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FADD/MORT1 and Caspase-8 Are Recruited to TRAIL Receptors 1 and 2 and Are Essential for Apoptosis Mediated by TRAIL Receptor 2

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Summary

Apoptosis induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/APO-2L) has been shown to exert important functions during various immunological processes. The involvement of the death adaptor proteins FADD/MORT1, TRADD, and RIP and the apoptosis-initiating caspases-8 and -10 in death signaling by the two death-inducing TRAIL receptors 1 and 2 (TRAIL-R1 and TRAIL-R2) are controversial. Analysis of the *native* TRAIL death-inducing signaling complex (DISC) revealed ligand-dependent recruitment of FADD/MORT1 and caspase-8. Differential precipitation of ligand-stimulated TRAIL receptors demonstrated that FADD/MORT1 and caspase-8 were recruited to TRAIL-R1 and TRAIL-R2 independently of each other. FADD/MORT1- and caspase-8-deficient Jurkat cells expressing only TRAIL-R2 were resistant to TRAIL-induced apoptosis. Thus, FADD/MORT1 and caspase-8 are essential for apoptosis induction via TRAIL-R2.

Introduction

Apoptosis is an essential process during the development of the immune system and for the maintenance of T and B cell homeostasis. Two apoptosis-inducing members of the tumor necrosis factor (TNF) family, TNF and CD95 ligand (CD95L/FasL/APO-1L), are involved in various immunological processes. These include inflammation, activation-induced T and B cell death, immune privilege, tumor evasion from the immune system, autoimmunity, and AIDS (Nagata, 1997; Krammer, 1999; Wallach et al., 1999). In addition, TNF and CD95L have been shown to kill various tumor cell lines in vitro. TNF and CD95L induce apoptosis upon binding to their cognate receptors capable of transmitting a caspase-activating signal due to the presence of a cytoplasmic death domain (DD) (Krammer, 1999; Peter et al., 1999).

TNF-related apoptosis-inducing ligand (TRAIL/APO-2L) was identified by sequence homology to CD95L and TNF (Wiley et al., 1995; Pitti et al., 1996). Interestingly, TRAIL induced apoptosis in about 60% of tumor cell lines, while most normal cells were resistant. Further, systemic administration of TRAIL/APO-2L suppressed tumor growth in SCID mice and nonhuman primates without being toxic to normal tissue, and TRAIL/APO-2L and chemotherapeutic drugs synergistically suppressed tumor growth in SCID mice (Ashkenazi et al., 1999; Gliniak and Le, 1999; Walczak et al., 1999). In addition, TRAIL/APO-2L was used successfully in locoregional treatment of glioblastoma xenografts in athymic mice (Roth et al., 1999). Thus, TRAIL may serve as a novel treatment for cancer.

The functional expression of TRAIL was recently discovered on the surface of different cells of the immune system previously known to induce apoptosis in target cells by an unidentified mechanism. Among them are type II interferon (IFN-γ) –stimulated monocytes (Griffith et al., 1999b), type I IFN- (IFN-α and IFN-β) or TCR-stimulated T cells (Kayagaki et al., 1999a; Musgrave et al., 1999), nonstimulated $CD4^+$ T cells (Mariani and Krammer, 1998; Thomas and Hersey, 1998; Kayagaki et al., 1999b; Martinez-Lorenzo et al., 1999), IFN-α and IFN-γ-stimulated as well as measles virus-infected dendritic cells (DC) (Fanger et al., 1999; Vidalain et al., 2000), and natural killer (NK) cells (Zamai et al., 1998; Johnsen et al., 1999; Kashii et al., 1999; Kayagaki et al., 1999c).

TRAIL interacts with five distinct receptors: TRAIL-R1 (DR4) (Pan et al., 1997b), TRAIL-R2 (DR5/TRICK2/KILLER) (MacFarlane et al., 1997; Pan et al., 1997a; Schneider et al., 1997a; Screaton et al., 1997; Sheridan et al., 1997; Walczak et al., 1997), TRAIL-R3 (DcR1/TRID/LIT) (Degli-Esposti et al., 1997a; Pan et al., 1997a; Schneider et al., 1997a; Sheridan et al., 1997; Mongkolsapaya et al., 1998), TRAIL-R4 (DcR2/TRUNDD) (Degli-Esposti et al., 1997b; Marsters et al., 1997; Pan et al., 1998), and Osteoprotegerin (OPG) (Emery et al., 1998). TRAIL-R1 and TRAIL-R2 contain an intracellular DD necessary for apoptosis induction upon TRAIL-mediated receptor ligation. In contrast, neither TRAIL-R3 nor TRAIL-R4 can mediate apoptosis due to complete or partial absence of an intracellular DD, respectively. OPG is a soluble receptor reported to bind OPG ligand (OPGL/RANKL/TRANCE/ODF) and TRAIL (Emery et al., 1998).

The biochemical events leading to apoptosis induction via TNF and CD95 have been analyzed in detail. Cross-linking of CD95 leads to the formation of a death-inducing signaling complex (DISC) (Kischkel et al., 1995). The death adaptor protein FADD/MORT1 (Boldin et al., 1995; Chinnaiyan et al., 1995) and the proteolytic enzyme caspase-8 (Boldin et al., 1996; Muzio et al., 1996) are recruited to the CD95 DISC (Kischkel et al., 1995). In a homotypic interaction, the DD of FADD/MORT1 binds to the DD of CD95. The death effector domain (DED) of FADD/MORT1 in turn interacts with the DED of pro-caspase-8 and thereby recruits this proenzyme to the

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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).

TNF induces apoptosis by cross-linking of its DD-containing receptor TNF-R1 (p55). Ligand-induced cross-linking of TNF-R1 leads to the recruitment of TRADD and RIP to the DD of the receptor. FADD/MORT1 is recruited to the TNF-R1 DISC via TRADD. In analogy to the CD95 system, FADD/MORT1 then recruits caspase-8 to the TNF-R1 DISC by homotypic interaction of their respective DEDs resulting in activation of caspase-8 and apoptosis. Signals emanating from RIP and TRADD-recruited TRAF2 result in activation of the NF-κB and Jun kinase pathways, respectively. These gene-inductive events are responsible for the inflammatory processes associated with triggering of TNF-R1 (Wallach et al., 1999).

