL-R1 or TRAIL-R2 with mAbs (10 μg/ml for 20 min at 25°C) against the respective receptors before DISC analysis was performed. The resulting complexes were separated by SDS–PAGE and analysed by western blotting. While precipitation of the unstimulated receptors shows no association of the proteins analysed (lane 1), under conditions where both receptors are stimulated (lane 2), caspase-10 associates with both TRAIL-R2 complexes (lane 3) and TRAIL-R1 complexes (lane 4). Blockage of both receptors completely abolishes DISC formation (lane 5).

detectable levels of caspase-10. Although K50 cells are devoid of detectable levels of caspase-10 (Figure 5A, upper panel), stimulation with CD95L or TRAIL resulted in normal apoptosis induction (Figure 5B). In addition, caspase-8 is recruited to both the CD95 and the TRAIL DISCs in this cell line, and is activated as indicated by the presence of the cleavage products p43 and p41 in the DISC (Figure 5C). Thus, caspase-10 is dispensable for activation of caspase-8 at the DISC and concomitant apoptosis induction.

Caspase-10 can not functionally substitute caspase-8, although it is recruited to the DISC in the absence of caspase-8

It has been shown previously by us and others that Jurkat cells deficient in caspase-8 are defective in apoptosis induction after stimulation with CD95L or TRAIL. However, analysis of expression of caspase-10 in this cell line revealed that the levels of caspase-10 are substantially reduced compared with the parental cell line (Figure 6A). To test whether caspase-10 can induce apoptosis in the absence of caspase-8, we established stable clones that expressed caspase-10d in the absence of caspase-8. To expand our analysis of the TRAIL DISC to TRAIL-R1, we first generated a caspase-8-deficient cell line stably transfected with a TRAIL-R1 expression plasmid, as the Jurkat caspase-8^{def} cell lines normally express only TRAIL-R2. A resulting clone was transfected with an expression plasmid for caspase-10. Several independent caspase-10-expressing clones were chosen for analysis. The amounts of caspase-10 expressed in these clones ranged from relatively low levels to amounts greatly exceeding that of caspase-10 present in the parental cell line, in the parental Jurkat caspase-8^{def} and also in primary T cells (data not shown). Expression of the TRAIL receptors and CD95 was comparable in the different cell lines, as determined by surface staining (data not shown). We next analysed TRAIL sensitivity of these cell lines in comparison to the parental clones. Surprisingly, apoptosis induction was not significantly higher in caspase-10-transfected versus non-transfected caspase-8-deficient cell lines (Figure 6B, upper panel).

Stimulation with CD95L led to the induction of cell death in the caspase-8-deficient cell lines, although much

