Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes

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Summary

Apoptosis induced by the death receptors Fas and TNF-receptor I (TNFR1) is proposed to proceed through recruitment of FADD and caspase-8 to the receptor complex. It is unclear, however, why Fas-induced cell death occurs within minutes, whereas several hours are required for the effector function of TNFR1. Here we report that TNFR1-induced apoptosis (in contrast to Fas) is a two-step process, that involves two sequential signaling complexes. The initial plasma membrane-bound complex (complex I) consists of TNFR1, TRADD, RIP1 and TRAF2 and rapidly signals activation of the transcription factor NF-κB. In this complex, TRADD and RIP1 undergo important posttranslational modifications and subsequently dissociate from the receptor. In a second step, TRADD and RIP1 associate with FADD and caspase-8, thereby forming a cytoplasmic complex (complex II). In surviving cells where NF-κB is activated by complex I, complex II harbors the caspase-8 inhibitor $FLIP_L$. In apoptosis sensitive, NF-κB signal-defective cells, substantial amounts of caspase-10 are found in complex II while $FLIP_L$ levels are highly reduced. Thus, TNFR1-triggered signal transduction includes a check-point, resulting in cell death (via signal complex II) in instances where the initial signal (via complex I, NF-κB) fails to be activated.

Introduction

Tumor necrosis factor (TNF) is a potent cytokine that exerts pleiotropic functions in immunity, inflammation, control of cell proliferation, differentiation and apoptosis (Ashkenazi and Dixit, 1998; Wallach et al., 1999). TNF is the prototypical member of a still growing family of cytokines that include, among others, TNF, lymphotoxin-α (LTα), Fas ligand (FasL), CD40 ligand (CD40L) and TNF-related apoptosis-inducing ligand (TRAIL). Although most of the TNF family members are potent inducers of the signaling pathways that lead to the activation of the transcription factor NF-κB, some of these ligands can also induce apoptosis by binding to so-called death receptors. These receptors (TNFR1, Fas (CD95), TRAMP (DR3), TRAIL-R1 (DR4), TRAIL-R2 (DR5), DR6 and EDAR) share not only the typical amino-terminal cysteine-rich domains (CRDs), which define their ligand specificity (Bodmer et al., 2002), but also a stretch of 60-70 amino acids called the death domain (DD) that is necessary for the induction of apoptosis (Ashkenazi and Dixit, 1998).

Signaling through Fas, TRAIL-R1 and TRAIL-R2 has been well characterized (Krammer, 2000). Engagement of these receptors delivers a powerful and rapid pro-apoptotic signal through a DD-mediated recruitment of the adapter protein FADD and the formation of the so-called death-inducing signaling complex (DISC) (Scaffidi et al., 1999). FADD in turn, via its death effector domain (DED), mediates the recruitment and the activation of pro-caspase-8, leading to the release of the active p18/p12 fragments. Cytoplasmic caspase-8 then activates downstream caspases that participate in the execution of the apoptotic process.

Recruitment of FLIP to the DISC inhibits the release of the active caspase-8 fragments from the complex (Irmler et al., 1997) and thus blocks cell death.

In contrast to Fas and TRAIL-receptors, the molecular mechanisms involved in TNFR1-induced cell death remain poorly defined, despite the fact that signaling through TNFR1 has long been studied (Chen and Goeddel, 2002; Rath and Aggarwal, 1999). It is currently believed that engagement of TNFR1 triggers the recruitment of the DD-containing adapter molecule TRADD followed by the DD-containing Ser/Thr kinase RIP1 (Ashkenazi and Dixit, 1998; Chen and Goeddel, 2002). This signaling complex is required for TRAF2/5 and c-IAP1 binding that leads to the triggering of NF-κB and JNK signaling pathways (Baud and Karin, 2001). TNFR1 occupation not only triggers these pathways, but can also induce apoptosis by instead binding the DD-containing adaptor FADD (via TRADD) that allows caspase-8 recruitment and activation (Hsu et al., 1996). In an alternative, though not exclusive model, the initial TRADD-RIP1 containing complex has been proposed to bind the adaptor protein RAIDD, resulting in caspase-2 dependent cell death activation (Duan and Dixit, 1997). However, both models are based on studies where interaction was demonstrated upon massive overexpression of proteins putatively present in the signaling complex and has yet to be confirmed under physiological conditions. Indeed, caspase-2 appears to be dispensable for TNF-induced apoptosis (Lassus et al., 2002). In contrast there is genetic evidence that FADD and caspase-8 are important for TNFR1-mediated apoptosis (Juo et al., 1998; Yeh et al., 1998). Moreover, expression of the inhibitor of caspase-8, $FLIP_L$, inhibits the TNF-induced apoptotic pathway (Micheau et al., 2001), further arguing for an important role of caspase-8. $FLIP_L$

expression is induced by NF-κB (Kreuz et al., 2001; Micheau et al., 2001), which may explain why death receptor-induced apoptosis is generally blocked in cells with active NF-κB.

