Modulation of Tumor Necrosis Factor Apoptosis-inducing Ligand-induced NF-κB Activation by Inhibition of Apical Caspases*

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Tumor necrosis factor (TNF) apoptosis-inducing ligand (TRAIL), a member of the TNF family, induces apoptosis in many transformed cells. We report TRAIL-induced NF-κB activation, concomitant with production of the pro-inflammatory cytokine Interleukin-8 in the relatively TRAIL-insensitive cell line, HEK293. In contrast, TRAIL-induced NF-κB activation occurred in HeLa cells only upon pretreatment with the caspase inhibitor, benzoylxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.fmk), indicating that this was due to a caspase-sensitive component of TRAIL-induced NF-κB activation. NF-κB activation was mediated by the death receptors, TRAIL-R1 and -R2, but not by TRAIL-R3 or -R4 and was only observed in HeLa cells in the presence of z-VAD.fmk. Receptor-interacting protein, an obligatory component of TNF-α-induced NF-κB activation, was cleaved during TRAIL-induced apoptosis. We show that receptor-interacting protein is recruited to the native TRAIL death-inducing signaling complex (DISC) and that recruitment is enhanced in the presence of z-VAD.fmk, thus providing an explanation for the potentiation of TRAIL-induced NF-κB activation by z-VAD.fmk in TRAIL-sensitive cell lines. Examination of the TRAIL DISC in sensitive and resistant cells suggests that a high ratio of c-FLIP to caspase-8 may partially explain cellular resistance to TRAIL-induced apoptosis. Sensitivity to TRAIL-induced apoptosis was also modulated by inhibition or activation of NF-κB. Thus, in some contexts, modulation of NF-κB activation possibly at the level of apical caspase activation at the DISC may be a key determinent of sensitivity to TRAIL-induced apoptosis.

Tumor necrosis factor apoptosis-inducing ligand (TRAIL) is a recently cloned member of the TNF ligand family. Unlike CD95L and TNF, which have a restricted tissue distribution, TRAIL is constitutively expressed, at least at the mRNA level, in a wide variety of tissues and cell types (1, 2). Due to this ubiquitous distribution, it was postulated that regulation of TRAIL-induced cell death may be mediated by restricted receptor expression. The TRAIL receptor family is unusually complex, comprising at least four membrane-bound members. TRAIL induces apoptosis through TRAIL-R1 (DR4) (3) and TRAIL-R2 (DR5/TRICK2/KILLER) (4–7), both of which contain a cytoplasmic death domain motif that displays homology to the death domains found in CD95 and TNF receptor 1 (TNF-R1). Two additional receptors, TRAIL-R3 (TR3/DR1/TRID/LIT) (8–11) and TRAIL-R4 (TR4/Dr2/TRUNDD) (12–14), are unable to signal for cell death and have been termed “decoy” receptors (9, 10). TRAIL-R3 lacks an intracellular domain and is a glycosylphosphatidylinositol-linked cell surface protein, whereas TRAIL-R4 contains a truncated intracellular domain and, thus, an incomplete death domain lacking residues critical for engaging apoptosis. Ectopic expression of TRAIL-R3 and -R4 protects cells from TRAIL-induced apoptosis, and it was hypothesized that they antagonize TRAIL-R1 and -R2 death signaling by either competing for limited TRAIL ligand (9, 10, 13) or, in the case of TRAIL-R4, by transduction of an anti-apoptotic signal (12). There is particular interest in the potential use of TRAIL as a novel anticancer agent as it appears to be selectively toxic to transformed and tumor cells but not to the majority of normal cells (1, 2, 15).

Although caspase-8 was identified as the apical caspase in TRAIL-induced apoptosis (16, 17), the mechanism of its recruitment and the adaptor molecule(s) involved have been subject to controversy. Early studies using overexpression of dominant-negative FADD produced conflicting results as to whether FADD and/or another adaptor was involved (9, 18–21). Recently, several studies report the presence of FADD in the native TRAIL death-inducing signaling complex (DISC) (16, 22, 23), suggesting that TRAIL utilizes a similar death-signaling pathway to those activated by CD95L and TNF. However, this does not explain the selective toxicity observed with TRAIL. Although TRAIL resistance sometimes correlates with the relative expression levels of death to decoy receptors, much evidence now points toward alternative models for TRAIL resistance (24), including the presence of intracellular anti-apoptotic molecules such as c-FLIP, which modulates TRAIL signaling (25, 26). Another mechanism of cellular resistance to members of the TNF family is through activation of the transcription factor, NF-κB (27). In many cell types, TNF negatively regulates its own cytotoxicity by up-regulation of NF-κB-regulated anti-apoptotic genes, such as c-IAP1 and c-IAP2 (28). Several studies show that TRAIL activates NF-κB (29, 30) and that this activation is mediated not only by the death receptors, TRAIL-R1 and -R2, but also...
by the truncated decoy receptor, TRAIL-R4 (12, 30).