Given its implication in various immunological processes, it is important to understand the biochemical mechanism of initiation of TRAIL-induced apoptosis. So far, this mechanism was addressed in a number of overexpression studies examining the role of different known apoptosis signaling proteins. In some overexpression systems, dominant-negative FADD/MORT1 (FADD-DN) inhibited TRAIL-induced apoptosis (Chaudhary et al., 1997; Schneider et al., 1997b; Walczak et al., 1997; Wajant et al., 1998). In other studies, FADD-DN overexpression did not prevent TRAIL-induced apoptosis (MacFarlane et al., 1997; Pan et al., 1997a, 1997b; Sheridan et al., 1997). Coimmunoprecipitations of overexpressed cytoplasmic domains of TRAIL-R1 or TRAIL-R2 with FADD/MORT1 supported a role for FADD/MORT1 in TRAIL-induced apoptosis (Chaudhary et al., 1997; Schneider et al., 1997b). Yet, opposite results were obtained in another study, as FADD/MORT1 was not coimmunoprecipitated with overexpressed TRAIL-R2 but only with CD95 (MacFarlane et al., 1997). Murine embryonic fibroblasts (MEF) from FADD/MORT1-deficient mice underwent apoptosis upon overexpression of human TRAIL-R1 (DR4) (Yeh et al., 1998). Thus, the role of FADD/MORT1 in TRAIL-induced apoptosis is still unclear.

The identity of the caspase(s) involved in the initiation of TRAIL-mediated apoptosis is also controversial. Under native conditions, early cleavage of caspase-8 has been observed following TRAIL stimulation (Griffith et al., 1998; Leverkus et al., 2000). Yet, overexpression studies with dominant-negative forms of caspase-8 and -10 either suggested caspase-10 alone (Pan et al., 1997a) or a combination of caspase-8 and -10 (MacFarlane et al., 1997) as initiator caspase(s) during TRAIL-induced apoptosis. In addition, TRAIL resistance of mature DC and activated peripheral T cells from ALPS II patients was suggested to be due to mutated nonfunctional caspase-10 and to be causative for the disease (Wang et al., 1999).

To dissect the molecular mechanisms of TRAIL-induced apoptosis initiation, we differentially analyzed the native TRAIL-R1 and TRAIL-R2 signaling complexes induced upon TRAIL stimulation of sensitive target cells. Our data indicate that, independently of each other, both TRAIL-R1 and TRAIL-R2 recruit FADD/MORT1 and caspase-8 in a ligand-dependent fashion. In addition, we show that FADD/MORT1 and caspase-8 are essential for TRAIL-R2-mediated apoptosis.

Results

BL60, BJAB, and CEM Cells Are Susceptible to TRAIL-R1- and TRAIL-R2-Induced Apoptosis, whereas Jurkat Cells Express Only Functional TRAIL-R2

In order to dissect the apoptosis-inducing events immediately following receptor stimulation by TRAIL in lymphoid cells, we chose the human B cell lines BL60 and BJAB and the human T cell lines CEM and Jurkat as model systems. We characterized these cell lines by determination of TRAIL sensitivity, analysis of the time course of caspase-8 activation, and their TRAIL receptor expression profile. We then analyzed the functionality of TRAIL-R1 and TRAIL-R2 signaling separately. All four cell lines were susceptible to LZ-TRAIL-induced apoptosis (Figure 1A). In accordance with studies on non-lymphoid cell lines (Griffith et al., 1998; Leverkus et al., 2000), stimulation of BL60, BJAB, CEM, and Jurkat cells with LZ-TRAIL led to early cleavage and activation of caspase-8 (Figure 1B) prior to cleavage of caspase-3 and PARP (data not shown). This supports an initiating role for caspase-8 during TRAIL-induced apoptosis also in lymphoid cells. While BL60, BJAB, and CEM cells expressed both apoptosis-inducing TRAIL receptors, only TRAIL-R2 was present on the surface of Jurkat cells (Figure 1C, top panels). TRAIL-R3 and TRAIL-R4 were not detectable on the surface of these cell lines (Figure 1C, bottom panels).

We next analyzed whether TRAIL-R1 and/or TRAIL-R2 were functionally expressed. After preincubation with soluble blocking monoclonal antibodies (mAbs) against TRAIL-R1, TRAIL-R2, or a combination thereof, we treated the different cells with LZ-TRAIL (Figure 1D). While the combination of TRAIL-R1- and TRAIL-R2-specific mAbs inhibited TRAIL-induced apoptosis in all four cell lines, preincubation with anti-TRAIL-R2 alone blocked TRAIL-induced apoptosis only in Jurkat cells. Preincubation with blocking anti-TRAIL-R1 alone did not inhibit TRAIL-induced apoptosis in any of the cell lines tested. Thus, BL60, BJAB, and CEM cells are susceptible to apoptosis mediated by ligand-induced cross-linking of either TRAIL-R1 or TRAIL-R2, whereas Jurkat cells can only be killed via TRAIL-induced cross-linking of TRAIL-R2. These data show that these antibodies are suitable for differential analysis of the individual protein complexes formed upon TRAIL-mediated cross-linking of the two different apoptosis-inducing TRAIL receptors under native conditions.

TRAIL-Dependent Recruitment of Caspase-8 and FADD/MORT1 to the TRAIL DISC in BL60 Cells

The active caspase-8 subunit p18 could be detected as early as 15 min after TRAIL stimulation (Figure 1B), suggesting that caspase-8 might be the caspase responsible for initiation of TRAIL-induced apoptosis. The *native* protein complex that initiates TRAIL-induced apoptosis was analyzed for presence of the various signaling proteins that have been suggested to play a role at