Here we demonstrate that, unlike the Fas signaling pathway, the TNFR1-induced pro-apoptotic signaling pathway requires the formation of two distinct signaling complexes. The rapidly formed plasma membrane-bound complex I is composed of TNFR1, TRADD, RIP, TRAF2 and c-IAP1, and triggers a NF-κB response, but no apoptosis. A second complex, which lacks TNFR1 but includes FADD and pro-caspases -8 and –10, subsequently forms in the cytoplasm. This secondary complex (complex II) initiates apoptosis, provided that the NF-κB signal from complex I fails to induce the expression of anti-apoptotic proteins such as $FLIP_L$.

Results

TNFR1 triggers apoptosis in the NF-κB unresponsive HT1080 I-kBmut cells

NF-κB can promote the expression of several anti-apoptotic genes such as TRAF1, TRAF2, cIAP-1, c-IAP-2 and notably $FLIP_L$, a potent inhibitor of death receptor-induced apoptosis (Micheau et al., 2001; Wang et al., 1998). We previously described the HT1080 fibrosarcoma cell line that is proficient in NF-κB activation (designated wt) and a variant of this cell line (designated IκBmut) that is defective in NF-κB activation □ □ □ to the expression of an undegradable form of the NF-κB inhibitor I-κBα (Micheau et al., 2001). NF-κB-induced upregulation of anti-apoptotic proteins protects the former but not the latter cell line from TNF-induced apoptosis (Micheau et al., 2001) (Fig. 1A). Determination of caspase-3 activity

demonstrated that TNF-induced apoptosis was slow and detectable only 4 h after TNF addition, and that it involved processing of caspase-8, caspase-3 and caspase-2 (Fig. 1B). Inhibition of the apoptotic process was achieved with zVAD-fmk (Fig. 1B) at concentrations as low as 5 µM. Moreover, TNF-induced cell death was specifically inhibited by an antagonistic anti-TNFR1 but not anti-TNFR2 antibody in a concentration dependent manner (Fig. 1C), indicating that cell death was mediated by TNFR1.

The TNFR1-membrane associated proximal complex (complex I) is devoid of caspase-8 and FADD

In an attempt to define the molecular mechanisms which govern the cell's decision to activate NF-κB or, alternatively, to undergo cell death, the composition of the signaling complexes in HT1080-wt and HT1080-IκBmut cells was initially investigated. Using either a Flag-tagged recombinant human TNF (Fig. 2A), Fc-TNF (Fig. 2B) or a specific anti-TNFR1 antibody (data not shown), we found no obvious divergence in protein composition of the precipitated signaling complex (complex I) obtained from either cell line. In the wt as well as in the I-κB mutant cells, RIP1, TRAF2, and the adaptor protein TRADD were co-immunoprecipitated with TNFR1 (Figs. 2A, B). Notably, c-IAP1 was primarily found in the signaling complex of TNF resistant but not sensitive cells. This finding is in agreement with the proposed role as an inhibitor of TNF-mediated apoptosis (Wang et al., 1998). During the course of stimulation (>30 min), a progressive and substantial loss in TNFR1-associated proteins was observed in both cell lines. A moderate reduction

of TNFR1 binding to the ligand was also observed, probably due to endocytosis of the engaged receptor (Schutze et al., 1999). As previously shown for RIP1 (Zhang et al., 2000)**,** TRADD and TNFR1 also underwent extensive post-translational modifications in complex I. These changes were not observed in total cell lysates and were detectable by independently raised antibodies (Fig. 2A, B). Ubiquitination is one of the modifications of RIP1 and TNFR1 (Legler et al., 2002), while the nature of the extensive TRADD modifications remains to be determined.

Surprisingly, neither FADD nor caspase-8 were detectable in complex I, even in the death-sensitive I-kBmut cell line (Fig. 2A, B), in which cleavage and activation of caspase-8 clearly occurred (see Fig. 1B). Likewise, RAIDD and caspase-2 were not found in the complex (data not shown). FADD and caspase-8 were, however, readily detectable in Fas ligand immunoprecipitates of both cell lines, using the same experimental protocol (Fig. 2C and data not shown). Identical data were obtained with Jurkat and U937 cells, which had been rendered sensitive to TNF-induced cell death by use of cycloheximide (not shown), suggesting that the FADD/caspase-8 interaction with complex I is either very weak or does not occur at all.

TNF stimulation triggers the formation of complexes of high molecular weight

TNFR1 stimulation leads not only to the recruitment and assembly of TRADD, RIP1 and TRAF2, but also of components of the downstream NF-κB machinery, such as I-κB, the protein kinases IKKα, β and the scaffold protein IKKγ (NEMO)

