Inhibition of Death Receptor-mediated Gene Induction by a Cycloheximide-sensitive Factor Occurs at the Level of or Upstream of Fas-associated Death Domain Protein (FADD)*

Harald Wajant‡‡, Elvira Haas‡, Ralph Schwenzer‡, Frank Mühlenbeck‡, Sebastian Kreuz‡, Gisela Schubert‡, Matthias Grell‡, Craig Smith‡, and Peter Scheurich‡

From the ‡Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany and §Immunex Research and Development Corp., Seattle, Washington 98101

In HeLa cells, induction of apoptosis and nuclear factor κB (NF-κB) activation initiated by TRAIL/Apo2L or the agonistic Apo1/Fas-specific monoclonal antibody anti-APO-1 require the presence of cycloheximide (CHX). Inhibition of caspases prevented TRAIL/anti-APO-1-induced apoptosis, but not NF-κB activation, indicating that both pathways bifurcate upstream of the receptor-proximal caspase-8. Under these conditions, TRAIL and anti-APO-1 up-regulated the expression of the known NF-κB targets interleukin-6, cellular inhibitor of apoptosis 2 (cIAP2), and TRAF1 (TRAF, tumor necrosis factor receptor-associated factor). In the presence of CHX, the stable overexpression of a deletion mutant of the Fas-associated death domain molecule FADD comprising solely the death domain of the molecule but lacking its death effector domain (FADD-(80–208)) led to the same response pattern as TRAIL or anti-APO-1 treatment. Moreover, the ability of death receptors to induce NF-κB activation was drastically reduced in a FADD-deficient Jurkat cell line. TRAIL-, anti-APO-1-, and FADD-(80–208)-initiated gene induction was blocked by a dominant-negative mutant of TRAP2 or the p38 kinase inhibitor SB203580, similar to tumor necrosis factor receptor-1-induced NF-κB activation. CHX treatment rapidly down-regulated endogenous cFLIP protein levels, and overexpression of cellular FLICE inhibitory protein (cFLIP) inhibited death receptor-induced NF-κB activation. Thus, a novel functional role of cFLIP as a negative regulator of gene induction by death receptors became apparent.

Cytokines of the tumor necrosis factor (TNF) ligand family are involved in the regulation of the immune system as well as in the maintenance of homeostasis. They act by multimerization and activation of one or more members of a complementary family of membrane receptors, the TNF receptor superfamily (1, 2). A subgroup of the TNF receptor superfAMILY can be defined by the capability of its members to induce cell death with the critical involvement of an ~100-amino acid intracellular motif, the death domain (3). At present, six human death domain-containing receptors have been identified: TNF-R1, Apo1/Fas, DR3 (TRAMP/Ws/Apo3/LARD), TRAIL-R1 (DR4), TRAIL-R2 (DR5/TRICK2/KILLER), and DR6 (3, 4). Stimulation of death domain-containing receptors leads to the recruitment of cytoplasmic death domain proteins and the enzymatic inactive proforms of caspase-8 and -10 (5–7). Oligomerization of the procaspases within the receptor signaling complexes may then lead to their autoproteolytic activation, culminating in the initiation of the apoptotic program of the cell (8, 9). Recruitment of caspase-8 into the death-inducing complex of Apo1/Fas is mediated by FADD (Fas-associating protein with a death domain) (10, 11). Whereas the carboxy-terminal death domain of FADD mediates association with the death domain of multimerized Apo1/Fas (10, 11), the amino-terminal death effector domain of FADD allows binding of caspase-8 and -10 (5–7). In the case of TNF-R1, FADD is indirectly recruited into the receptor signaling complex via death domain-mediated interaction with another cytoplasmic death domain protein called TRADD (12) that directly interacts with the death domain of TNF-R1 (13). Moreover, as fibroblasts from FADD knockout mice are completely protected against the cytotoxic action of TNF-R1, Apo1/Fas, and DR3 (14, 15), the latter should also mediate apoptosis under critical involvement of FADD. However, the coupling of TRAIL-R1 and TRAIL-R2 to the apoptotic program is rather undefined. As transient transfection of TRAIL-R1 leads to an apoptotic response in FADD fibroblasts, it appears that FADD has no major role in TRAIL-R1-induced apoptosis (14). Nevertheless, a role of FADD in TRAIL-R2-induced apoptosis and/or a FADD-related molecule in TRAIL-R1 and TRAIL-R2-induced apoptosis is conceivable, as overexpression of a dominant-negative mutant of FADD was shown to interfere with TRAIL-mediated apoptosis (16–19). In fact, direct binding of FADD has been shown in transient overexpression studies for both receptors (16, 17). In addition, TRADD was found in immunoprecipitates of TRAIL-R1 and TRAIL-R2 when coexpressed with FADD, whereas TRADD binding was not observed in the absence of coexpressed FADD (17). Moreover, in FADD-deficient Jurkat cells, TRAIL-R2-mediated apoptosis is completely blocked (20).