To further examine the role NF-κB plays in TRAIL signaling, we employed a reporter gene system to study TRAIL-induced NF-κB activation and its relationship to TRAIL-induced apoptosis. We demonstrate that TRAIL-induced NF-κB activation is mediated by TRAIL-R1 and TRAIL-R2 and that this activation is a caspase-sensitive event as it occurs in TRAIL-sensitive cells only in the presence of the cell-permeable broad spectrum caspase inhibitor, benzylxoycarbonyl-Val-Ala-Asp (Ome) fluoromethyl ketone (z-VAD.fmk). In parallel, we show that the receptor-interacting protein (RIP), which is responsible for TNF-α-induced NF-κB activation, is cleaved during TRAIL-induced apoptosis and that this is inhibited by z-VAD.fmk. We demonstrate that RIP is recruited to the native TRAIL DISC and this recruitment is enhanced in the presence of z-VAD.fmk, thus providing evidence for a direct link between TRAIL receptor engagement and an obligatory component of the NF-κB signaling pathway. Because the C-terminal product of RIP cleavage inhibits NF-κB activation (31, 32), we propose that the ability of z-VAD.fmk to reveal an NF-κB component of TRAIL signaling is mediated in part by its ability to inhibit RIP cleavage and, thus, maintain NF-κB activation in TRAIL-sensitive cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human TRAIL (residues 95–281) was produced as previously described (5). Recombinant human TNF-α was obtained from Sigma (Poole, UK). The caspase inhibitor z-VAD.fmk was from Enzyme Systems Inc. (Dublin, CA). Anti-FADD and anti-RIP monoclonal Abs were obtained from BD Transduction Laboratories and BD Pharmingen (Heidelberg, Germany), respectively. Anti-TRAIL-R2 and anti-TRAIL-R4 monoclonal Abs were gifts from Immunex Corp. (Seattle, WA), anti-poly(ADP-ribose) polymerase (PARP) monoclonal Ab C2–10 was a gift from Dr. G. Pourier (Laval University, Quebec, Canada), anti-Bid Ab was a gift from Dr. X. Wang (University of Texas Southwestern Medical Center, Dallas, Texas), and anti-caspase-3 Ab was a gift from Dr. D. Nicholson (Merck Frosst, Quebec, Canada). A rabbit polyclonal anti-caspase-8 Ab has been described previously (33), and a mouse monoclonal antibody to caspase-8 (C15) (34), used for DISC analysis, was a gift from Dr. F. H. Krammer (German Cancer Research Center, Heidelberg, Germany). Horseradish peroxidase-conjugated secondary antibodies, goat-anti-mouse and goat-anti-rabbit, were obtained from Sigma and Dako (Cambridge, UK), respectively.

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produced high basal levels of IL-6, which were not significantly affected by treatment with TRAIL but were markedly increased by TNF-α (data not shown). To further implicate the role of NF-κB activation in TRAIL signaling, cells were transfected with an IκB-α (S32A/S36A) mutant, which completely blocks NF-κB activation in response to a number of stimuli (38). In both cell lines, this mutant completely abolished both TNF-α- and TRAIL-induced reporter gene activity together with IL-6 and IL-8 production (data not shown). This suggested that IL-6 and IL-8 production by TNF-α and TRAIL was regulated by NF-κB. To investigate whether there was any correlation between NF-κB activation in 293 and HeLa cells and the sensitivity of these cells to TRAIL-induced apoptosis, the cleavage of PARP, a substrate for caspase-3 and -7, was assessed. In apoptotic cells, PARP is cleaved at a DEVD motif to yield a characteristic 85-kDa fragment (41). After exposure to TRAIL, PARP was cleaved to an 85-kDa fragment in HeLa but not in 293 cells, indicating that HeLa but not 293 cells were sensitive to TRAIL-induced apoptosis (Fig. 1C). Taken together these data suggested that the resistance of 293 cells to TRAIL-induced apoptosis may, in part, be linked to their ability to activate NF-κB.