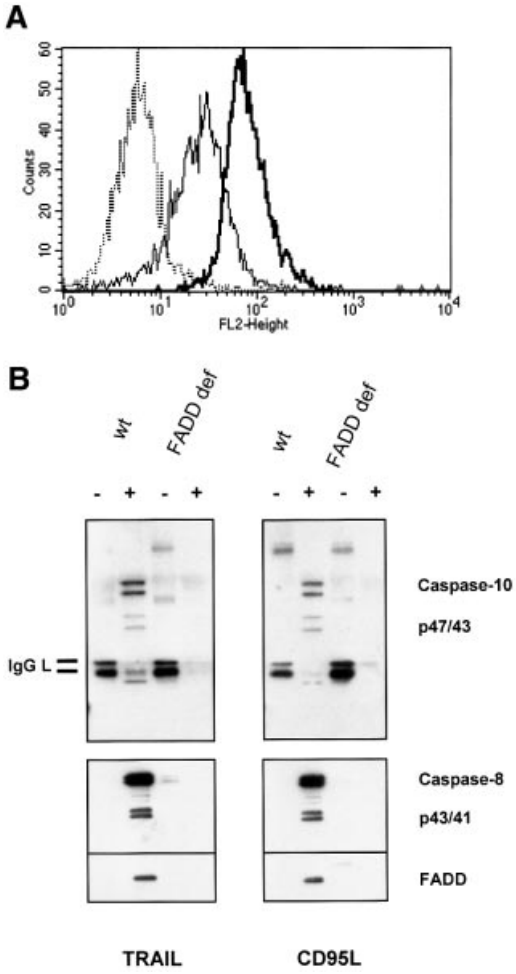

Fig. 4. FADD is the adapter protein for caspase-10 recruitment to TRAIL-R1, TRAIL-R2 and CD95. (**A**) FACS stain of the FADD-deficient cell line FADD^{def}-TR1/2. A surface stain for TRAIL-R1 (thin line) and TRAIL-R2 (thick line) shows expression of both TRAIL-R1 and TRAIL-R2. Dotted line, IgG control. (**B**) DISC analysis was performed with TRAIL and CD95L on wild-type and FADD^{def}-TR1/2 Jurkat cells. Western blot analysis of the precipitated protein complexes shows that caspase-8, caspase-10 and FADD are efficiently recruited to the TRAIL DISC and the CD95 DISC in the parental wild-type (wt) cells. In the absence of the adapter protein FADD in the cell line Jurkat FADD^{def}-TR1/2 (FADD def), caspase-10 as well as caspase-8 are not recruited to either the TRAIL DISC or to the CD95 DISC.

higher amounts of CD95L were needed when comparing caspase-8-deficient cell lines with the caspase-8-expressing control cell line (Figure 6B, lower panel). Recently, cell death induction by CD95L in caspase-8-deficient Jurkat cells was described (Holler *et al*., 2000; Matsumura *et al*., 2000). However, as for stimulation with TRAIL, overexpression of caspase-10 did not result in a sensitization to CD95L-induced apoptosis when compared with the parental caspase-8-deficient cell line. The inability of caspase-10 to substitute for caspase-8 could be due to its inability to be activated at the DISC complexes in the absence of caspase-8. Yet, analysis of the DISC shows that caspase-10 is also recruited to and activated at the CD95 DISC in the absence of caspase-8 (Figure 6C). Together with the findings presented in Figure 6B, this result shows that although caspase-10 is recruited to and activated at the DISC, it can not functionally substitute for caspase-8 in apoptosis induction.