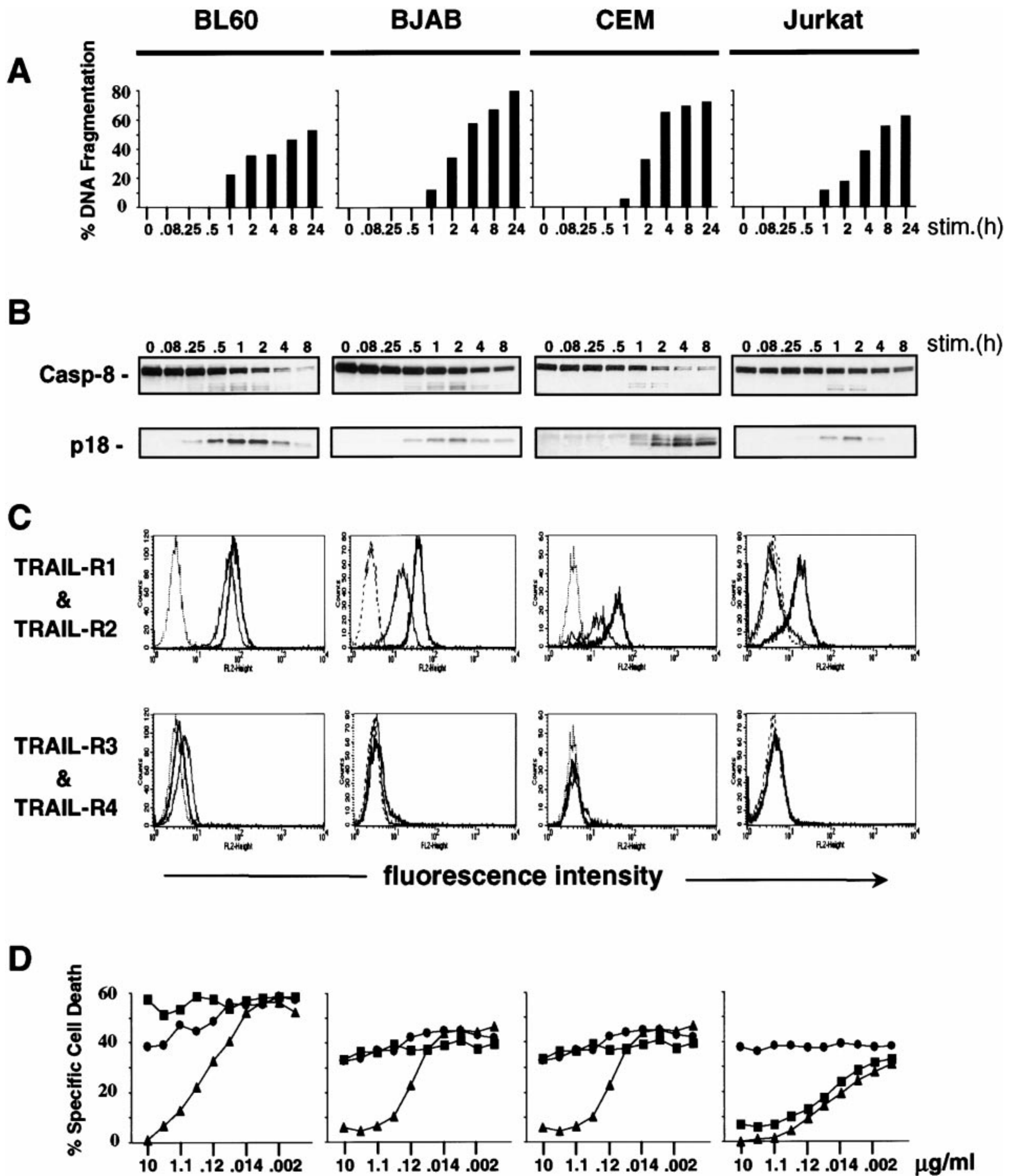

Figure 1. Analysis of Kinetics of TRAIL-Induced Apoptosis, Caspase-8 Cleavage, Receptor Expression Profile, and Functional Expression of TRAIL-R1 and TRAIL-R2 in BL60, BJAB, CEM, and Jurkat Cells

(A and B) Cells were stimulated with 1 μg/ml LZ-TRAIL for the indicated time periods followed by analysis of percentage of cells with subdiploid DNA content (A) and Western blot analysis of caspase-8 (p55/53) and its p18 cleavage product (B).

(C) FACS analysis of surface expression of TRAIL-R1 (upper panels, solid lines); TRAIL-R2 (upper panels, solid bold lines); TRAIL-R3 (lower panels, solid lines); and TRAIL-R4 (lower panels, solid bold lines) as compared to an isotype-matched control mIgG1 mAb (dashed lines).

(D) Identification of TRAIL-R1- and TRAIL-R2-inhibiting mAbs. Cells were treated with 100 ng/ml LZ-TRAIL after 30 min preincubation with or without blocking TRAIL-R1- and/or TRAIL-R2-specific mAbs at the indicated concentrations. The percentage of cell death was determined by FSC/SSC analysis and plotted against the concentration of mAbs specific for TRAIL-R1 (closed circle), TRAIL-R2 (closed square), or both apoptosis-inducing TRAIL receptors (closed triangle). Percentage of specific cell death was calculated as follows: 100 × (% experimental cell death − % spontaneous cell death)/(100 − % spontaneous cell death). Spontaneous cell death was below 10% in all samples. One of four representative experiments is shown.

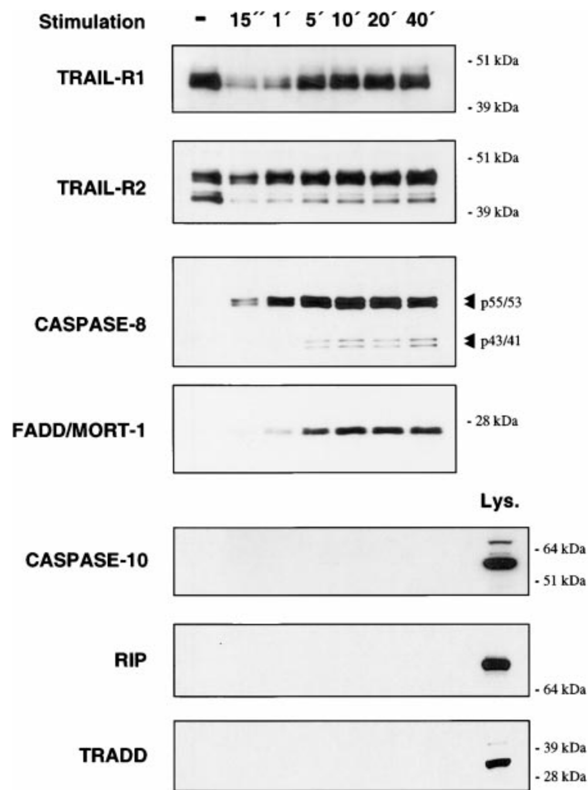

Figure 2. Kinetics of DISC Assembly in BL60 Cells

BL60 cells were either stimulated with 1 μg/ml biotinylated LZ-TRAIL (Bio-LZ-TRAIL) for the indicated time periods or left untreated (−) before cell lysis. Bio-LZ-TRAIL (1 μg/ml) was added to the lysates in the untreated control. Bio-LZ-TRAIL-bound protein complexes were analyzed by SDS-PAGE and Western blot following affinity precipitation with Streptavidin beads. Lysates prepared from BL60 cells (Lys.) are shown as positive controls for caspase-10, RIP, and TRADD antibodies. The upper doublet band detected by the TRAIL-R2 antibody corresponds to the two different splicing variants reported (Screaton et al., 1997), while the lower doublet likely represents proteolytically processed forms of the the two TRAIL-R2 variants (Walczak et al., 1997).

the onset of TRAIL-induced apoptosis (Figure 2). BL60 cells were incubated either in the absence or presence of biotinylated LZ-TRAIL (Bio-LZ-TRAIL) for various time periods. Both TRAIL-R1 and TRAIL-R2 were precipitated with Bio-LZ-TRAIL already after 15 s of stimulation. When analyzing the precipitated protein complex for the presence of caspase-8 and FADD/MORT1, we found that both signaling proteins were recruited as early as 15 s to 1 min after stimulation with Bio-LZ-TRAIL. In addition, cleavage fragments of caspase-8 (p43/41), indicative of activation of the proform of this enzyme (p55/53), were detectable within 1–5 min of stimulation. In contrast, caspase-8 and FADD/MORT1 did not associate with nonstimulated TRAIL receptors that were precipitated with Bio-LZ-TRAIL from the lysates of mock-treated cells (Figure 2, left lane). Thus, recruitment of FADD/MORT1 and caspase-8 was dependent on ligand-induced receptor cross-linking. The amount of FADD/MORT1 and caspase-8 recruited to the TRAIL receptors increased in a time-dependent manner reaching maximum levels between 5 min and 20 min of stimulation. We conclude that TRAIL stimulation leads to the formation of an apoptosis-initiating protein complex, the TRAIL DISC. FADD/MORT1, caspase-8, and the apoptosis-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2, are integral components of this signaling complex. However, although prominently present in the lysates of BL60 cells, we could not detect caspase-10, RIP, and TRADD associated with the TRAIL DISC at any time point analyzed (Figure 2, bottom panels).