**Fig. 1. Activation of NF-κB in HEK293 but not HeLa cells correlates with their sensitivity to TRAIL-induced apoptosis.** A, HEK293 and HeLa cells were transfected with 0.1 μg of NF-κB-alkaline phosphatase reporter construct and 0.1 μg of β-lactamase reporter construct. Fresh medium was added 16 h after transfection, and the cells were treated with recombinant TNF-α (10 ng/ml) or recombinant TRAIL (1 μg/ml). Reporter gene activity was measured 24 h later, and results were normalized using β-lactamase expression levels. B, medium was also assayed for production of the NF-κB-regulated gene product, IL-8. Data are presented as fold-increase above control from three independent experiments. C, treated cells were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting as described under “Experimental Procedures.” Membranes were probed with a mouse monoclonal antibody against PARP. The arrows represent intact or cleaved PARP.

**Fig. 2. Inhibition of NF-κB sensitizes 293 cells to TRAIL-induced apoptosis, whereas up-regulation of NF-κB attenuates TRAIL-induced apoptosis in HeLa cells.** A, 293 cells were transfected with 0.1 μg of NF-κB reporter, 50 ng of a β-galactosidase-containing construct, pRSC, together with 0.1 μg of pCMV-IκB-ALK (S32A/S36A). Medium was removed 16 h after transfection, fresh medium was added, and the cells were then treated with TRAIL (1 μg/ml) or TNF (10 ng/ml). Where indicated z-VAD.fmk (20 μM) was used as a 1-h pretreatment. After 24 h, medium was removed, and cells were stained with X-gal. The percentage apoptosis was assessed by comparing total blue cells in each well with the total number of blue cells displaying apoptotic morphology. B, HeLa cells were transfected with 0.1 μg of NF-κB reporter and 50 ng of a β-galactosidase-containing construct pRSC together with 5 ng of pcDNA3-NIK. 0.1 μg of pCMV-IκB-ALK (S32A/S36A) was included where indicated to block NIK-induced NF-κB activation. Medium was removed 16 h after transfection and assayed for reporter activity. Fresh medium was added, and cells were further incubated for 2 h either alone or in the presence of TRAIL (1 μg/ml). Cells were then stained with X-gal, and apoptosis was assessed by comparing the total number of normal blue cells in each well to the number of blue cells displaying morphological features of apoptosis such as membrane blebbing and nuclear condensation. All transfections were carried out in duplicate, and the data presented represent three independent experiments. 

**Inhibition of NF-κB Sensitizes HEK293 Cells to TRAIL-induced Apoptosis**—To assess whether there is a relationship between the activation of NF-κB by TRAIL in 293 cells (Fig. 1A) and their relative insensitivity to TRAIL-induced apoptosis (Fig. 1C), we overexpressed the IκB-α (S32A/S36A) mutant, which blocks NF-κB signaling, thereby sensitizing cells to TNF-induced apoptosis (27). Transfected cells were treated with TNF-α, TRAIL, or vehicle alone for 24 h before apoptosis measurements. In control vector-transfected cells, TNF-α or TRAIL induced a small amount of apoptosis, only ∼5% above that observed in untreated control-transfected cells (Fig. 2A). However, in IκB-α (S32A/S36A)-transfected cells, both TNF-α and TRAIL induced marked apoptosis in 50–60% of transfected cells, which was completely abrogated by z-VAD.fmk (Fig. 2A). These data demonstrate that blocking the NF-κB...
pathway in 293 cells sensitizes them to both TNF- and TRAIL-induced apoptosis.

**Up-regulation of NF-κB Protects against TRAIL-induced Apoptosis**—To determine the potential role of NF-κB in ameliorating TRAIL-induced apoptosis, HeLa cells were transfected with reporter constructs and then pretreated with the caspase inhibitor, z-VAD.fmk (20 μM) for 1 h before treatment with TNF (10 ng/ml) or TRAIL (1 μg/ml) (A). Reporter gene activity was measured 24 h after treatment, and results were normalized using β-lactamase expression levels. B, medium from transfected 293 and HeLa cells was also analyzed for IL-8 content. Data presented represent three independent experiments, and the error bars are the mean ± S.E.