(Poyet et al., 2000; Zhang et al., 2000). IKKs assemble in a complex with a molecular mass of approximately 700 kDa (Karin and Lin, 2002). Complex formation correlates with the activation of the two kinases that act on their I-κB substrate. We therefore analyzed the size of signaling complexes formed in the HT1080-IκBmut cells. Examination of the elution profile of proteins following TNF stimulation for 5 min indicated that the IKKs were present in a high apparent molecular weight complex of approximately 700-1200 kDa as expected (Fig. 3). Interestingly, a proportion of TNFR1, RIP1, TRADD and TRAF2 were also present in a high mw complex (Fig. 3). Sixteen hrs after stimulation, IKKβ, TNFR1 and to a lesser extent TRADD, TRAF2 and RIP1 returned to an elution peak indicative of the status observed in untreated cells. Analysis of the elution profiles of caspase-8, -10 and FADD proved particularly interesting. These proteins also rapidly shifted to high molecular weight after TNF stimulation, but in contrast to IKKβ, a substantial portion remained in a complex even after 16 hrs. This is consistent with the observation that caspase-8, -10 and FADD were not associated with immunoprecipitated TNF-complexes (see Fig. 2). In addition, a proportion of caspase-8, -10 and FADD co-eluted with RIP1, TRADD and TRAF2, 16 hrs after TNF stimulation. Together with the observation that neither caspase-3 nor Fas partitioned in these high molecular mass fractions upon stimulation (Fig. 3), these data suggest that a novel, long-lived complex (designated complex II), comprising most of the components of complex I except TNFR1, is formed

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Evidence for a novel, caspase-8 and FADD containing complex II

Upon TNF binding, TNFR1 is internalized (Schutze et al., 1999). Indeed, in our experimental system, a decrease in TNFR1 surface accessibility was observed in both wt or I-kBmut cells upon TNF stimulation (see Fig. 2 , also assessed by flow cytometry, not shown). However, the moderate loss of surface expression only partly correlated with the loss of TNFR1 associated proteins observed in Fig. 2, suggesting that these proteins dissociate from TNFR1. FADD and caspase-8 were previously shown to be essential for TNF-mediated cell death (Juo et al., 1998; Yeh et al., 1998). Both proteins are also constituents of a high molecular weight complex which is formed upon TNFR1 engagement (Fig. 3). We therefore decided to further investigate the putative pro-apoptotic complex further downstream in the signaling pathway, by immunoprecipitating caspase-8. In both resistant wt- and sensitive HT1080-I-κBmut cells stimulated with TNF, caspase-8 immunoprecipitates were found to incorporate not only FADD, but also TRADD, RIP1 and TRAF2 in a time- and stimulation-dependent manner (Fig. 4A). Association started 30 to 60 min post-stimulation, increased over time and was strongest after 4 to 8 hrs, corresponding to the time of onset of apoptosis (Fig. 4A). Association of TRADD and RIP1 coincided with their disappearance from the primary complex, suggesting that TRADD and RIP1 dissociated from complex I. This notion is supported by the observation that both RIP1 and TRADD carried higher molecular weight modifications that are induced upon TNFR1 binding (see Fig. 2). Most importantly, analysis of the caspase-8 co-immunoprecipitates revealed differences between the resistant and sensitive cell lines. RIP1, TRADD

and TRAF2 recruitment to caspase-8 was transient in resistant cells, peaking 2-4 hrs after TNF stimulation. In contrast, these three proteins remained associated with caspase-8 even 8 hrs post-stimulation in the sensitive cell line. In addition, both caspase-8 and caspase-10 were cleaved and activated in complex II of the sensitive cell line, whereas substantially less processing was evident in the resistant cell line. This correlates with the differences in levels of the long form of FLIP ($FLIP_L$) found in complex II. Higher levels of $FLIP_L$ were detectable in resistant cells. Importantly, $FLIP_L$ was predominantly found in its uncleaved form in resistant cells, in contrast to sensitive cells where caspase-8 associated $FLIP_L$ was almost completely processed to produce its 43/41 kDa cleavage products. It has been shown previously that complete processing of $FLIP_L$ occurs only in cells that undergo apoptosis (Tschopp et al., 1998). Complex II of resistant but not sensitive cells also contained TRAF1 and c-IAP1, which is expected as TRAF1 and c-IAP-1 expression are dependent on NF-κB signals (Wang et al., 1998). Remarkably, however, TNFR1 was absent from this secondary complex in both cell lines (Fig. 4A). To make sure that the protocol used to immunoprecipitate the signaling complex with caspase-8 permitted precipitation of the receptors, it was applied to cells that had been stimulated with Fas ligand. In this case, caspase-8 immunoprecipitates revealed an association with Fas in addition to FADD and $FLIP_L$ (Fig 4B), demonstrating the validity of the method used and reinforcing the differences in the signaling behavior of Fas and TNFR1.

To corroborate these data, we investigated the composition of complex II 16 hrs after stimulation, at a time when almost all cells had undergone apoptosis. At this late stage, the composition of complex II in wt and I-kBmut cells was definitely

different. In wt cells, $FLIP_L$ and TRAF-1 were still associated with caspase-8, but little caspase-10 was co-immunoprecpitated (Fig 5). In the sensitive HT1080-I-kBmut cell line however, little or no TRAF1 was present in complex II, and $FLIP_L$ was not detectable at all. In contrast, caspase-10 was abundant and was immunoprecipitated in its processed form. These data suggested that recruitment of $FLIP_L$ and caspase-10 to complex II are mutually exclusive. To corroborate this notion, we expressed $FLIP_L$ in sensitive I-kBmut cells at a level comparable to that found in wt cells (Fig 5), which rendered these cells again resistant to TNF-mediated apoptosis (data not shown). While $FLIP_L$ levels in complex II were high in these transfected cells, the amount of caspase-10 was again low. Moreover, we overexpressed caspase-8 and caspase-10 in 293T cells and found that caspase-8 was present in caspase-10 immunoprecipitates (Fig. 5B). Co-expression of $FLIP_L$ reduced caspase-8 association in a dose-dependent manner, indicating that caspase-8 and caspase-10 interaction can be modulated by $FLIP_L$.