A broad range of non-apoptotic cellular responses have been described for TNF-R1. In contrast, Apo1/Fas, TRAIL-R1, and
TRAIL-R2 have been predominantly studied with respect to their death-inducing capabilities. Nevertheless, gene induction may also be a function of these receptors. Indeed, some reports have shown the capability of these receptors to activate the transcription factor NF-κB (16, 17, 21–23).

In this report, we demonstrate that both TRAIL (TNF-related apoptosis-inducing ligand) and an agonistic Apo1/Fas-specific antibody have a capacity similar to TNF with regard to NF-κB activation, IL-6 production, and up-regulation of cIAP2 as well as TRAF1 mRNA. Moreover, a dominant-negative TRAF2 mutant and the p38 kinase inhibitor SB203580 interfere with TNF-, TRAIL-, and anti-APO-1-induced activation of NF-κB, arguing for the utilization of common or at least related gene-inducting pathways. However, in contrast to TNF-R1, gene induction by TRAIL and anti-APO-1 likely occurs via a FADD-dependent pathway that is negatively regulated by a CHX-sensitive factor in HeLa cells. Interestingly, we found that the expression of FLIP, a known inhibitor of death receptor-induced apoptosis, is reduced upon CHX treatment and inhibits death receptor-mediated NF-κB activation.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The Kym-1 cell line was generously supplied by M. Sekiguchi (University of Tokyo) and maintained in Click-RPMI 1640 medium (Biochrom, Berlin) containing 10% heat-inactivated fetal calf serum. HeLa cells as well as transfectants derived thereof and HEK293 cells were grown in RPMI 1640 medium (Biochrom) containing 5% fetal calf serum. The HeLa and HEK293 cell line were obtained from American Type Culture Collection (Manassas, VA). Recombinant human TNF (2 107 units/mg) was kindly provided by I.-M. von Broen (Knoll AG, Ludwigshafen, Germany). Z-VAD-fmk was purchased from Bachem AG (Bubendorf, Switzerland). The murine Fas-specific monoclonal antibody Jo2 as well as fluorescein isothiocyanate-labeled Jo2 were from Pharmingen (Hamburg, Germany). The Apo1/Fas-specific mAb anti-APO-1 was a kind gift from Marcus Peter (Deutsches Krebsforschungs Zentrum, Heidelberg, Germany), and the anti-caspase-8 mAb was a kind gift from Prof. Klaus Schulze-Osthoff (University of Tübingen, Tübingen, Germany). The expression plasmids for murine Fas and murine FasΔ (pEBB-Myc-Fas and eEBB-Myc-FasΔ, respectively) were kindly supplied by David Baltimore (California Institute of Technology, Pasadena, CA). The FLIP-specific antiserum and the FLIPΔ expression plasmid (pCR3-FLIPΔ) were from Jürg Tschopp (University of Lausanne, Lausanne, Switzerland). The FADD-deficient mutant Jurkat cell line was a kind gift from John Blenis and Peter Juo (Harvard Medical School, Boston).