**Fig. 3. Inhibition of TRAIL-induced apoptosis reveals an NF-κB component of TRAIL signaling.** 293 and HeLa cells were transfected with reporter constructs and then pretreated with the caspase inhibitor, z-VAD.fmk (20 μM) for 1 h before treatment with TNF (10 ng/ml) or TRAIL (1 μg/ml) (A). Reporter gene activity was measured 24 h after treatment, and results were normalized using β-lactamase expression levels. B, medium from transfected 293 and HeLa cells was also analyzed for IL-8 content. Data presented represent three independent experiments, and the error bars are the mean ± S.E.
Fig. 4. Overexpression of TRAIL-R1 and TRAIL-R2 activates NF-κB in 293 cells, but in HeLa cells, activation occurs only in the presence of z-VAD.fmk. To study the NF-κB signaling component of the individual TRAIL receptors, 293 (A) and HeLa (B) cells were transfected with 0.1 μg of each reporter construct together with 0.2 μg of the indicated TRAIL receptor construct in the presence or absence of z-VAD.fmk (20 μM). A wsl-1-containing construct (0.2 μg) was used as a positive control for NF-κB activation, and control transfections were supplemented with 0.2 μg of empty control vector. The medium was changed 16 h after transfection and, where indicated, fresh z-VAD.fmk was added, and then reporter gene assays were performed after a further 24 h. Results were normalized using β-lactamase expression levels. Data presented represent three independent experiments, and the error bars represent the mean ± S.E.

Fig. 5. Overexpression of TRAIL-R4 does not activate NF-κB in 293 cells. A, 293 cells were co-transfected with reporter constructs together with increasing amounts of TRAIL-R4 as indicated. Control transfections were supplemented with empty vector. After 16 h, fresh medium was added where indicated in the presence of TRAIL (1 μg/ml), and reporter gene assays were performed 24 h later. B, transfected 293 cells were harvested at the time of reporter assay and subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with an anti-human TRAIL-R4 monoclonal antibody. Data presented represent three independent experiments, and the error bars represent the mean ± S.E.

significant increase in reporter gene activity in HeLa cells (Fig. 4B). NF-κB activation by WSL-1 was also potentiated by z-VAD.fmk. Transfection of TRAIL-R1 and -R2 in 293 cells in the presence of z-VAD.fmk caused a large potentiation in reporter gene activity (Fig. 4A) together with IL-8 production (data not shown) when compared with untreated cells. NF-κB activation induced by WSL-1 was also potentiated but to a lesser extent. Although 293 cells are relatively resistant to TRAIL-induced apoptosis (Fig. 1C), overexpression of death receptors such as TNF-R1 and TRAIL-R1 and -R2 resulted in ligand-independent receptor trimerization and, thus, extensive apoptosis (5, 47), irrespective of the inherent TRAIL sensitivity of these cells. No NF-κB activation was evident in either cell line in response to TRAIL-R3 or -R4 overexpression in the presence or absence of z-VAD.fmk (Fig. 4, A and B). This inability of z-VAD.fmk to reveal an NF-κB component of TRAIL-R3 and -R4 signaling is presumably because these receptors do not induce caspase recruitment or activation. Taken together these data provide indirect evidence that TRAIL-R1- and -R2-induced NF-κB activation is, in part, a caspase-sensitive process.

A TRAIL-R2 Partial Death Domain Mutant Does Not Activate NF-κB—The lack of NF-κB activation in either 293 or HeLa cells by TRAIL-R4 (Figs. 4, A and B) is in contrast to a number of other reports. To ensure that sufficient TRAIL-R4 had been expressed, increasing concentrations of TRAIL-R4 (200–1000 ng) were transfected into 293 cells. Under these conditions, TRAIL-R4 was unable to induce NF-κB activation even in the presence of exogenous TRAIL (Fig. 5A) and despite there being a concomitant increase in protein levels in these cells (Fig. 5B). One mechanism by which TRAIL-R4 is purported to act as a decoy receptor is through activation of an NF-κB-mediated survival pathway (12, 30). However, TRAIL-R4 contains only a partial death domain lacking 56 amino acids at the N terminus, which are present in the death domain of both TRAIL-R1 and -R2 and are believed to be responsible for engaging downstream signaling pathways. To test the hypothesis that this N-terminal region of the death domain may be responsible for NF-κB activation, we created a TRAIL-R2 mutant lacking this region (Fig. 6A). Western blotting with a TRAIL-R2-specific monoclonal Ab clearly showed that the mutant was expressed (data not shown). When overexpressed in 293 cells in the presence of z-VAD.fmk, mutant TRAIL-R2 (ΔSer-324–Ser-369) failed to induce NF-κB activation, whereas both wild-type TRAIL-R2 and WSL-1 produced marked activation (Fig. 6B). These results suggested that motifs required for adaptor binding and/or NF-κB activation by wild-type TRAIL-R2 were no longer present in mutant TRAIL-R2 (ΔSer-324–Ser-369).