TNF-induced signaling complex I and complex II localize to different sub-cellular compartments

In order to determine the subcellular localization of the TNFR1 complexes I and II, respectively, confocal and cell fractionation experiments were performed. Subcellular localization of RIP1 and active (processed) caspase-8 was monitored by confocal microscopy in HT1080 I-κBmut cells stimulated by TNF for 5 or 360 min, respectively. In untreated cells, RIP1 was expressed mainly in the cytoplasm and no active caspase-8 was detected (Fig. 6). Five min after stimulation, RIP1 was found occasionally at the membrane level (Fig. 6), but the majority of the

RIP1 proteins was present in the cytoplasm, which is in agreement with the observation that at most 5% of RIP1 undergoes post-translational modifications (and thus recruitment to TNFR1) upon TNF stimulation (see Fig. 2). Active caspase-8 staining was not observed after 5 min, but after 360 min a relatively small number of cells remaining attached at the end of the staining procedure became positive (Fig. 6). In these cells, we found active caspase-8 in the cytoplasm. The apoptotic phenotype of the cells did not allow us to determine the precise sub-cellular localization of active caspase-8.

We next performed cell fractionation experiments prior to immunoprecipitation of TNFR1 or caspase-8, as described above. HT1080 I-κBmut cells were first treated with TNF for different time periods, and then disrupted by hypotonic lysis. Membrane enriched and soluble fractions were separated by high speed centrifugation, and complex I and complex II immunoprecipitated after detergent solubilization of the two fractions. As expected, assembly of complex I occurred principally in the membrane enriched P100 (pellet) fraction (Fig. 7A), in which TNFR1 and other transmembrane receptor such as transferrin receptor were present. In agreement with results shown in Fig. 2, caspase-8 was not found in complex I. In contrast, complex II partitioned principally in the cytosolic fraction (Fig 7B), although in this case, substantial amounts were also detectable in the membrane fraction. This may suggest that a proportion of complex II may be associated with cytoskeletal proteins.

Complex II formation requires FADD

As noted previously, there is convincing evidence that FADD is required for TNF-induced apoptosis (Juo et al., 1999; Yeh et al., 1998). We therefore asked whether the dominant negative version of FADD (FADD-DN) or the absence of FADD would impair the formation of complex II. As expected, HT1080-I-kBmut cells overexpressing FADD-DN were resistant to TNF-induced apoptosis (Fig. 8A). No or little RIP1 was found in anti-caspase-8 immunoprecipitates after TNF treatment in FADD-DN cells (Fig. 8B), indicating that RIP1 binding to caspase-8 is dependent on the presence of FADD. Similar data were obtained in the FADD-deficient Jurkat cell line I2.1, in which RIP1 association with caspase-8 was severely impaired, as evidenced by the absence of modified RIP1 (Fig. 8C). Transfection with wt FADD restored RIP1 association (Fig. 8C). In contrast to complex II, FADD-deficient and -reconstituted Jurkat I2.1 cells showed comparable amounts of RIP1 in complex I (Fig. 8D), demonstrating that RIP1 recruitment to TNFR1 is not dependent on the presence of FADD. Taken together these data indicate that in complex II, procaspase-8 interaction with RIP1 is dependent on FADD.

Discussion

Although the molecular mechanisms of TNF-induced activation of pro-survival pathways (NF-κB, JNK) have been reasonably well elucidated (Baud and Karin, 2001; Devin et al., 2001), the principle deciding on whether TNF signals cell survival or cell death remains largely unknown. Our data now provide evidence that the decision is not made at the level of the rapidly formed complex assembling around the ligand-bound TNFR1 at the plasma membrane. Commitment to cell

death is slow and is dependent on a complex that dissociates from TNFR1 (complex II) and which is found mostly in the cytoplasm.

The results presented in this paper are compatible with the model outlined in Fig. 9. TNFR1 stimulation leads to the rapid assembly of a complex (complex I) comprising the receptor itself, TRADD, RIP1, TRAF2, c-IAP1 and possibly other known (c-IAP2, FAN etc.) or yet unidentified proteins. Complex I is, however, devoid of FADD and caspase-8. Complex I triggers the NF-κB signaling pathway via recruitment of the IKK complex (Zhang et al., 2000) whereas JNK is activated via TRAF2-mediated activation of MAP3-kinases (Chen and Goeddel, 2002). Assembly of complex I occurs in lipid rafts (Legler et al., 2002) where posttranslational modifications of several complex-associated proteins are likely to occur. For example, complexed TNFR1, which in its non-stimulated state exhibits an apparent molecular mass of 48 kDa, forms molecular species with apparent mw ranging from 48 kDa to up to 150 kDa. Also, up to 50% of TRADD present in complex I undergoes modifications that increase its molecular mass from 35 kDa to approximately 44 kDa and 55 kDa. Finally, complex I-associated RIP1 migrates as a smear with apparent mw ranging from 78 kDa to 120 kDa.