FADD/MORT1 and Caspase-8 Form Part of the TRAIL DISC in Various Lymphoid Cell Lines

We next studied whether FADD/MORT1 and caspase-8 recruitment to the TRAIL DISC can also be observed in other cell lines that express both apoptosis-inducing TRAIL receptors. Therefore, we compared the formation of the TRAIL DISC in BL60 cells with the BJAB and CEM TRAIL DISC. We incubated the cells for 20 min in the presence or absence of Bio-LZ-TRAIL before analyzing the proteins complexed with the biotinylated ligand. TRAIL-R1 and TRAIL-R2 associated with Bio-LZ-TRAIL in BJAB and CEM cells, and both FADD/MORT1 and caspase-8 were recruited to the TRAIL DISC (Figure 3). This association was stimulation dependent, since Bio-LZ-TRAIL added after cell lysis precipitated the nonstimulated receptors but not caspase-8 and FADD/MORT1. Thus, these two signaling proteins, previously identified as integral components of the native CD95 DISC (Kischkel et al., 1995; Muzio et al., 1996), also form part of the native TRAIL DISC in various cell lines.

Jurkat cells only express TRAIL-R2 on their surface (Figure 1C). Thus, Jurkat cells served to test whether cross-linking of TRAIL-R2 in the absence of TRAIL-R1 may also lead to recruitment of FADD/MORT1 and caspase-8. Stimulation of Jurkat cells with Bio-LZ-TRAIL induced recruitment of TRAIL-R2, caspase-8, and FADD/MORT1 in the absence of TRAIL-R1 (Figure 3). Under nonstimulatory conditions, only TRAIL-R2 was bound to TRAIL, indicating that recruitment of FADD/MORT1 and caspase-8 to TRAIL-R2 is dependent on TRAIL-induced cross-linking of TRAIL-R2 on intact Jurkat cells. Thus, homomeric TRAIL-R2 complexes are sufficient for recruitment of FADD/MORT1 and caspase-8 in the absence of TRAIL-R1.

Homomeric TRAIL-R1 and TRAIL-R2 DISCs Recruit FADD/MORT1 and Caspase-8 in BL60 Cells

Since both apoptosis-inducing TRAIL receptors associated with TRAIL upon stimulation, FADD/MORT1 and caspase-8 might have been recruited to homomeric TRAIL-R1, homomeric TRAIL-R2, or heteromeric complexes containing TRAIL-R1 and TRAIL-R2. To differentiate between the proteins associated with homomeric TRAIL-R1 and TRAIL-R2 DISC, respectively, we stimulated the two receptors separately. This was achieved by preincubating BL60 cells in the presence or absence of blocking mAbs against TRAIL-R1 and TRAIL-R2, respectively, before stimulation with Bio-LZ-TRAIL (Figure 4, odd numbered lanes). In the unstimulated controls (Figure 4, even numbered lanes), Bio-LZ-TRAIL added after cell lysis could precipitate not only cell surface–bound but also intracellularly stored TRAIL-R1 and TRAIL-R2, a finding recently described in melanoma

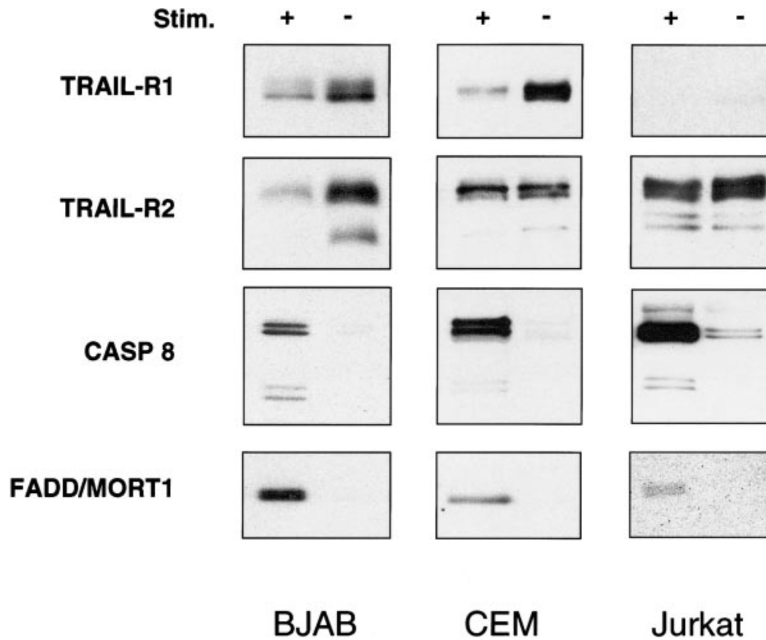

Figure 3. TRAIL DISC Analysis in BJAB, CEM, and Jurkat Cells

BJAB, CEM, and Jurkat cells were left untreated or were treated for 20 min with 1 μg/ml Bio-LZ-TRAIL before cell lysis. Bio-LZ-TRAIL (1 μg/ml) was added to lysates from unstimulated cells for precipitation of unstimulated TRAIL receptors. Bio-LZ-TRAIL-bound protein complexes were analyzed for presence of TRAIL-R1, TRAIL-R2, FADD/MORT1, and caspase-8 by SDS-PAGE and Western blot analysis following affinity precipitation from the lysates with Streptavidin beads.

cells (Zhang et al., 2000). Western blot analysis revealed that in the presence of TRAIL-R2 blocking mAb only TRAIL-R1 was precipitated upon stimulation with Bio-LZ-TRAIL (Figure 4, lane 3, top two panels) and vice versa (Figure 4, lane 5, top two panels). Thus, both homomeric TRAIL-R1 and TRAIL-R2 DISCs were formed upon stimulation with Bio-LZ-TRAIL.