RIP Is Cleaved in HeLa Cells during TRAIL-induced Apoptosis—Our results with z-VAD.fmk in HeLa cells implicated the presence of a protein, whose cleavage by caspases prevented TRAIL-induced NF-κB activation (Fig. 3A). In TNF-R1 signaling, RIP, a death domain-containing kinase, has been implicated in NF-κB activation (48). In addition, cells derived from RIP−/− mice were unable to activate NF-κB in response to TNF-α, and these mice were hypersensitive to the cytotoxic effects of TNF (49). RIP is also cleaved during TNF-induced apoptosis by caspase-8 to produce a dominant-negative fragment, which inhibits TNF-induced NF-κB activation (31, 32).
By analogy, cleavage of RIP during TRAIL-induced apoptosis could explain our observation that activation of NF-κB in TRAIL-sensitive HeLa cells only occurred when TRAIL-induced apoptosis was blocked (Fig. 3A). We therefore studied the cleavage of RIP as well as the activation of caspase-8 in HeLa cells. Treatment with TRAIL resulted in the loss of the proform of caspase-8 accompanied by processing to its p43/41 and p18 forms (Fig. 7). TRAIL treatment also resulted in the cleavage of RIP to a 42-kDa immunoreactive fragment, corresponding to the reported RIP cleavage product (31) and cleavage of PARP to its well characterized 85-kDa product. Processing of caspase-8 and cleavage of RIP and PARP were completely inhibited by z-VAD.fmk (Fig. 7). No cleavage of caspase-8, PARP, or RIP was observed in 293 cells under the same conditions, in agreement with the relative resistance of these cells to TRAIL-induced apoptosis (Fig. 7). These data demonstrated that a caspase-dependent cleavage of RIP was associated with TRAIL-induced apoptosis in HeLa cells and supported the hypothesis that extensive RIP cleavage prevented concomitant TRAIL-induced NF-κB activation within the same cell.

To determine whether the cleavage of RIP was mediated entirely by caspase-8 as proposed (31, 32) or whether effector caspases such as caspase-3 also played a role, we utilized MCF-7 cells, which do not express functional caspase-3 (35). In MCF-7 cells, TRAIL induced a time-dependent processing of caspase-8 to its p41/p43 and p18 fragments as well as cleavage of Bid (Fig. 8 lanes 1–7), a preferred caspase-8 substrate (50), both of which were completely inhibited by z-VAD.fmk (Fig. 8, lane 8). This was consistent with the ability of z-VAD.fmk to inhibit death receptor-induced apoptosis by inhibiting the proc-
TRAIL DISC in Both HeLa and 293 Cells—It was possible that the difference between the ability of TRAIL to induce NF-κB activation and/or apoptosis in 293 and HeLa cells was due to differences in their ability to form a functional TRAIL DISC. We therefore examined the extent of native TRAIL DISC formation in these two cell lines both in the presence and absence of z-VAD.fmk. In order that a valid comparison be made between the two cell lines, the TRAIL DISC samples obtained were analyzed from identical exposures after Western blot analysis. The addition of Bio-TRAIL induced the time-dependent formation of a TRAIL DISC with lower levels of TRAIL-R2 precipitated from HeLa cells compared with 293 cells (Fig. 9). In unstimulated cells, the two splice forms of TRAIL-R2 (6) were present. Although the larger form predominated in both cell lines, there appeared to be no preferential recruitment of either form within the TRAIL DISC (Fig. 9). Bio-TRAIL induced a time-dependent recruitment of both FADD and caspase-8 (Fig. 9), which was TRAIL stimulation-dependent since neither was present when TRAIL was added after cell lysis. Interestingly, less FADD was recruited to the TRAIL DISC in 293 cells compared with HeLa cells even though higher levels of TRAIL-R2 had been precipitated by TRAIL. In parallel with this, the FADD-dependent recruitment of procaspase-8 to the TRAIL DISC was also significantly reduced in 293 cells compared with HeLa cells, and by 60 min after TRAIL treatment very little caspase-8 was present in the TRAIL DISC from 293 cells. In contrast, in HeLa cells there was clearly a continual recruitment of procaspase-8 to the TRAIL DISC. Both the p55 and p53 zymogen forms of procaspase-8 were present in the DISC, corresponding to caspases-8a and -8b (34), and activation at the DISC resulted in their partial processing to the intermediate p43 and p41 forms, which arise after cleavage between the large and small subunits. When cells were isolated in the presence of z-VAD.fmk, procaspase-8 was retained within the DISC with no significant inhibition of its initial processing to p41 and p43.