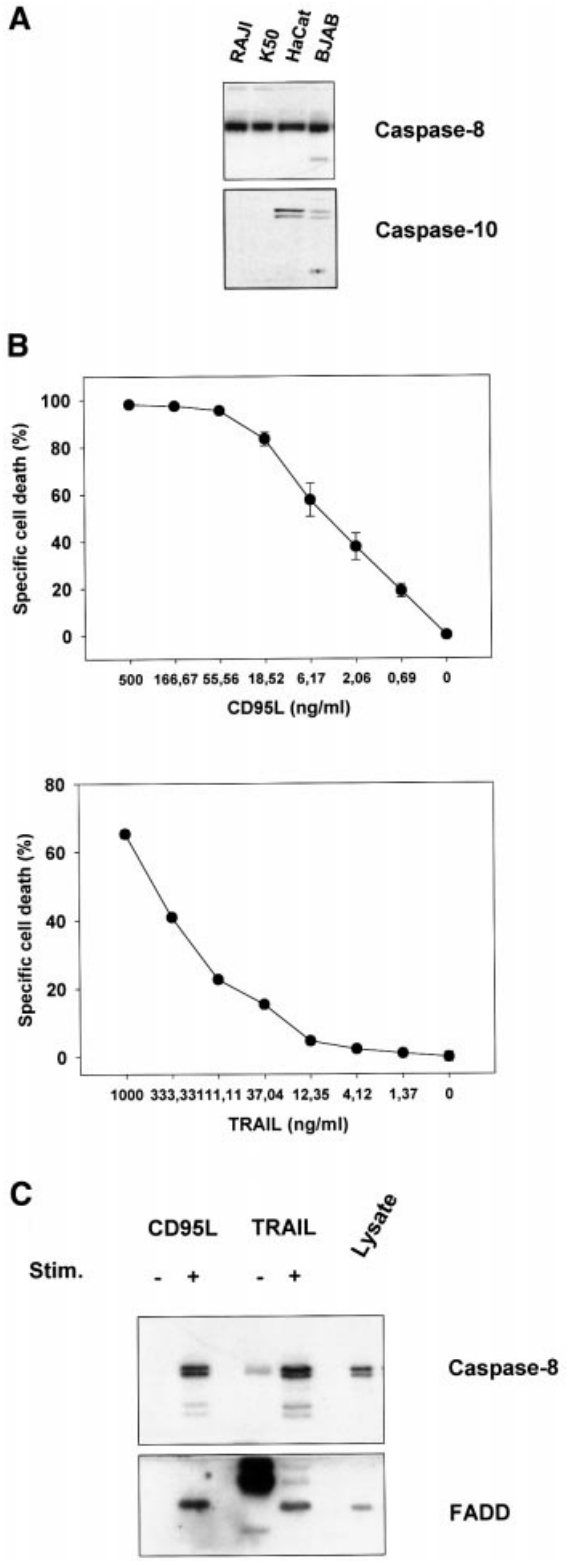

Fig. 5. Caspase-10 is not required for TRAIL- and CD95L-mediated cell death and activation of caspase-8. (**A**) Western blotting of lysates of different cell lines shows that some cell lines do not express detectable levels of caspase-10. (**B**) Cells from the cell line K50 were incubated in CD95L or TRAIL at the concentrations indicated. Cell death was measured after 16 h by pI exclusion and FSC/SSC analysis. Both ligands induced cell death in the absence of detectable levels of caspase-10. (**C**) Analysis of the TRAIL and CD95 DISCs in K50 cells shows that caspase-8 is recruited and activated at both DISC complexes in the absence of caspase-10. The DISCs were precipitated and analysed as in Figure 4B.