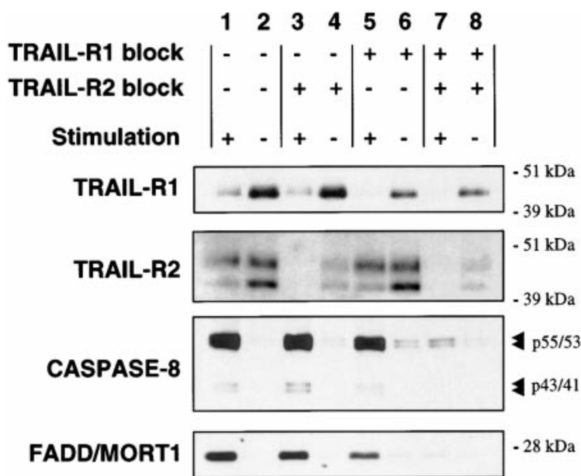

Figure 4. TRAIL Stimulation Induces Recruitment of FADD/MORT1 and Caspase-8 to Homomeric TRAIL-R1 and TRAIL-R2 DISCs in BL60 Cells

After 30 min preincubation with 10 μg/ml blocking TRAIL-R1- and/or TRAIL-R2-specific mAbs, cells were either stimulated with 1 μg/ml Bio-LZ-TRAIL (+) for 20 min or left untreated (−) before cell lysis. Bio-LZ-TRAIL (1 μg/ml) was added to the unstimulated samples for precipitation of unstimulated TRAIL receptors from lysates. Bio-LZ-TRAIL-bound protein complexes were analyzed for presence of TRAIL-R1, TRAIL-R2, FADD/MORT1, and caspase-8 by SDS-PAGE and Western blot analysis following affinity precipitation from the lysates with streptavidin beads. After the longer exposure times necessary to reveal the p43/41 forms of caspase-8, background levels of caspase-8 can be seen in some unstimulated controls.

It is noteworthy that higher amounts of TRAIL-R1 and TRAIL-R2 were detected in the control precipitate when compared to the stimulated condition (Figure 4, lanes 1 and 2). In addition, although antibody blockage before stimulation completely inhibited precipitation of the blocked receptor (Figure 4, lanes 3, 5, and 7), significant amounts of TRAIL-R1 and TRAIL-R2 were precipitated in the unstimulated controls (Figure 4, lanes 4, 6, and 8). The incomplete blockage observed under nonstimulatory conditions indicates that, similar to melanoma cells (Zhang et al., 2000), also BL60 cells contain substantial intracellular amounts of TRAIL-R1 and, to a lesser extent, TRAIL-R2.

Stimulated homomeric TRAIL-R1 and TRAIL-R2 recruited pro-caspase-8 (p55/53), cleavage intermediates of caspase-8 (p43/41), and FADD/MORT1 (Figure 4, lanes 3 and 5). This recruitment was dependent upon ligand stimulation of TRAIL receptors on intact cells since caspase-8 and FADD/MORT1 were not bound to unstimulated TRAIL receptors (Figure 4, lanes 2, 4, 6, and 8). In addition, no other FADD/MORT1 and caspase-8 binding TRAIL receptors were present on BL60 cells as concomitant blockage of TRAIL-R1 and TRAIL-R2 resulted in reduction of ligand-induced precipitation of caspase-8 and FADD/MORT1 to background levels (Figure 4, lane 7). Thus, FADD/MORT1 and caspase-8 are integral components of both the native homomeric TRAIL-R1 DISC and the native homomeric TRAIL-R2 DISC in BL60 cells.

FADD/MORT1 and Caspase-8 Are Essential for TRAIL-R2-Induced Apoptosis in Jurkat Cells

The mere presence of a protein in the DISC does not imply functional importance at the initiation of apoptosis. Other proteins have been shown to associate with CD95 or TNF-R1 (Krammer, 1999; Wallach et al., 1999). However, for the CD95 system, an essential function could so far only be attributed to FADD/MORT1 and

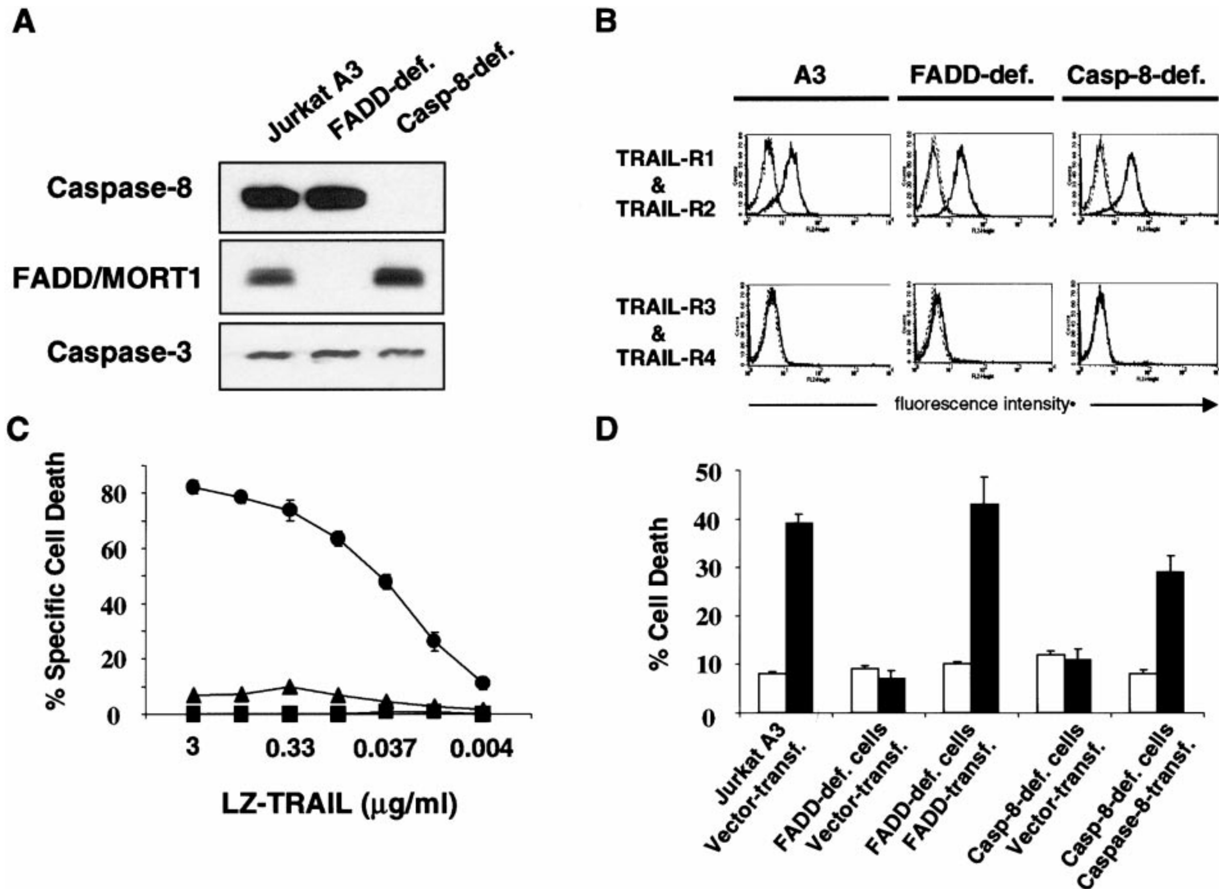

Figure 5. FADD/MORT1 and Caspase-8 Are Necessary for TRAIL-R2-Induced Apoptosis

(A) Western Blot analysis for expression levels of FADD/MORT1, caspase-8, and caspase-3 in Jurkat A3, FADD/MORT1^{def}, and caspase-8^{def} Jurkat cells. Migration positions of the detected proteins are indicated.