The procaspase-8 homologue, c-FLIP, exists as a long (c-FLIPL) and a short (c-FLIPS) splice variant, both of which are capable of protecting cells from death receptor-induced apoptosis (51). Both 293 and HeLa cells expressed significant levels of only the c-FLIPL isoform, and after TRAIL stimulation, c-FLIPL was recruited to the DISC (Fig. 9). Interestingly, in the stimulated but not the unstimulated cells most of the c-FLIPL was cleaved to a p43 fragment. This represents the product obtained after removal of the C-terminal p12 subunit of c-FLIP, and like c-FLIPL, the p43 fragment can inactivate the DISC by preventing further recruitment of procaspase-8 into the complex (52). In contrast with HeLa cells, where a continual time-dependent recruitment of c-FLIPL was observed, the TRAIL DISC in 293 cells appeared to have a decreased capacity for the continual recruitment of c-FLIPL. As early as 30 min after TRAIL stimulation, no further recruitment of c-FLIPL was detected in 293 cells, and all of the c-FLIPL that had initially been recruited was processed to its p43 form. Interestingly, in the TRAIL DISC from 293 cells, the ratio of c-FLIPL and its cleaved product to caspase-8 was much higher than in HeLa cells. This balance would clearly favor a much greater inhibition of TRAIL-induced apoptosis in 293 cells than in HeLa cells.

Surprisingly, the adaptor protein RIP was associated with unstimulated TRAIL receptors isolated from both 293 and HeLa cells. After TRAIL stimulation, there was a time-dependent increase in the recruitment of RIP to the TRAIL DISC (Fig. 9). As early as 60 min after the addition of TRAIL, some cleavage of RIP was apparent in the DISC formed in both cell lines. In the presence of z-VAD.fmk, this cleavage was com-

FIG. 8. Enhanced processing of RIP in caspase-3 transfected MCF-7 cells. Mock-transfected (MCF-7 (Vector)) and caspase-3-transfected (MCF-7 (Caspase-3)) MCF-7 cells were treated with TRAIL (1 μg/ml) for the indicated time periods and subjected to Western blotting using antibodies to caspase-8, the caspase-8 substrate Bid, RIP, PARP, and FADD. z-VAD.fmk (20 μM) was included where indicated as a 1-h pretreatment. The adaptor protein FADD was used as a protein loading control.
Caspase Inhibition Potentiates TRAIL-induced NF-κB Activation

**Fig. 9. RIP is recruited to the TRAIL DISC in 293 and HeLa cells.** 293 and HeLa cells (3 × 10⁵) were treated with biotinylated TRAIL (Bio-TRAIL) for up to 60 min, and where indicated, cells were pretreated for 60 min with z-VAD.fmk (20 μM). Unstimulated receptor controls (u/s) represent the addition of Bio-TRAIL to an equivalent volume of lysate isolated from unstimulated cells. TRAIL receptor complexes were precipitated with streptavidin-conjugated agarose beads and analyzed by Western blotting for the known TRAIL DISC components, TRAIL-R2, FADD, and caspase-8. Precipitates were also analyzed for the presence of c-FLIP, RIP, and as a negative control, caspase-3. Lysates isolated from unstimulated control cells were included as a positive control for the expression of all these proteins in both 293 and HeLa cells. To enable comparison of the relative amounts of each component recruited to the DISC, equivalent exposures are shown. The asterisk indicates a minor nonspecific band detected by the TRAIL-R2 antibody.

**DISCUSSION**

**TRAIL-induced Apoptosis Can Be Modulated by NF-κB**—In most cell types the predominant downstream signaling event of TNF is not apoptosis but NF-κB activation. TNF can negatively regulate its own cytotoxic ability through the up-regulation of NF-κB-regulated anti-apoptotic genes (28), and inhibition of NF-κB activation restores its cytotoxicity (27). In this study we show that NF-κB can similarly modulate TRAIL-induced apoptosis. Increased NF-κB activation, by overexpression of NIK, markedly decreased TRAIL-induced apoptosis in HeLa cells (Fig. 2), similar to the protection reported previously in transformed keratinocytes by IL-1-induced NF-κB activation (53).