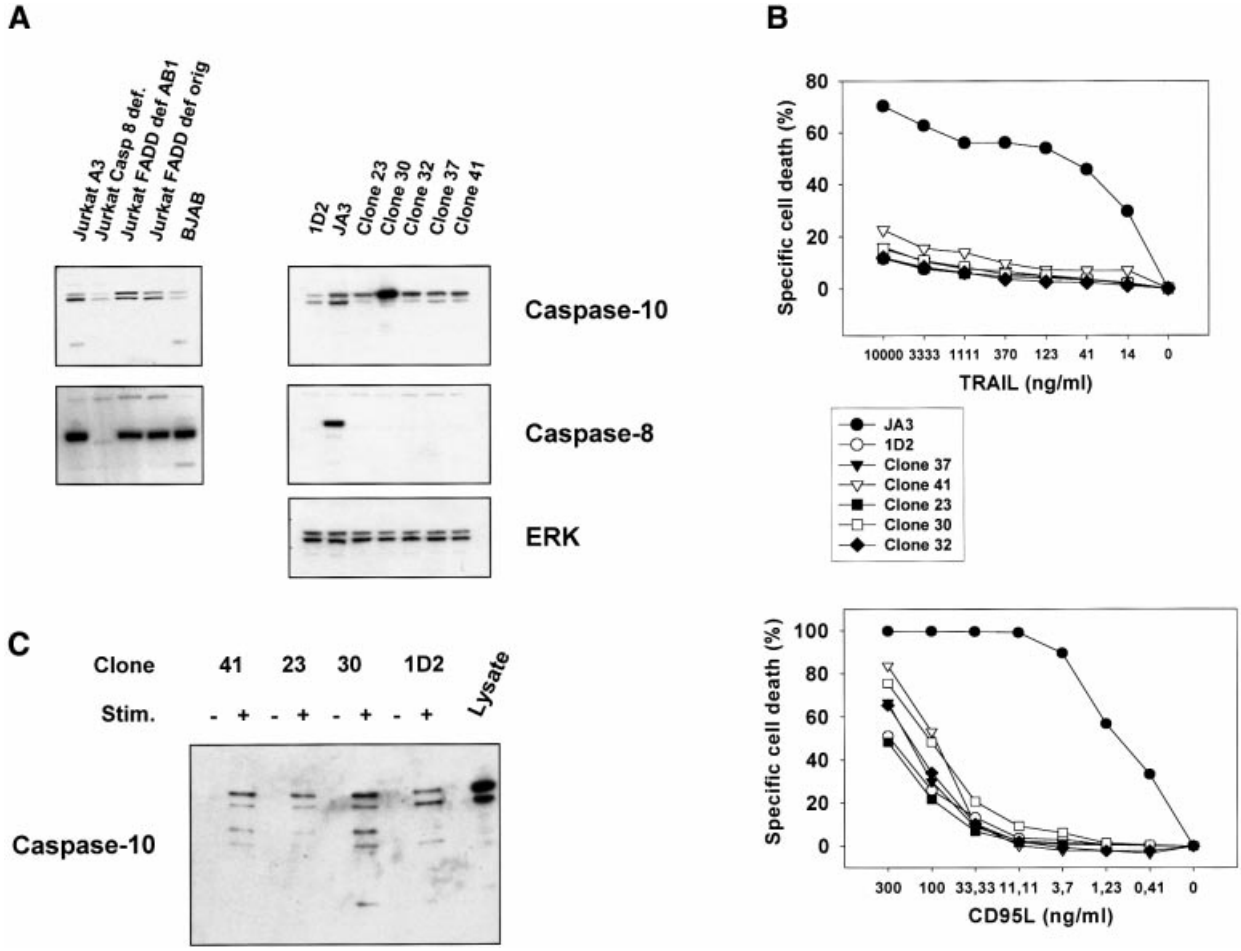

Fig. 6. Caspase-10 can not functionally substitute loss of caspase-8. (**A**) Comparison of the expression levels of caspase-10 in different cell lines and clones stably transfected with caspase-10. Cell lysates from the cell lines indicated were resolved by SDS–PAGE and blotted. The blots were probed with antibodies against caspase-10 and caspase-8. (**B**) High expression levels of caspase-10 do not sensitize caspase-8-deficient cell lines to TRAIL- or CD95L-induced apoptosis. Different cell lines were incubated with either TRAIL (upper panel) or CD95L (lower panel) at the concentrations indicated. Cell death was assessed after 24 h by measuring the decrease in FSC/SSC and pI exclusion. (**C**) Caspase-10 is recruited to and activated at the CD95 DISC in the absence of caspase-8. Cells were stimulated with CD95L at 100 ng/ml for 15 min. After lysis, the DISC complexes were precipitated with an antibody against CD95 (anti-APO-1), resolved by SDS–PAGE and blotted. The blots were probed for caspase-10, showing recruitment of caspase-10 in the caspase-8-deficient cell line 1D2 and in the caspase-10-transfected caspase-8-deficient cell lines (clones 41, 23 and 30). Caspase-10 is recruited to the CD95 DISC in all cell lines even in the absence of caspase-8. Also, in all cases, caspase-10 is activated in these complexes, as indicated by the appearance of the p47/p43 fragments. Lysate denotes the lane containing control lysate from unstimulated clone 41.

Caspase-10 is expressed in primary human T cells and is cleaved upon CD95-induced apoptosis of pre-activated human T cells

Mutations in caspase-10 have been shown to result in the human ALPS II that is characterized by a defect in T-cell and dendritic cell apoptosis (Wang *et al*., 1999). These results prompted us to test whether caspase-10 might play a role in apoptosis induction in primary cells. We therefore analysed whether caspase-10 is cleaved during CD95-induced apoptosis of pre-activated human T cells. This analysis showed that caspase-10a, -10d and -10c were present in lysates obtained from these cells, and that all caspase-10 isoforms were cleaved following CD95 stimulation (Figure 7). The observed cleavage pattern was essentially identical to the pattern observed in BJAB and Jurkat cells (Figure 2). The proteolytic activation of caspase-10 during apoptosis of activated T cells is indicative of a role for this caspase in the transmission of signals emanating from death receptors not only in transformed cell lines but also in primary cells of the immune system.

Discussion

One regulatory step during death-receptor-mediated apoptosis is the assembly of the apical caspase-activating protein complex, the DISC. To understand the mechanism of death-receptor-mediated apoptosis, a detailed analysis of the composition of the DISC complex is essential. The recent description of patients with ALPS II raised a number of interesting questions (Wang *et al*., 1999). In the two patients described in the study, a mutation in the caspase-10 gene was proposed to be causative for the disease. One of the two identified mutants, which completely abrogated caspase-10 function (L242F), was shown to exert a clear dominant-negative effect upon overexpression on TRAIL-induced apoptosis and, interestingly, also on CD95-induced apoptosis. The other mutated form of