Formation of complex I is transient since a large portion of TRADD, RIP1 and TRAF2 dissociate from TNFR1 within an hour, at a time when TNFR1 starts to undergo endocytosis. Dissociation of TRADD was suggested to be dependent on TNFR1 endocytosis (Jones et al., 1999), although based on our data, endocytosis and dissociation do not strictly correlate. Our data also do not reveal whether or not the extensive modifications seen cause dissociation. In any case, after dissociation from TNFR1, the DD of TRADD (and RIP1) previously engaged in

the interaction with the DD of TNFR1 becomes available for interaction with other DD-containing proteins. FADD is a likely interaction partner for TRADD, since TRADD and FADD were previously shown to interact via their respective DD (Hsu et al., 1996; Thomas et al., 2002; Varfolomeev et al., 1996). Although a RIP1-FADD- interaction was also described (Varfolomeev et al., 1996), it is less likely to be of importance for complex II formation since TNFR1-induced apoptosis still proceeds in RIP1-deficient Jurkat cells (Holler et al., 2000). Thus, similar to the DD of Fas, the DD of modified TRADD may act as a central platform for the recruitment and activation of FADD, leading to the subsequent binding of caspase-8.

After recruitment of FADD and caspase-8, the decision as to whether TNF acts to promote gene transcription or apoptosis has to be made. Indeed, in contrast to complex I, the composition of complex II in apoptosis-resistant and sensitive cells differs. In resistant cells, complex II comprises increased amounts of the two anti-apoptotic proteins c-IAP1 and $FLIP_L$ and the expression of which is regulated by the transcriptional activity of NF-κB (Micheau et al., 2001; Wang et al., 1998). Inhibition of the pro-apoptotic activity of caspase-8 is more likely to occur through $FLIP_L$, since enforced expression of FLIP but not c-IAP1 potently blocks TNF-mediated cell death (Micheau et al., 2001). Moreover, FLIP-/- embryonic fibroblasts are highly sensitive to TNF-induced apoptosis and show rapid induction of caspase activities (Yeh et al., 2000). In keeping with this observation, sixteen hrs after TNFR1 stimulation, complex II is devoid of $FLIP_L$ in sensitive cells, while it contains increased quantities of caspase-10. Caspase-8 is known to interact with itself, caspase –10 and with FLIPs, although the preferred interaction

partner is $FLIP_L$ (Irmler et al., 1997; Krueger et al., 2001; Wang et al., 2001). Thus, in cells with high $FLIP_L$ content, caspase-10 has limited access to caspase-8 within complex II, while in cells expressing low quantities of $FLIP_L$, high amounts of caspase-10 are found associated with caspase-8. Whether $FLIP_L$ and caspase-10 compete for the same site on caspase-8 or whether $FLIP_L$ indirectly competes with caspase-10 remains to be determined. Moreover, it is not known whether caspase-10 is an essential component in the pro-apoptotic complex II, since the role of caspase-10 in TNF-mediated or in Fas-and TRAIL–mediated apoptosis is uncertain (Kischkel et al., 2001; Sprick et al., 2002).

$FLIP_L$ availability at the moment complex II is formed is dependent on a signal previously triggered by complex I (Kreuz et al., 2001; Micheau et al., 2001). If NF-κB-activation promotes the expression of $FLIP_L$, the pro-apoptotic activity of caspase-8 is inhibited. In contrast, if complex I-triggered NF-κB activation is not productive, the amount of available $FLIP_L$ will rapidly diminish and the proapoptotic activity of caspase-8 will not be stopped. Such a model predicts that $FLIP_L$ plays two important roles; on the one hand it regulates whether or not TNF triggers apoptosis, and on the other hand it is also able to act as a sensor for the fidelity of the signal emanating from complex I.

This model has interesting, more general implications as it predicts that the transcriptional activity of the NF-κB signaling pathway is controlled by (a) checkpoint(s), similar to checkpoints controlling the integrity of cell cycle progression. This control mechanism is triggered immediately after TNFR1 engagement but is operational only a few hours later, at a time when the success of the transcriptional activity of NF-κB can be assessed. Cells with defective NF-κB

signals (and thus having low quantities of FLIP and other anti-apoptotic proteins) will be eliminated through TNF-induced apoptosis.

The formation of complex II may also explain the different kinetics of apoptosis induced by TNFR1 and Fas. Fas recruits FADD directly to the plasma membrane, and subsequent activation of the two DED-containing upstream caspases is rapid and can occur within minutes. In contrast, TNFR1 is unable to recruit FADD directly but instead recruits adaptor proteins which upon dissociation can bind FADD in a second step. Complex II formation is clearly FADD-dependent, as demonstrated using FADD-DN or FADD-deficient cells. Interestingly, point mutations in FADD, inhibiting the association with Fas but not with TRADD nor caspase-8, have been identified (Thomas et al., 2002). Reconstitution of Jurkat FADD-deficient cells with FADD constructs carrying these mutations severely impair Fas-induced apoptosis, but restore TNF-induced apoptosis (Thomas et al., 2002). Moreover, overexpression of TRADD leads to FADD-dependent cell death (Yeh et al., 1998) placing FADD downstream of TRADD. Recent results even suggest that in promyeolytic cells, TRADD is able to trigger cell death from within the nucleus (Morgan et al., 2002).