Conversely, inhibition of NF-κB activation by overexpression of an IκB-α (S32A/S36A) mutant sensitized 293 cells to TRAIL-induced apoptosis (Fig. 2). This mutant also sensitizes TRAIL-resistant primary leukemic and melanoma cells (29, 54) but did not sensitize HeLa-TL-R cells that had become resistant after long term culture in TRAIL (30). This suggests that the ability of NF-κB to modulate TRAIL sensitivity may be model-dependent. It is unclear whether TRAIL modulates its own cytotoxicity by activation of NF-κB in a manner similar to that reported for TNF or whether resistant cells may have a high constitutive NF-κB activity that offers protection. Taken together these data demonstrate that modulation of NF-κB activation is a key determinant of the sensitivity of some cells to TRAIL-induced apoptosis.

**Activation of NF-κB by TRAIL Is Mediated by TRAIL-R1 and TRAIL-R2 but Not by TRAIL-R4**—Another mechanism by which cells may modulate their sensitivity to TRAIL is through the expression of the putative decoy receptors TRAIL-R3 and -R4. These decoy receptors are purported to act by either tri-merizing with TRAIL-R1 or -R2 to form inactive signaling complexes or by sequestering ligand from TRAIL-R1 or -R2. In this study we found that TRAIL-R1 and -R2 mediated TRAIL-induced NF-κB activation in a ligand-independent manner. However, contrary to some other reports (12, 30), we were unable to demonstrate TRAIL-R4-mediated NF-κB activation even upon gross overexpression of TRAIL-R4 and the subsequent addition of TRAIL (Fig. 5). Previous studies have proposed a model for the protection of cells by TRAIL-R4 via the activation of a NF-κB-mediated survival pathway (12). This model is not easily explained because TRAIL-R4 contains a truncated death domain, which lacks a number of key residues conserved throughout the TNF-R family that have been implicated in cytotoxicity signaling (55). In the present study a TRAIL-R2 mutant, containing a truncated death domain resembling that found in TRAIL-R4, was unable to activate NF-κB (Fig. 6). This suggested that residues or motifs required for NF-κB activation were absent in this TRAIL-R2 mutant. When one such residue, Ile-225, is mutated to Asn in CD95, it is responsible for the lymphoproliferative (lpr) phenotype in mice (56). This residue is conserved in both TNF-R1 and TRAIL-R2 (Leu-351 and Leu-334, respectively) and, when similarly mutated, results in loss of receptor cytotoxicity (19, 55). Interestingly, this mutation has also previously been demonstrated to abolish TRAIL-R2-mediated NF-κB activation (19).

The lack of any NF-κB activation by TRAIL-R4 observed in this study is in agreement with an earlier study (13) and a very recent study (57), which demonstrated that TRAIL-R4 is capable of protecting colon carcinoma cells from TRAIL-R2- and p53-mediated apoptosis. This protective effect was localized to the first 43 amino acids of the cytoplasmic domain and not within the remaining portion of the death domain (57). Thus, TRAIL-R4 may mediate as yet unknown signaling pathways that protect against TRAIL-induced apoptosis.

**Caspase Inhibition Potentiates TRAIL-induced NF-κB Activation and Enhances Recruitment of RIP to the Native TRAIL DISC**—TRAIL-induced NF-κB activation appeared to require a molecule(s) that was inactivated after caspase cleavage, since no NF-κB activation was apparent in HeLa cells with TRAIL or
TRAIL-R1 or -R2 overexpression unless z-VAD.fmk was present (see Fig. 3A and Fig. 4B). RIP is clearly such a candidate molecule, based on the observations that TRAIL induced a caspase-dependent cleavage of RIP in HeLa cells (Fig. 7) that, when prevented by z-VAD.fmk, led to a marked increase in NF-κB activation (Figs. 3 and 4). RIP has been implicated in receptor-mediated NF-κB activation through direct interaction with the IκB kinase signalosome complex component, NEMO/IκB kinase γ (58). Although RIP has previously been shown to associate with TRAIL receptors after overexpression of various components of the TRAIL signaling pathway (19, 20), we now show for the first time that RIP is a component of the native TRAIL DISC in both HeLa and 293 cells (Fig. 9). A recent study has also provided evidence that RIP may be absolutely required for TRAIL-mediated NF-κB activation, because no activation was observed in TRAIL-treated RIP−/− cells (59). The observation that RIP was pre-associated with the unstimulated receptor control in both cell lines was unexpected, and the significance of this, if any, remains to be elucidated. In the TNF-R system, RIP and FADD are both recruited through the intermediate adaptor TNFR-associated death domain in a stimulation-dependent manner (48). In previous studies, TNFR-associated death domain was not found to be a component of the TRAIL DISC (22, 23), and whether RIP requires such an intermediate adaptor or directly associates with TRAIL-R2 remains to be elucidated. Some cleaved as well as full-length RIP was present within the TRAIL DISC isolated from both cell lines, compatible with some RIP cleavage occurring within the DISC. In HeLa cells, where caspase-3 was also activated, some processing of RIP could almost certainly have occurred outside the DISC, thereby diminishing the pool of full-length RIP available for NF-κB activation. The implications of extensive RIP cleavage became more evident from studies where the DISC was isolated in the presence of z-VAD.fmk. Under these conditions caspase-dependent cleavage of RIP was blocked, and as a consequence, more RIP accumulated within the TRAIL DISC (Fig. 9). Taken together, these data provided a potential mechanism for the potentiation of TRAIL-induced NF-κB signaling observed in the presence of z-VAD.fmk.