(B) FACS analysis of surface expression of TRAIL-R1 (upper panels, solid lines); TRAIL-R2 (upper panels, solid bold lines); TRAIL-R3 (lower panels, solid lines); and TRAIL-R4 (lower panels, solid bold lines) as compared to an isotype-matched control mIgG1 mAb (dashed lines) on Jurkat A3, FADD^{def}, and casp-8^{def} cells.

(C) Jurkat A3 (closed circle), FADD^{def} (closed square), and casp-8^{def} (closed triangle) cells were treated with the indicated concentrations of LZ-TRAIL or were left untreated. Cell death was determined 12 hr after stimulation. Percentage of specific cell death was calculated as follows: $100 \times$ (% experimental cell death $-$ % spontaneous cell death)/(100 $-$ % spontaneous cell death). Spontaneous cell death was below 10% in all samples. All data points are the mean ($\pm$ SD) of six independent experiments.

(D) Jurkat A3, FADD^{def}, and casp-8^{def} cells were transfected with an expression plasmid coding for a spectrin–GFP fusion protein (Kalejta et al., 1997) together with an expression plasmid coding for the respective missing signaling component or together with empty vector in the controls. Cells were incubated for 10 hr in the absence (open bars) or presence (closed bars) of 1 μg/ml LZ-TRAIL before cell death was determined by FSC/SSC analysis in the GFP-positive cell population. Mean $\pm$ SD of two independent experiments is shown.

caspase-8 as cells deficient for one of these two signaling proteins were resistant to CD95-mediated apoptosis. In addition to experiments with cells from mice deficient for FADD/MORT1 (Yeh et al., 1998; Zhang et al., 1998) or caspase-8 (Varfolomeev et al., 1998) that showed the requirement of these two signaling proteins, respectively, it was shown that Jurkat cells deficient for either one of these two proteins did not undergo CD95-mediated apoptosis (Juo et al., 1998, 1999). These FADD/MORT1-deficient (FADD^{def}) and caspase-8-deficient (casp-8^{def}) Jurkat cells expressed normal levels of caspase-3 but showed the expected deficiencies in FADD/MORT1 and caspase-8 expression, respectively (Figure 5A). In addition, the TRAIL receptor surface expression pattern of the deficient clones was identical to that of Jurkat A3 control cells as TRAIL-R2 was expressed while TRAIL-R1, -R3, and -R4 were not present (Figure 5B). In order to determine whether caspase-8 and FADD/MORT1 are necessary for TRAIL-R2-induced apoptosis in Jurkat cells, we treated FADD^{def}, casp-8^{def}, and Jurkat A3 control cells with LZ-TRAIL. Both the FADD^{def} and casp-8^{def} Jurkat cells were resistant to TRAIL-induced apoptosis, while Jurkat A3 cells underwent TRAIL-induced apoptosis in a dose-dependent manner (Figure 5C).

FADD/MORT1 and caspase-8 deficiency do not necessarily constitute the only defects of these mutated cell lines. Therefore, we tested whether ectopic expression of FADD/MORT1 or caspase-8 resensitized the respective Jurkat clones for TRAIL-induced apoptosis. While Jurkat A3 cells transfected with vector control were killed by LZ-TRAIL, both Jurkat FADD^{def} and Jurkat casp-8^{def} remained TRAIL resistant when transfected with vector alone. However, reconstitution of the miss-

ing protein resensitized both cell lines for TRAIL-induced apoptosis (Figure 5D). Since TRAIL resistance was overcome by reexpression of FADD/MORT1 in Jurkat-FADD^{def} and by reexpression of caspase-8 in the caspase-8^{def} Jurkat cells, TRAIL resistance was due to deficiency in the respective signaling proteins. Therefore, we conclude that FADD/MORT1 and caspase-8 are essential for TRAIL-R2-induced apoptosis and cannot be substituted for by other endogenous proteins in Jurkat cells.

Discussion

Thus far, it has been controversial whether the adaptor protein FADD/MORT1 and caspase-8 play a key role in TRAIL-induced apoptosis (Ashkenazi and Dixit, 1999). Our data demonstrate that endogenous FADD/MORT1 and caspase-8 are recruited to the native TRAIL DISC within seconds following stimulation with TRAIL. Subsequently, caspase-8 is cleaved and DNA fragmentation typical for apoptosis is observed in BL60, BJAB, CEM, and Jurkat cells. These data imply that FADD/MORT1 and caspase-8 are likely to play an important role during TRAIL-induced apoptosis.

However, FADD/MORT1-deficient murine embryonic fibroblasts (MEF) were shown to undergo apoptosis upon overexpression of human TRAIL-R1 (Yeh et al., 1998). In the light of our data showing essential recruitment of FADD/MORT1 and caspase-8 to the TRAIL-R2 DISC, there are three possible explanations for this finding by Yeh and colleagues. TRAIL-R1-induced apoptosis in MEFs could be due to overexpression-related unspecific recruitment of an adaptor protein different from FADD/MORT1. Alternatively, cell type–specific differences between the lymphoid cell lines studied here and MEFs could be responsible for the differential results. The most attractive explanation, however, is that TRAIL-R1 and TRAIL-R2 may be differentially regulated. TRAIL-R1-induced apoptosis may, therefore, use an additional, as of yet unidentified, FADD/MORT1-independent apoptotic mechanism (Yeh et al., 1998) apart from the FADD/MORT1 involving pathway (Figure 4), whereas TRAIL-R2-induced apoptosis may be entirely dependent on FADD/MORT1 (Figures 3, 4, and 5). Thus, the observed coexpression of the two apoptosis-inducing TRAIL receptors on certain cell types would allow for fine-tuned regulation of TRAIL-induced apoptosis.

DN-caspase-10 and not DN-caspase-8 (Pan et al., 1997a) or both DN-caspase-10 and DN-caspase-8 (MacFarlane et al., 1997) were reported to associate with overexpressed TRAIL-R1 and TRAIL-R2 and to inhibit apoptosis induced by overexpression of these two receptors. In addition, caspase-10 was suggested to play a role in TRAIL-induced apoptosis of mature DC and peripheral activated T cells. Deletion of these cells was reported to be inhibited in ALPS II patients carrying mutated caspase-10, which supposedly caused TRAIL resistance of T cells and DCs in these patients (Wang et al., 1999). However, recently one of the caspase-10 variants was identified as a common polymorphism in the Danish population with an allele frequency of 6.8% (Gronbaek et al., 2000). These data cast doubt on the role of caspase-10 mutations as the sole causative factor for ALPS II. In the light of our data on native TRAIL DISC composition, the concept that caspase-10 and not caspase-8 initiates TRAIL-induced apoptosis may not be generally applicable.