EMSA Analysis of NF-κB Activation—Kym-1 or HeLa cells (106) were seeded in 60-mm cell culture dishes and cultured overnight. The next day, the cells were stimulated for various times with the indicated reagents in PBS and lysed in 10% deionized chloroform. The samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blocked with 5% nonfat dry milk in PBS/Tween 20 (0.05%) overnight. The membrane was incubated with anti-caspase-8, anti-caspase-2 (Pharmingen), or anti-FLIP antiserum (diluted 1:1000) for 1 h. After washing three times with PBS/Tween 20, membranes were incubated with anti-mouse (casapse-8, caspase-2) or anti-rabbit (FLIP) alkaline phosphatase antibody (0.1 μg/ml; Sigma, Deisenhofen, Germany) for 1 h. After four washes with PBS/Tween 20, the blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

RESULTS

Activation of NF-κB by TRAIL Receptors and Apo1/Fas—We have recently found that HeLa and Kym-1 cells are TRAIL-responsive with respect to induction of apoptosis and activation of JNK (25). We therefore analyzed the gene-inductive properties of TRAIL in these cells in greater detail. In Kym-1 cells, TRAIL treatment led to a significant activation of the transcription factor NF-κB as revealed by EMSA analysis (Fig. 1A); concomitantly, cell death was also induced (data not shown). When the onset of apoptosis was completely blocked using the broad range caspase inhibitor Z-VAD-fmk, NF-κB activation was even enhanced (Fig. 1A). For these and the following experiments, a leucine zipper-tagged form of TRAIL (18) was used. Different results were obtained in HeLa cells. Although mRNA (25) and protein (data not shown) of TRAIL-R1 and TRAIL-R2 were detectable in HeLa cells, TRAIL failed to induce NF-κB activation in EMSAs (Fig. 1B) and reporter gene assays (Fig. 1C). As HeLa cells are sensitive to TRAIL-induced apoptosis only in the presence of CHX (25), we also looked for TRAIL-induced NF-κB activation under these conditions. In fact, when protein synthesis was blocked by CHX, induction of apoptosis was blocked by Z-VAD-fmk, stimulation with TRAIL led to a significant activation of NF-κB in terms of nuclear translocation (Fig. 1B) and NF-κB-dependent gene induction (Fig. 1C). Hence, it seems that in HeLa cells, not only is TRAIL-induced apoptosis blocked by a CHX-sensitive factor(s), but also TRAIL-induced activation of NF-κB. Similarly, activation of NF-κB by Apo1/Fas in Apo1/Fas-responsive HeLa cells (1.5 106) were treated with the reagents of interest for 5 h; and subsequently, total RNA was isolated with an RNA INSTAPURE kit (Euromedex, Seraing, Belgium) according to the manufacturer’s recommendations. The presence of transcripts of xIAP, TRAF1, TRAF2, TRAF3, TRAF4, neuronal apoptosis inhibitory protein (NAIP), cIAP1, and cIAP2 as well as that of the internal controls L32 and glyceraldehyde-3-phosphate dehydrogenase were analyzed using the human Apo5a Multi-Probe template set (Pharmingen). Probe synthesis, hybridization, and RNase treatment were performed with the RiboQuant Multi-Probe RNase Protection assay system (Pharmingen) according to the manufacturer’s recommendations. Finally, protected transcripts were resolved by electrophoresis on denaturing polyacrylamide gels (5%) and quantified on a PhosphoImager with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). To correct signals of protected transcripts for background intensities, the latter were determined for each individual lane in close proximity to the respective mRNA signal and subtracted from the value of the protected transcript. Background intensities for TRAF1, cIAP2, and xIAP were determined in the area between the location of the xIAP and TRAF1 bands, and background intensities for glyceraldehyde-3-phosphate dehydrogenase were taken directly below the glyceraldehyde-3-phosphate dehydrogenase band.