Upstream caspases have to be brought in close proximity for their activation (Boatright et al., 2003). Assembly of death receptors upon ligand binding as well as Apaf-1 complexes upon cytochrome c leads to the formation of ideal platforms for caspase activation. It is likely that TRADD remains oligomerized upon dissociation from TNFR1 and thus brings caspase-8/10 into close proximity after recruitment of FADD. The TRADD-induced type II complex is the first example of a soluble, cytoplasmic complex that leads to caspase-8/10 activation. Cells

deficient in TRADD however, need to be studied to conclusively draw this conclusion.

Experimental Procedures

Cell Culture. HT1080 (human fibrosarcoma) cell lines wt, I-κBmut and the 293T human embryonic kidney cell line were cultured in Dulbecco's modified Eagle's medium Gibco BRL (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin (50 µg/ml of each) and grown in 5% CO_2 at 37°C. HT1080 wt or I-κBmut cells expressing $FLIP_L$ were described previously (Micheau et al., 2001). FADD deficient Jurkat (I2.1) were maintained in RPMI-1640 (Life Sciences, Basel, Switzerland) supplemented with 10% FCS and antibiotics. HT1080 expressing FADD-DN (aa 80-203) or Jurkat I2.1 expressing wt FADD (1-209) stable cell populations were generated, essentially as described (Micheau et al., 2001), by use of the viral vectors pBABE or pMSCV (Clonetech), respectively.

Antibodies and Materials. Rabbit polyclonal anti-TRAF2 (C20), anti-Nemo (FL-419), anti-Fas (C20), mouse anti-TRAF-1 (H3), anti-TNFR1 (H5) and anti-caspase-8 (C20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-RIP1, anti-TRADD, and anti-FADD were from Transduction Lab (Lexington, KY). Mouse anti-caspase-8 (IgG 2b) and caspase-10 were from MBL (Naka-ku, Japan), anti-transferrin receptor was from Zymed (San Francisco, CA). Anti-caspase-8 (IgG2a), anti-caspase-3 and anti-caspase-2

antibodies were from Pharmingen (San Diego, CA), anti-FLIP (Dave II) was from Apotech (San Diego), anti-IκBα from Biolabs (Beverly, MA). Anti-Flag (M2) antibody was from Sigma (St Louis, MO). Rabbit polyconal anti-human active caspase-8 was kindly provided by T. Momoi (National Institute of Neuroscience, Japan), anti-TNFR1 antagonistic antibody (MAB 225) was from R&D. Anti-TNFR2 antagonistic antibody (UTR1) was a gift from Dr. Brockhaus, Hoffmann La Roche. Human recombinant ligands (Fas ligand, TNF) were obtained from Apotech (San Diego).

Cell Death and Viability Assays. Fibrosarcoma HT1080 cells or transfectants derived therefrom (1.5×10^4 per well) were seeded in 96-well microtiter plates in the presence of the indicated reagents for 48 h and viability was determined by the methylenee blue colorimetric assay (Micheau et al., 1999). In some experiments, the number of apoptotic cells was determined by Hoechst staining. Caspase activity was assayed by incubating 10 µl NP40 lysates with 100 µl of a caspase reaction buffer (100 mM HEPES, pH 7.0, 10% glycerol, 1 mM EDTA, 0.1% CHAPS, 1 mM dithiothreitol) containing 50 µM of DEVD-AMC (Apotech). The mixture was incubated for 60 min in an ELISA titer plate and the fluorescence was measured using a Fluoroscan ELISA reader (excitation 355 nm, emission 460 nm). Caspase-3 activity is expressed as the ratioo of fluorescence increase relative to non-treated cells. Background was subtracted using a the lysis-buffer only. Values were normalized with respect to protein content.

Western blotting. Cell lysates were prepared in lysis buffer (20 mM Tris-HCl pH7.4, 150 mM NaCl, 10% glycerol, Nonidet NP40 0.2%, supplemented with a

protease inhibitor cocktail (Roche Biochemicals, Basel, Switzerland). Cell debris and nuclei were removed by centrifugation at 10,000 g for 10 min and the protein concentration was determined by the Bradford assay (Pierce, Rockford, IL). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes by electroblotting and non-specific binding sites were blocked by incubation in TBS containing 0.5 % Tween-20 and 5 % (w/v) dry milk. Immunoblot analyses were performed with the indicated antibodies. Bound primary antibodies were visualized with horseradish peroxidase-conjugated goat anti-rabbit-IgG, goat anti-rat-IgG or goat anti-mouse-IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and ECL (Amersham, Freiburg, Germany). For TNFR1 complex analysis, the horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a and IgG2b from Southern Biotechnology Associates (Birmingham, AL) were preferentially used.