The Ratio of c-FLIP to Caspase-8 in the DISC May Determine Sensitivity to TRAIL-induced Apoptosis—Analysis of the TRAIL DISC also provided a potential explanation for the differential sensitivity of 293 and HeLa cells to TRAIL-induced apoptosis. Although 293 cells were not sensitive to TRAIL-induced apoptosis (Fig. 7), a TRAIL DISC was formed that contained small amounts of FADD and some processed caspase-8 (Fig. 9). However, when compared with HeLa cells, the recruitment of FADD to the TRAIL DISC in 293 cells was clearly less efficient even though higher levels of TRAIL-R2 were precipitated from these cells. Levels of c-FLIP may in some cells determine resistance to CD95-induced apoptosis (52), since in the presence of c-FLIP, procaspase-8 is no longer able to replace the cleavage products at the DISC and become activated. We now show that in 293 cells, the ratio of c-FLIPL to caspase-8 within the TRAIL DISC is much greater than in HeLa cells and may contribute to inactivation of the TRAIL DISC, as evidenced by the lack of caspase-8 or RIP processing detected in 293 whole cell extracts (Figs. 7 and 9). Taken together our data suggest that the differential sensitivity of 293 and HeLa cells to TRAIL-induced apoptosis may be in part explained by the efficiency of recruitment and activation of integral DISC components such as FADD and procaspase-8.

The differential sensitivity of these cells to TRAIL-induced NF-κB activation may also be, in part, explained by the differential activation of apoptotic signaling molecules within their TRAIL DISCs. For example, the extensive caspase-dependent processing of the NF-κB-activating kinase RIP in TRAIL-treated HeLa cells could significantly inhibit the capacity of these cells to activate NF-κB and, thus, could provide an explanation for the lack of NF-κB activation detected in these cells in the absence of caspase inhibitors. Interestingly, c-FLIP has also recently been shown to possess some NF-κB-activating activity (60–62), and in CD95-treated cells both c-FLIPL and its cleavage fragment interact with RIP. It is therefore possible that c-FLIPL may also contribute to TRAIL-induced NF-κB activation. Therefore, in cells where comparable levels of c-FLIPL and caspase-8 are recruited to the TRAIL DISC, then c-FLIP, in addition to inhibiting caspase-8 activation, may also signal for cell survival through concomitant activation of NF-κB.

In conclusion, we have shown that sensitivity to TRAIL-induced apoptosis can be modulated by activation or inhibition of NF-κB. TRAIL activates NF-κB only when its apoptotic signaling arm is blocked either by use of a caspase inhibitor or via endogenous resistance mechanisms. Analysis of the TRAIL DISC in sensitive and resistant cells revealed that a high ratio of c-FLIP to caspase-8 may explain the resistance of some cells to TRAIL-induced apoptosis. We demonstrate for the first time the recruitment of the NF-κB-activating kinase RIP to the native TRAIL DISC and propose that caspase-mediated cleavage of RIP can inhibit the capacity of TRAIL-sensitive cells to activate NF-κB. By contrast, in cells that are relatively resistant to TRAIL-induced apoptosis, the predominant TRAIL-signaling event is NF-κB activation. Whether recruitment of other as yet unidentified components and/or additional adaptors are required for TRAIL-induced NF-κB activation is under investigation.

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