Western Blot Analysis—Cell lysates were prepared in radioimmune precipitation assay buffer containing 0.1 volume of a protease inhibitor mixture stock solution (Roche Molecular Biochemicals). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blocked with 5% nonfat dry milk in PBS/Tween 20 (0.05%) overnight. The membrane was incubated with anti-caspase-8, anti-caspase-2 (Pharmingen), or anti-FLIP antiserum (diluted 1:1000) for 1 h. After washing three times with PBS/Tween 20, membranes were incubated with anti-mouse (casapse-8, caspase-2) or anti-rabbit (FLIP) alkaline phosphatase antibody (0.1 μg/ml; Sigma, Deisenhofen, Germany) for 1 h. After four washes with PBS/Tween 20, the blots were developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Gene Induction by TRAIL Receptors and Fas
Fas-transfected HeLa cells (HeLa-Fas cells) also depended on the presence of CHX and inhibition of apoptosis (Fig. 1D).

Induction of IL-6, TRAF1, and cIAP2 by TRAIL-LZ and Anti-APO-1—To verify that TRAIL-LZ- and anti-APO-1-mediated NF-κB activation in HeLa cells results in induction of endogenous genes, we analyzed the biosynthesis of the NF-κB-regulated gene products IL-6, cIAP2, and TRAF1. Treatment of HeLa or HeLa-Fas cells with TRAIL or anti-APO-1, respectively, led to the up-regulation of IL-6 production only when CHX was added and concomitantly induced apoptosis was blocked. TNF treatment, however, did not require reduction of protein synthesis to induce this response (Fig. 2A). As expected, reduction of protein synthesis was necessary and sufficient for TRAIL-induced IL-6 production in HeLa cells stably transfected with the crmA (cytokine response modifier A) gene of cowpox virus (Fig. 2B). The CrmA protein is an efficient inhibitor of caspase-1 and, more important in this context, caspase-8 and renders cells resistant to the apoptotic effects of TNF, TRAIL, and anti-APO-1. Hence, CHX has to affect gene products acting upstream of caspase-8 or on a pathway that bifurcates upstream of this molecule to allow gene induction. TRAIL (Fig. 3A) and anti-APO-1 (Fig. 3B) efficiently induced the production of IL-6 in HeLa cells in a dose-dependent manner in the presence of CHX when induction of apoptosis was blocked. In contrast, TNF-induced IL-6 production was already induced in the absence of CHX (Fig. 3C), but addition of CHX together with inhibition of cell death shifted the dose-response curve of TNF-mediated IL-6 production strongly toward lower concentrations (Fig. 3C). Remarkably, IL-1-induced IL-6 production was not or only moderately affected by CHX/Z-VAD-fmk treatment (Fig. 3D). Concentrations of CHX between 2 and 5 μg/ml were sufficient to allow half-maximal activation of IL-6 production upon TRAIL and anti-APO-1 treatment and were also sufficient to significantly enhance TNF-induced IL-6 production (Fig. 3E). In contrast, there was no effect of CHX on IL-1-induced IL-6 production even at concentrations up to 50 μg/ml. The modest CHX concentrations used in our experiments had almost no effect on the viability of the cells and did not activate NF-κB (Fig. 1) or JNK (data not shown).

Using RNase protection assays, we have previously shown that cIAP2 and TRAF1 are transcriptionally up-regulated by NF-κB-inducing reagents (26). Moreover, cIAP2 has been identified as an NF-κB-regulated gene (27). In line with our previous results, transcripts of both genes were up-regulated after treatments had almost no effect on the viability of the cells and did not activate NF-κB—