Other adaptors like RIP and TRADD, although prominently present in the cell lysates, could not be detected in the BL60 TRAIL DISC (Figure 2). Although we cannot rule out that these proteins may be associated as minor components or in a more transient fashion than FADD/MORT1 and caspase-8, they are probably less important for the initiation of TRAIL-induced apoptosis. In the case of TNF-R1 signaling, RIP and TRADD association have been shown to activate the gene inductive JNK and NF-κB pathways (Wallach et al., 1999). As we have only investigated TRAIL-sensitive cells, other proteins including RIP and TRADD, reported to associate with TRAIL receptors upon overepression, may serve anti- rather than proapoptotic functions. Analysis of TRAIL-resistant cells will provide further insight into these mechanisms.

So far, it was unclear whether TRAIL-induced apoptosis may be differentially regulated by the triggering of the two different apoptosis-inducing TRAIL receptors. Here we show that TRAIL-induced cross-linking of either TRAIL-R1 or TRAIL-R2 leads to the recruitment of both FADD/MORT1 and caspase-8 to the individual receptor-specific DISCs. Apart from the homomeric receptor complexes, heteromeric complexes consisting of TRAIL-R1 and TRAIL-R2 also may exist. We cannot exclude that these complexes may signal apoptosis via an additional pathway different from the FADD/MORT1 and caspase-8 involving pathway. However, these complexes and their associated signaling proteins would be part of the TRAIL DISC in BL60 cells. Since we did not detect TRADD, RIP, and caspase-10 in the BL60 TRAIL DISC (Figure 2), it is unlikely that these proteins associate with heteromeric complexes and may, thus, be common mediators of TRAIL-induced apoptosis.

Finally, we could show that TRAIL-resistant FADD/MORT1- and caspase-8-deficient Jurkat cells expressing TRAIL-R2 regained TRAIL sensitivity upon reexpression of these two proteins. Thus, caspase-8 and FADD/MORT1 are essential for TRAIL-R2-induced apoptosis, as they are for CD95-mediated apoptosis (Juo et al., 1998, 1999; Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 1998). These data clearly establish the importance of FADD/MORT1 and caspase-8 in TRAIL-induced apoptosis. FADD/MORT1 and caspase-8 have previously been shown to be implicated in apoptosis induction via CD95, TNF-R1, and TRAMP (Ashkenazi and Dixit, 1999; Krammer, 1999). Thus, the recruitment of endogenous FADD/MORT1 and caspase-8 to the native TRAIL-R1 and TRAIL-R2 provides evidence that these two proteins play a central role in death receptor–mediated apoptosis.

There is an apparent discrepancy between our data on the composition of the native TRAIL DISC and other reports showing no association of FADD/MORT1 with TRAIL-R1 or TRAIL-R2. In addition, in some studies overexpression of FADD-DN failed to inhibit apoptosis induced by TRAIL or overexpressed TRAIL-R1 or TRAIL-R2. While our studies were performed in cell lines expressing native levels of the respective proteins, all previous experiments were performed under conditions where at least one of the putatively interacting proteins

was overexpressed. This overexpression of DD/DED containing proteins might result in nonspecific aggregation. In addition, the homeostasis of different interacting proteins and, thus, assembly of the signaling complexes could be disturbed.

As shown in Figure 2, caspase-8 and FADD/MORT1 coprecipitate only with stimulated TRAIL receptors. Several of the previous studies relied on coprecipitation of proteins with overexpressed receptors without prior ligand-induced cross-linking. Although overexpression of TRAIL receptors induces apoptosis, this does not necessarily mimick ligand-induced receptor oligomerization. In conclusion, it seems that overexpression studies alone are not sufficient to define molecular interactions in apoptosis signaling.

TRAIL-induced apoptosis seems to utilize a pathway similar to the one used by CD95 (Krammer, 1999; Peter et al., 1999). Yet, most normal cells are resistant to TRAIL, whereas CD95 agonists have been shown to kill various normal cells (Nagata, 1997; Krammer, 1999) and, in contrast to TRAIL (Ashkenazi et al., 1999; Walczak et al., 1999), are toxic upon systemic administration (Ogasawara et al., 1993; Walczak et al., 1999). Thus, in addition to the similarities between the CD95 and TRAIL receptor pathways, there must also be differences between them. Studying TRAIL signaling in resistant cells will be likely to give insight into the mechanisms that regulate sensitivity versus resistance to TRAIL-induced apoptosis. The identification of the native TRAIL apoptosis-inducing signaling complex presented here provides the basis for such analyses.

Experimental Procedures

Cell Lines

The human B cell lines BL60 and BJAB and the human T cell lines CEM and Jurkat were maintained in RPMI 1640 (GIBCO-BRL, Karlsruhe, Germany) containing 10% fetal calf serum (GIBCO-BRL). The mutated Jurkat FADD^{def}, Jurkat casp-8^{def}, and the Jurkat A3 control cells were cultured as described elsewhere (Juo et al., 1998, 1999).

Antibodies and Reagents

Monoclonal antibodies (mAb) against FADD/MORT1, TRADD, RIP, and caspase-3 were purchased from Transduction Laboratories (San Diego, CA). Anti-caspase 10 mAb (Zytomed, Berlin, Germany) was raised against the p17 subunit of caspase 10. The mAb anti-FLICE C15 recognizes the p18 subunit of caspase-8 (Scaffidi et al., 1997), whereas the anti-caspase-3 mAb recognizes the p17 subunit of caspase-3 but not the p12 subunit. Leucine zipper (LZ) –TRAIL is a stable trimer of TRAIL and induces apoptosis upon binding to TRAIL-sensitive cells (Walczak et al., 1997). The antibodies specific for the different TRAIL receptors were described elsewhere (Griffith et al., 1999a). We used anti-TRAIL-R1 M272 and TRAIL-R2 M413 for FACS staining and anti-TRAIL-R1 M271 and anti-TRAIL-R2 M413 for receptor blockade. Combinations of anti-TRAIL-R1 mAbs (M270 and M272) or anti-TRAIL-R2 mAbs (M414 and M415) were used for Western blot detection of TRAIL-R1 and TRAIL-R2, respectively. Horseradish peroxidase (HRPO) –conjugated goat anti-mouse IgG1, IgG2a, and IgG2b polyclonal antibodies (pAb) were obtained from Southern Biotechnology Associates (Birmingham, AL). HRPO-goat anti-rat IgG was from Jackson ImmunoResearch (Dianova, Hamburg, Germany). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO).