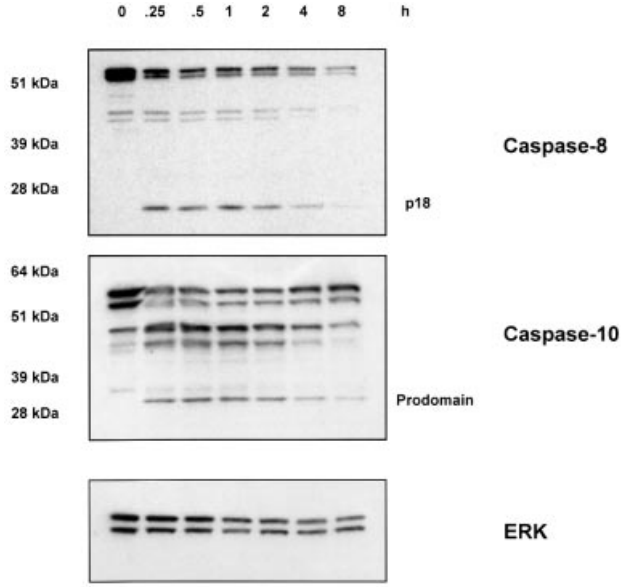

Fig. 7. Caspase-10 is expressed and rapidly processed in primary T cells after stimulation with CD95L. Activated primary T cells were stimulated with CD95L (500 ng/ml) for the time periods indicated, lysed and subjected to western blot analysis. Processing of caspase-10 and caspase-8 can be detected simultaneously at 15 min after stimulation, as indicated by the appearance of the p18 subunit of caspase-8 (upper panel) or the processed prodomain of caspase-10 (lower panel), respectively. The cleavage fragments of caspase-10 p47, p43 and the prodomain p25 correspond to the pattern detected in BJAB and Jurkat cell lines (Figure 2).

caspase-10 (V410I), which was found during this study, less severely affected caspase-10 function and exerted this effect only in the individual who was homozygous for the mutated allele. Later, this V410I mutation was described to be a common polymorphism in the Danish population (Gronbaek *et al*., 2000), although, to date, besides the ALPS II patient described by Wang *et al*. (1999), no other individual homozygous for the V401I caspase-10 variant has been identified.

Four different isoforms of caspase-10 (a–d) have been described (Fernandes-Alnemri *et al*., 1996; Vincenz and Dixit, 1997; Ng *et al*., 1999). Previous studies on the function of caspase-10 relied on overexpression of the different isoforms or catalytically inactive variants. Thus far, analysis of the function and expression of caspase-10 was hampered by the unavailability of specific antibodies. We identified an antibody specific for the prodomain of caspase-10 that is capable of recognizing all theoretically existing isoforms of caspase-10. We show here that, as with caspase-8, two large isoforms of caspase-10 are expressed. We identified these isoforms as caspase-10a (Mch4; Fernandes-Alnemri *et al*., 1996) and caspase-10d (Ng *et al*., 1999). However, we were unable to detect the previously reported caspase-10b (FLICE-2; Vincenz and Dixit, 1997) in any of the cell lines tested, although it should be readily detectable with the antibody used. In addition, a smaller isoform, caspase-10c, can be detected. This isoform yields a catalytically inactive form that is reminiscent of the CAP3 isoform of caspase-8 (Kischkel *et al*., 1995). The function and significance of the small isoforms of the two caspases for regulation of DISC function remain to be determined. However, the conserved pattern of presence of these caspase-8 and caspase-10 short isoforms is indicative of a functional role. Taken together, caspase-8 and caspase-10 are not only structurally homologous, but are also similar in their isoform pattern, with two large forms and a truncated form being expressed.

Thus far, it has been controversial whether the DED-containing caspase-10 is recruited to the signalling complex formed upon ligand-induced cross-linking of death receptors. Our data demonstrate that the three different isoforms of endogenous caspase-10 are all recruited to the native TRAIL and CD95 DISC upon stimulation with the respective ligands, resulting in activation of caspase-10 at the DISC. The sequence of activation at the DISC appears to be comparable with that of caspase-8. In the first cleavage step, the small subunit is cleaved, then the large subunit is cleaved from the remaining DISC-bound p43 and p47 fragments, respectively.

The two death receptors for TRAIL, TRAIL-R1 and TRAIL-R2, can induce apoptosis independently from each other (Sprick *et al*., 2000). In search of potentially different signalling properties, we determined whether a difference in recruitment of caspase-10 exists between both receptors. However, differential DISC analysis revealed that both receptor complexes recruit and activate caspase-10 independently. Thus, as differential caspase-10 recruitment could not be found, it can not account for possible biochemical and functional differences between TRAIL-R1- and TRAIL-R2-initiated apoptosis pathways. However, in cells expressing both TRAIL-R1 and TRAIL-R2, we found a much lower amount of the DISC components FADD, caspase-8 and caspase-10 recruited to TRAIL-R2 upon stimulation. Whether the lower amount of DISC complexes recruited to TRAIL-R2 alone corresponds to a reduced ability of this receptor to recruit these DISC components, or merely reflects lower amounts of TRAIL-R2 expressed, is currently unknown. The co-occurrence of caspase-10 and caspase-8 in the CD95 and the TRAIL DISCs raises the question whether caspase-10 may act in the same pathway as caspase-8, either in a redundant or an enhancing fashion in death-receptor-mediated apoptosis, or whether caspase-10 may act as a transmitter of signals other than apoptosis induction that emanate from TRAIL death receptors and CD95.