TNFR1 complex analysis by immunoprecipitation. The complexes initiated upon TNFα stimulation were analysed either by use of Flag-tagged TNFα (Apotech) or Fc-TNFα (Holler et al., 2003) or by the use of anti-caspase-8 antibodies (C20 from Santa Cruz or Pharmingen). HT1080 cells, (5×10^7 cells/ml) were stimulated for the indicated times with 2 µg TNFα and lysed in 1 ml lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Nonidet P40, 10% glycerol and complete protease inhibitor cocktail) for 15 min on ice. Lysates were precleared with 20 µl Sepharose-6 B (Sigma-Aldrich) for 0.5-2 h at 4 °C and immunoprecipitated with 20 µl protein-G–Sepharose CL-4B (Amersham) for 4h to overnight at 4 °C with 2 µg anti-Flag M2 or 2 µg anti-caspase-8. Beads were

recovered by centrifugation and washed four times with 500 µl of lysis buffer before analysis by SDS–PAGE and western blotting.

Co-immunoprecipitation experiments. 293T cells were plated overnight in 10 cm dishes and transfected with the indicated pCRII-based expression vectors for HA-tagged caspase-8, VSV-tagged $FLIP_L$, as previously described (Thome et al., 1997) or a FLAG-tagged caspase-10 for 24 hrs. Cells were collected, washed in PBS and lysed in a buffer containing 0.1% Nonidet-P40, 50mM Tris pH7.8, 150mM NaCl, 5mM EDTA, protease inhibitor cocktail. Complete lysis was ensured by three subsequent quick steps of freezing-thawing were done to complete the lysis. Preclearing was achieved with 20 µl Sepharose 6B for 1 hour, and lysates were incubated overnight at 4^oC in the presence of protein G Sepharose and 1 µg anti-FLAG antibody before analysis by western blotting.

Subcellular fractionation. Cells were stimulated or not with TNFα, washed twice in cold PBS and resuspended in 1 ml homogenisation buffer (250 mM sucrose, 20 mM Tris-Hcl pH 7.4, 1 mM $MgCl_2$, 1 mM $MnCl_2$, plus complete protease inhibitor cocktail (Roche Biochemicals)) for 20 min on ice. Cells were disrupted with 15 strokes of a tight-fitting pestle in a dounce homogenizer. Nuclei and unbroken cells were removed by low-speed centrifugation (1000 g for 10 min at 4°C) and homogenates were centrifuged at 100,000 g for 1 h at 4°C. The pellets containing cellular membranes were resuspended in 10 mM Tris-HCl pH7.4, 150 mM NaCl, 0.2 % NP40. Both fractions were used for further immunoprecipitation as described above.

Size exclusion chromatography. HT1080 I-kBmut cells (10^8) were stimulated with 100 ng/ml TNFα for 5 or 960 min, washed twice in cold phosphate-buffered

saline, and lysed in CHAPS containing lysis buffer (14 mM CHAPS, 150 mM NaCl, 20 mM Tris-Hcl pH 7.4) plus complete protease inhibitors. Lysates were loaded onto a Superdex-200 HR10/30 column previously equilibrated in CHAPS lysis buffer. Proteins were eluted at 1 ml/min. Fractions (1 ml) were maintained at 4°C and precipitated using chloroform / methanol. Samples were analyzed by western blotting for TNFR1, RIP1, TRADD, TRAF2, IKKβ, IKKγ, caspase-8, -3, -10, FADD and Fas using appropriate antibodies, and apparent molecular weight evaluated after column calibration with standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Immunostaining and Confocal Laser Scanning Microscopy. Cells were seeded in Lab-Tek tissue culture chamber slides 24 hours before TNFα (100 ng/ml) stimulation for the indicated time. Immunostaining was carried out on 2% paraformaldehyde fixed cells using the anti-RIP1 (Transduction Laboratories) and a rabbit anti-active Caspase-8, kindly provided by Dr. T. Momoi, University of Tokyo). Subsequently, cells were incubated with secondary antibodies, anti-mouse Alexa-488 or anti-rabbit CY5. Nuclei were stained by Hoechst (10 µg/ml) for 5 minutes and slides were mounted with FluorSave reagent (Calbiochem) before analysis on a Zeiss Axiovert 100 microscope (Zeiss Laser Scanning Microscope 510).

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Figure legends

Fig. 1. TNFR1 induces slow apoptosis in the NF-κB unresponsive HT1080 I-kBmut cells

(A) The HT1080 fibrosarcoma cell lines, wt (circles) or I-κBmut (stably transfected with a mutated, nondegradable version of IκBα, squares) were treated with increasing quantities of TNF and cell viability assessed after 48 hrs.

(B) Wt or I-κBmut HT1080 cells were treated with 50 ng/ml TNF for the indicated period of time, in the presence or absence of 50 μM zVAD-fmk. Cell extracts were analyzed for caspase-8, caspase-2, and caspase-3 content by western blotting. Caspase-3 activity was quantified in wt (empty bars) and I-κBmut (filled bars) cells by use of the substrate DEVD-AMC.

(C) TNF-induced apoptosis of HT1080 I-κBmut cells is mediated by TNFR1. Apoptosis was quantified 24 hrs after TNF treatment, in cells preincubated or not (1 hr) with TNFR1 or TNFR2-antagonistic antibodies.