FACS Analysis

Cells were incubated with mAbs of the same isotype (mIgG1) against the four surface-expressed TRAIL receptors (M272 anti-TRAIL-R1, M413 anti-TRAIL-R2, M430 anti-TRAIL-R3, M444 anti-TRAIL-R4) or control mIgG1 followed by biotinylated secondary goat anti-mouse antibodies (Southern Biotechnology Associates) and Streptavidin-PE (Pharmingen, Hamburg, Germany). Surface staining was determined on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany). Specificity of the respective anti-TRAIL-R mAbs used here was determined by staining of TRAIL-R1 to TRAIL-R4 on CV1/EBNA cells transfected with expression vectors coding for the individual surface-bound TRAIL receptors (data not shown).

Quantitation of Apoptotic Cell Death

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified essentially as described (Nicoletti et al., 1991). Briefly, 2.5×10^5 cells were incubated in 24-well plates (Costar, Cambridge, MA) with or without apoptotic stimuli in 0.5 ml medium at 37°C. Cells were collected by centrifugation at $600 \times g$ for 10 min at 4°C, washed twice with PBS, and then resuspended in 100 μl lysis solution containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium citrate, and 50 μg/ml propidium iodide (PI). Apoptosis was quantitatively determined by flow cytometry after incubation at 4°C in the dark for at least 24 hr as cells containing nuclei with subdiploid DNA content. Alternatively, apoptosis was determined by a drop in the forward to sideward scatter (FSC/SSC) profile of apoptotic in comparison to living cells.

Preparation of Cell Lysates

Cells were harvested by centrifugation at $300 \times g$ for 10 min at 4°C and washed twice with PBS, and lysates were prepared by resuspending the resulting cell pellets in 100 μl lysis buffer per 1×10^7 cells (30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% Glycerol, 1% Triton X-100) supplemented with Complete protease inhibitors (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. After 30 min incubation on ice, the lysates were centrifuged once at $15{,}000 \times g$ at 4°C to remove nuclei. In the case of lysate preparation for ligand affinity precipitations, an intermediate centrifugation step ($600 \times g$ for 15 min at 4°C) was added after lysis in order to remove cellular debris.

Western Blot Analysis

For Western blot analysis, the resulting postnuclear supernatants or ligand affinity precipitates were supplemented with 2-fold concentrated standard reducing sample buffer (2× RSB). Subsequently, lysate 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% nonfat dry milk (NFDM) in PBS/Tween (PBS containing 0.05% Tween-20) for at least 2 hr, washed with PBS/Tween, and incubated in PBS/Tween containing 3% NFDM and primary antibodies against caspase-3, caspase-10, FADD/MORT1, TRADD, RIP, TRAIL-R1 (M270 and M272), TRAIL-R2 (M414 and M415) (all at 1 μg/ml), or caspase-8 (1:10-diluted C15 hybridoma supernatant [Scaffidi et al., 1997]). Specificity of the respective anti-TRAIL-R mAbs used here was determined by Western blot analysis of TRAIL-R1 to TRAIL-R4 in lysates prepared from CV1/EBNA cells transfected with expression vectors coding for the individual TRAIL receptors (data not shown). After six washes for 5 min each in PBS/Tween, the blots were incubated with HRPO-conjugated isotype-specific secondary antibody diluted 1:20,000 in PBS/Tween. After washing six times for 5 min with PBS/Tween, the blots were developed by enhanced chemoluminescence (ECL) following the manufacturer's protocol (Amersham Pharmacia Biotech). For stripping, blots were either incubated for 30 min in a buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol at 60°C or in 50 mM glycine HCl (pH 1.9) for 20 min at room temperature when only secondary antibodies needed to be removed. Subsequently, blots were washed six times for 10 min in PBS/Tween and blocked again.

Ligand Affinity Precipitation

We performed ligand affinity precipitations by using biotinylated LZ-TRAIL (Bio-LZ-TRAIL) in combination with Streptavidin beads (Pierce). Bio-LZ-TRAIL was prepared by incubation of purified LZ-TRAIL at 1 mg/ml with Sulfo-NHS-LC-Biotin at 1 mg/ml (Pierce) for 1 hr on ice before the reaction was stopped by adding 1/10 volume of 1 M Tris-HCl at pH 7.5. Unincorporated biotin was removed from Bio-LZ-TRAIL preparations by buffer exchange into 150 mM NaCl, 30 mM HEPES (pH 7.5) on PD-10 columns (Amersham Pharmacia Biotech). Protein preparations were checked for purity and incorporation of biotin by SDS-PAGE. The biological activity of Bio-LZ-TRAIL was determined by its apoptosis-inducing capacity and found to be comparable to nonbiotinylated LZ-TRAIL.

For ligand affinity precipitation, 3×10^8 cells were used per sample. Cells were washed twice with 50 ml RPMI medium at 37°C and subsequently incubated for the indicated time periods at 37°C and a cell density of 1×10^8/ml in the presence of 1 μg/ml Bio-LZ-TRAIL or, for the unstimulated control, in the absence of Bio-LZ-TRAIL. In the case of differential TRAIL receptor DISC analysis, we preincubated the cells with 10 μg/ml TRAIL-R1 and/or TRAIL-R2-blocking mAbs for 15 min before stimulation with Bio-LZ-TRAIL. DISC formation was stopped by addition at least 15 volumes of ice-cold PBS. Cells were then washed twice with 50 ml ice-cold PBS before cell lysates were prepared by addition of 4.5 ml lysis buffer per 3×10^8 cells. The resulting protein complexes were precipitated from the lysates by coincubation with 20 μl Streptavidin Beads (Pierce) for 2–4 hr on an end-over-end shaker at 4°C. For the precipitation of the nonstimulated receptors, Bio-LZ-TRAIL was added to the lysates prepared from nonstimulated cells at 1 μg/ml to control for protein association to nonstimulated receptor(s). Ligand affinity precipitates were washed four times with lysis buffer before the protein complexes were eluted from the beads by addition of 15 μl 2× standard reducing sample buffer. Subsequently, proteins were separated on SDS-PAGE before presence or absence of antigens was determined in the different precipitates by Western blot analysis.

Transfection and Complementation

Jurkat A3 control, $FADD^{def}$, and $FLICE^{def}$ cells were transfected essentially as described (Juo et al., 1998) with slight modifications. In brief, 6×10^6 cells were transfected with a total of 20 μg DNA by electroporation with an expression plasmid coding for a spectrin–GFP fusion protein (Kalejta et al., 1997) together with an expression plasmid coding for the respective missing signaling component, or together with empty vector in the controls, at a ratio of 3 to 1 (0.4 cm cuvette, 250 V, 950 μF, Bio-Rad Gene-Pulser). The expression plasmids pRSV-HA-FADD, pRSV-caspase-8, or empty control vector are described elsewhere (Juo et al., 1998, 1999). Cells were purified over a Ficoll gradient 1 hr after transfection in order to remove dead cells. After each transfection, half of the cells were either left untreated or stimulated with 1 μg/ml LZ-TRAIL. After 10 hr of incubation, apoptosis was quantified by FSC/SSC analysis of the GFP-positive population on a FACSscan cytometer (Beckton Dickinson).

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