While this manuscript was under revision, two additional reports appeared showing that caspase-10 is recruited to the DISC (Kischkel *et al*., 2001; Wang *et al*., 2001). In one of these studies, cell death was observed in caspase-8-deficient cells after prolonged stimulation with TRAIL or CD95L and was proposed to be mediated by caspase-10 (Kischkel *et al*., 2001).

This is in line with the results shown in Figure 5 that both death ligands can induce cell death in the caspase-8-deficient Jurkat cells. However, titration of TRAIL and CD95L shows that high concentrations of these ligands are needed for death induction when compared with the caspase-8-expressing Jurkat cells (Figure 6). Nevertheless, this residual cell death could have been mediated by caspase-10, which is also recruited to both DISCs in these cell lines.

The reduced sensitivity of the caspase-8-deficient cells to TRAIL and CD95L has been attributed to lower expression levels of caspase-10 in these cells (Kischkel

et al., 2001; Wang *et al*., 2001). In both studies, transient transfection of caspase-8-deficient cells with caspase-10 sensitized these cells for CD95L- and TRAIL-induced apoptosis, arguing for redundant roles of caspase-10 and caspase-8 in death-receptor-mediated apoptosis.

We analysed several independent caspase-10-over-expressing clones that we had established from the caspase-8-deficient cell line. In these clones, stable expression of caspase-10 did not increase the apoptosis susceptibility when these cells were stimulated with TRAIL or CD95L. The levels of caspase-10 expression we observed exceeded the level found in wild-type Jurkat cells (Figure 6) and in several primary cells analysed (data not shown) by several fold. These data strongly argue against a role of caspase-10 as a second redundant initiator caspase in the TRAIL and CD95 system. Possibly, the transient expression system utilized in the previous reports leads to caspase-10 levels that are much higher than those observed in our clones. These levels may surpass the physiological levels that can be tolerated by the cells, explaining the observed increase in sensitivity to TRAIL and CD95L. In addition, caspase-10 is not necessary for normal apoptosis to proceed, as cell lines deficient in caspase-10 show no apparent defect in apoptosis.

Taken together, we have shown that caspase-10 is recruited to the CD95 and the TRAIL DISCs, and that caspase-10 is cleaved during TRAIL- and CD95-mediated apoptosis. Importantly, while some cell death is observed in the absence of caspase-8, caspase-10 is unable to fully substitute for a loss of caspase-8, even at high expression levels, suggesting that it serves a function different from that of caspase-8. As caspase-10 can not fully substitute for a loss of caspase-8 in apoptosis induction, it might serve different functions during apoptosis induction. It may function in a non-apoptosis pathway that is triggered by death receptor cross-linking. Alternatively, caspase-10 may diversify the apoptotic signal that emanates from the CD95 and the TRAIL death receptors by targeting a different set of proteins for proteolysis. Thus, the precise function of caspase-10 and the nature of the signals it transmits await identification of its specific substrates in a cellular context.

Materials and methods

Cell lines

The human B-cell line BJAB, the human T-cell line Jurkat A3, the caspase-8-deficient Jurkat clone, the Jurkat FADD^{def} clone (Juo *et al*., 1999) and its derivative, the TRAIL-R1-expressing Jurkat FADD^{def}-TR1/2 clone, were maintained in RPMI 1640 (Gibco-BRL, Karlsruhe, Germany) containing 10% fetal calf serum (FCS; Gibco-BRL). The CHO cell line was maintained in Ham's F12 medium with 10% FCS.

Cloning of caspase-10 isoforms

Plasmids coding for the caspase-10 isoforms were obtained by amplification of the different caspase-10 open reading frames by PCR with *Pfu* polymerase. Primer sequences for amplification were CASP10UP (5′-ATGAAATCTCAAGGTCAACATTGGTATTCC-3′) and CASP10LOW (5′-CTATATTGAAAGTGCATCCAGGGGCAC-AGG-3′). PCR fragments were ligated into pEFV5His-TOPO vector (Invitrogen, Karlsruhe, Germany) for eukaryotic expression. The stop codon contained in the caspase-10 cDNA was included to generate an untagged version of caspase-10. Inserts containing vectors were fully sequenced and verified to contain the desired products without mutations.

Bacterial expression of the caspase-10 prodomain

The caspase-10 prodomain was amplified by PCR from a vector containing full-length caspase-10d and subcloned into pCRT7NT-TOPO (Invitrogen). His-tagged prodomain was expressed in *Escherichia coli* BL21 and purified on a Ni^{2+} column under denaturing conditions.