Fig. 2. The TNFR1-membrane associated complex I is devoid of caspase-8 and FADD

(A) Time course of recruitment of the proximal signaling complex (Complex I) to TNFR1. Wt or I-kBmut HT1080 cells were stimulated for the indicated time with Flag-tagged human-TNF at 37°C and immunoprecipitated using anti-Flag antibodies. Samples were analyzed by western blotting using antibodies directed against TNFR1, TRADD, RIP1, TRAF2, FADD, caspase-8 or c-IAP1. Cellular extracts (CE) are shown that correspond to 1/200th of the cell lysate that was used

to perform the immunoprecipitation. The filled arrowheads point to cleaved proteins.

(B) TNFR1-membrane associated complex I was analyzed as above using a modified version of human recombinant TNF fused to the Fc portion of human IgG_1. Where indicated (0+), TNF was added to untreated cell lysates before immunoprecipitation. Modified proteins are indicated with open arrowheads.

(C) FADD and caspase-8 recruitment to the Fas DISC was analyzed as above (A) in HT1080 I-κBmut cells 5 and 15 min after the addition of Flag-tagged FasL.

Fig. 3. RIP1, TRADD, TRAF2, IKKβ, FADD and Caspase-8 exhibit high apparent molecular weights after TNF stimulation

I-kBmut HT1080 cells were stimulated or not for 5 or 960 min with TNF, and subsequently lysed in CHAPS lysis buffer. After fractionation on a S200 gel filtration column, TNFR1, RIP1, TRADD, TRAF2, IKKβ, caspase-3, caspase-8, caspase-10, FADD and Fas protein content was analyzed by western blotting. The elution position of molecular weight markers (in kDa) are indicated at the top of the figure.

Fig. 4. The caspase-8 containing complex II does not contain TNFR1.

(A) WT or I-kBmut HT1080 cells were stimulated as described in Fig 2, but immunoprecipitations were performed using an anti-caspase-8 antibody. Samples were analyzed by western blotting using antibodies to the indicated proteins. Cleaved proteins are indicated with filled arrowheads, while open arrowheads

point to the respective proteins and their modified forms.

(B) Time course of Fas DISC formation in the HT1080 I-kBmut cells after Fas ligand stimulation and immunoprecipitation using either an anti-Flag antibody or the anti-caspase-8 antibody. Samples were analyzed by western blotting using antibodies to Fas, FADD, $FLIP_L$ and caspase-8.

Fig. 5. Analysis of TNFR1-induced complex II in FLIP-expressing cells

(A) Complex II was analyzed as in Fig. 4 after an overnight incubation with TNF in wt, I-kBmut or I-kBmut cells stably transfected with $FLIP_L$.

(B) Flag-tagged caspase-10, HA-tagged caspase-8 and and increasing quantities of VSV-$FLIP_L$ were overexpressed in 293 T cells and caspase-10 immunoprecipitates (anti-Flag) and cell extracts (CE) analyzed for the presence of Caspase-10, caspase-8, and $FLIP_L$. Asterix: non-specific band. Filled arrowhead: cleaved proteins.

Fig. 6. Subcellular localization of RIP1 and caspase-8 upon TNF-stimulation

I-kBmut HT1080 cells were stimulated or not with 100 ng/ml TNF for the indicated time and fixed in 2% PFA before staining with anti-RIP1 or an anti-active caspase-8 antibody. Nuclei were counterstained with Hoechst. Slides were analyzed by confocal microscopy.

Fig. 7. Complex I and Complex II localize in different subcellular

compartments

(A) Subcellular fractionation and immunoprecipitation analysis of the TNFR1-induced proximal complex I. HT1080 I-kBmut cells were stimulated as above and lysis was performed by use of a tight-fitting pestle in a dounce homogenizer (see Experimental Procedures). Soluble (S100) and membrane-enriched (P100) were separated by ultracentrifugation and analyzed for the presence of TNFR1, RIP1, TRADD and Transferrin receptor. (B) Complex I was immunoprecipitated from the S100 and P100 fraction using Flag-TNF and analyzed by western blotting. (C) The subcellular localisation of Complex II was analyzed as in (B) by use of the anti-caspase-8 antibody and western blotting using antibodies to RIP1, FADD, caspase-10 or caspase-8.

Fig. 8. FADD is required for the formation of complex II

(A) I-kBmut HT1080 cells overexpressing (filled squares) or not (open squares) FADD-DN were treated with increasing amounts of TNF. Cell viability was quantified after 48 hrs. The inset shows the expression levels of FADD in the two cell populations. (B) Cells were stimulated for the indicated time with TNF, and analysis of Complex II formation was performed by immunoprecipitation as described Fig. 5. (C) Complex II and (D) Complex I were analyzed in Jurkat FADD-deficient or FADD-deficient cells reconstituted with FADD. Asterix: non-specific band.

Fig. 9. Model TNFR1-mediated apoptosis

After binding of TNF to TNFR1, rapid recruitment of TRADD, RIP1 and TRAF2 occurs (Complex I). Subsequently TNFR1, TRADD and RIP1 become modified (◆) and dissociate from TNFR1. The liberated death domain (DD) of TRADD (and /or RIP1) now binds to FADD, resulting in caspase8/10 recruitment (forming complex II) and resulting in apoptosis. If NF-κB activation triggered by complex I is successful, cellular $FLIP_L$ levels are sufficiently elevated to block apoptosis and cells survive.