Transient transfection of CHO cells

CHO cells were transiently transfected with the expression plasmids for caspase-10a, -10d or a control vector with Fugene transfection reagent according to the manufacturer's instructions (Roche, Mannheim, Germany). Twenty-four hours after transfection, cell lysates were collected and analysed.

Stable transfection of caspase-10 and TRAIL-R1 in Jurkat caspase-8-deficient cells

First, a caspase-8-deficient cell line stably expressing TRAIL-R1 was generated by transfecting the parental caspase-8-deficient cell line with the expression vector pCDNA3 Hygro coding for TRAIL-R1. After initial selection in hygromycin B (200 μg/ml), single clones were obtained by two rounds of limiting dilution cloning. Clones were analysed by surface staining for expression of TRAIL-R1 and one clone, denoted clone 1D2, was chosen.

Caspase-8-deficient cell lines stably overexpressing caspase-10d were generated by transfecting the caspase-8-deficient cell line with the pEFV5His-TOPO-Casp-10d vector using Xtreme-GeneQ2 (Roche). After initial selection in blasticidin S (10 μg/ml), single clones were obtained by limited dilution cloning. To exclude the introduction of mutations in caspase-10 during the generation of the stable clones, the cDNA encoded by the plasmid was re-isolated from the single clones, sequenced and found to be without mutations.

2D gel analysis

For 2D analysis, cells were directly lysed for 1 h in 2D sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 mM Tris–butylphosphine), supplemented with complete protease inhibitors (Roche). Subsequently, the samples were clarified by centrifugation (50 000 *g* for 30 min). For the first dimension, protein samples were diluted in 2D rehydration buffer [2D sample buffer supplemented with bromophenol blue, 1% ampholytes pH 5–8 (Bio-Rad, München, Germany), 10% (v/v) glycerol]. The first dimension isoelectric focusing (IEF) was performed with ReadyStrip precast IPG strips pH 5–8 essentially according to the manufacturer's instructions (Bio-Rad). After electrofocusing, the strips were incubated twice for 15 min in equilibration buffer [50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% (w/v) SDS, 10 mg/ml dithiothreitol (DTT)]. The second dimension was run on pre-cast 4–12% NuPage Bis-Tris gels (Invitrogen) with MOPS running buffer, and subsequently, gels were blotted onto nitrocellulose membrane.

Establishment of the cell line FADD^{def}-TR1/2

The TRAIL-R1- and -R2-expressing FADD-deficient Jurkat cell line TR1/2 (clone 6D2-1D2) was derived from the original Jurkat FADD^{def} cell line by transfection with an expression plasmid coding for the complete TRAIL-R1 open reading frame. The transfected pool was subjected to two rounds of limiting dilution cloning, choosing the cell line with the highest TRAIL-R1 surface expression, as evaluated by FACS staining with a TRAIL-R1-specific mAb (HS102).

Antibodies and reagents

Monoclonal antibodies against FADD were purchased from Transduction Laboratories (San Diego, CA). Anti-caspase-10 mAb (Clone 4C1) was from MBL International (Watertown, MA) and anti-ERK polyclonal serum from Santa Cruz. Antibodies that were tested for reactivity with caspase-10 on western blots of lysates of transfected CHO cells and found to be non-specific were PharMingen (Hamburg, Germany) 67041N, Upstate Biotechnology 06-836 and Oncogene Sciences anti-caspase-10 Ab-2. The mAb MAB834 from R&D Systems recognized the transfected caspase-10, but was unsuited for analysis of endogenous proteins in cell lysates as it strongly cross-reacted with several proteins. The mAb anti-FLICE C15 recognizes the p18 subunit of caspase-8 (Scaffidi *et al*., 1997). Anti-FLAG M2 was purchased from Sigma (Taufkirchen, Germany). The mAbs specific for the different TRAIL receptors were generated by immunizing mice with the respective TRAIL receptor–Fc fusion proteins. Specificity of the antibodies was confirmed by staining cells transfected with expression plasmids for TRAIL-R1–TRAIL-R4. Anti-TRAIL-R1 (clone HS101) and anti-TRAIL-R2 (clone HS201), both of the mIgG1 isotype, were used for FACS staining and receptor

blockage. The antibodies are available from Alexis (Gruenberg, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1, IgG2b and mouse κ light-chain-specific polyclonal antibodies (pAb) were obtained from Southern Biotechnology Associates (Birmingham, AL). FLAG-CD95L was from Alexis. FLAG-TRAIL was expressed as described previously (Keogh *et al*., 2000). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma.

FACS analysis

Cells (5×10^5) were incubated with mAbs against TRAIL-R1 (HS101), TRAIL-R2 (HS201), or control mIgG1 at 10 μg/ml, followed by biotinylated secondary goat anti-mouse antibodies (Southern Biotechnology Associates, CA) and streptavidin–PE (PharMingen). Surface staining was analysed on a FACS-Calibur (Becton Dickinson, Heidelberg, Germany).

Preparation of cell lysates

Cells were harvested by centrifugation at 300 *g* for 10 min at 4°C, washed twice with ice-cold phosphate-buffered saline (PBS) and lysates prepared by resuspending the resulting cell pellets in 100 μl of lysis buffer per 1×10^7 cells (30 mM Tris–HCl pH 7.5, 120 mM NaCl, 10% glycerol, 1% Triton X-100) supplemented with Complete™ protease inhibitors (Roche) according to the manufacturer's instructions. After 30 min incubation on ice, the lysates were centrifuged once at 15 000 *g* at 4°C to remove nuclei. For the lysis of primary T cells, the protease inhibitor concentration was increased five-fold.

Western blot analysis

For western blot analysis, the resulting post-nuclear supernatants or DISC precipitates were supplemented with 2-fold concentrated standard reducing sample buffer (2× RSB). Subsequently, lysates containing 20 μg of protein, as determined by the BCA method (Pierce, Rockford, IL), or proteins eluted from beads after ligand affinity immunoprecipitation were separated on 4–12% NuPage Bis-Tris gradient gels (Novex, San Diego, CA) in MOPS buffer according to the manufacturer's instructions. After protein transfer onto nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by electroblotting, membranes were blocked with 5% non-fat dry milk (NFDM) in PBS/Tween (PBS containing 0.05% Tween-20) for at least 1 h, washed with PBS/Tween, and incubated in PBS/Tween containing 3% NFDM and primary antibodies against caspase-10, FADD or caspase-8 (1:10-diluted C15 hybridoma supernatant). After five washes, for 3 min each, in PBS/Tween, the blots were incubated with HRP-conjugated isotype-specific secondary antibody diluted 1:20 000 in PBS. After washing five times, for 3 min, with PBS/Tween, the blots were developed by enhanced chemiluminescence using SuperSignal West Dura substrate following the manufacturer's protocol (Perbio Science, Bonn, Germany). For stripping, blots were incubated in 50 mM glycine–HCl and 500 mM NaCl pH 2.3 for 20 min at room temperature. Subsequently, blots were washed twice for 10 min in PBS/Tween and blocked again.

DISC analysis

We performed DISC analysis by using FLAG-tagged TRAIL (FLAG-TRAIL) or FLAG-tagged CD95L (FLAG-CD95L) in combination with streptavidin beads (Pierce). For ligand affinity precipitation, $1–5 \times 10^7$ cells were used per sample. Cells were washed with 50 ml of RPMI medium at 37°C and subsequently incubated for the indicated time periods at 37°C and a cell density of 5×10^7/ml in the presence of 1 μg/ml FLAG-TRAIL pre-complexed with 2 μg/ml anti-FLAG M2 for 15 min, or, for the unstimulated control, in the absence of FLAG-TRAIL. In the case of differential TRAIL receptor DISC analysis, we pre-incubated the cells with 10 μg/ml TRAIL-R1 and/or TRAIL-R2-blocking mAbs for 15 min before stimulation with FLAG-TRAIL. DISC formation was stopped by addition of at least 15 vols of ice-cold PBS. Cells were then washed twice with 50 ml of ice-cold PBS before cell lysates were prepared. Cells were lysed by addition of 1 ml of lysis buffer. The resulting protein complexes were precipitated from the lysates by co-incubation with 20 μl of protein G beads (Roche) for 2–4 h on an end-over-end shaker at 4°C. For precipitation of the non-stimulated receptors, FLAG-TRAIL plus M2 was added to the lysates prepared from non-stimulated cells at 200 ng to control for protein association with non-stimulated receptor(s). Ligand affinity precipitates were washed five times with lysis buffer before the protein complexes were eluted from the beads by addition of 15 μl of 2× RSB. Subsequently, proteins were separated by SDS–PAGE before detection of DISC components by western blot analysis.

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