FIG. 2. TRAIL and anti-APO-1 induce up-regulation of IL-6, TRAF1, and cIAP2 in HeLa cells: requirement for CHX and caspase inhibition. A, HeLa or HeLa-Fas cells (1.5 × 10^5) were cultured overnight in 96-well assay plates. The next day, HeLa cells were incubated for 18 h with TNF (10 ng/ml) or TRAIL-LZ (100 ng/ml) and HeLa-Fas cells with the agonistic Apo1/Fas-specific mAb anti-APO-1 (100 ng/ml) in the presence of the indicated reagents (20 μg Z-VAD-fmk (ZVAD) and 2 μg/ml CHX). Finally, IL-6 concentrations in supernatants were measured using a commercially available ELISA kit. B, HeLa-CrmA cells were cultured as described for A. Cells were then treated for an additional 18 h with TNF (10 ng/ml) and TRAIL-LZ (100 ng/ml) alone or in the presence of the indicated combinations of 2 μg/ml CHX and 20 μg/ml Z-VAD-fmk. Again, IL-6 concentrations in supernatants were measured using a commercially available ELISA kit. con., control.
FIG. 3. Effect of CHX and apoptosis inhibition on IL-6 production by TRAIL, anti-APO-1, TNF and IL-1. HeLa and HeLa-Fas cells were cultured as described in the legend to Fig. 2. HeLa cells were then treated for an additional 18 h with TRAIL-LZ (A), TNF (C), or IL-1 (D), and HeLa-Fas cells were incubated with anti-APO-1 (B) alone (○) or in the presence of 2 μg/ml CHX (●). IL-6 concentrations in supernatants were measured using a commercially available ELISA kit. In E, HeLa cells (1.5 × 10⁴) were cultured overnight in 96-well assay plates. The next day, cells were incubated for an additional 18 h with TNF (10 ng/ml; □), TRAIL-LZ (100 ng/ml; ◆), IL-1 (10 ng/ml; ▼), or medium alone (●) in the presence of Z-VAD-fmk (20 μM) and the indicated concentrations of CHX. Finally, IL-6 production was measured as already described in the legend to Fig. 2. In F are shown the results from the RNase protection assay analysis of various members of the TRAF and IAP protein families in HeLa and HeLa-Fas cells upon TRAIL-LZ and anti-APO-1 treatment, respectively. Cells were treated with TRAIL-LZ (100 ng/ml) or anti-APO-1 (100 ng/ml) for 5 h in the presence of the indicated reagents (20 μM Z-VAD-fmk (Z) and 2 μg/ml CHX (X)). Please note that isolation of RNA from CHX/TRAIL- and CHX/anti-APO-1-treated cells failed due to extended cell death. Whole RNAs were isolated after treatment, and 10 μg of each RNA was analyzed with the human Apo5a Multi-Probe template set to detect the indicated mRNAs. Relative expression levels were calculated as described under “Experimental Procedures.” GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 4. The p38 kinase inhibitor SB203580 and TRAF2-(87–501) interfere with TRAIL- and anti-APO-1-mediated gene induction. A, overexpression of TRAF2-(87–501) interferes with TRAIL- and Apo1/Fas-mediated NF-κB activation. HeLa and HeLa-Fas cells (1 × 10⁵ cells/well in a 24-well plate) were transiently transfected with an increasing amount of an expression vector encoding TRAF2-(87–501) together with a 3xNF-κB/luciferase reporter plasmid and an SV40 promoter-driven β-galactosidase expression plasmid. To adjust the DNA to the same amount per well, empty vector was added. On the following day, cells were incubated for 8 h with 2 mg/ml CHX and Z-VAD-fmk with or without addition of TRAIL-LZ (100 ng/ml) and anti-APO-1 (100 ng/ml). Finally, cell lysates were prepared and assayed for luciferase and galactosidase activities.

B, TNF-, TRAIL-LZ-, and anti-APO-1-mediated IL-6 production is blocked by the p38 kinase inhibitor SB203580. HeLa-Fas or HeLa cells (1.5 × 10⁴) were cultured overnight in 96-well assay plates. The next day, cells were incubated for an additional 18 h with TNF (10 ng/ml), TRAIL-LZ (100 ng/ml), or the agonistic Apo1/Fas-specific mAb anti-APO-1 (100 ng/ml) alone in the presence of 2 μg/ml CHX and 10 μM Z-VAD-fmk or in combination with the indicated concentrations of the p38 kinase inhibitor SB203580 (SB; black bars) or the carrier Me₂SO (DMSO; hatched bars). IL-6 concentrations in supernatants were measured using a commercially available ELISA kit. C, SB203580 interferes with TRAIL-LZ-induced up-regulation of TRAF1. RNase protection assay analyses of the indicated TRAF and IAP family members in HeLa cells upon TRAIL-LZ stimulation for 5 h in the presence of 20 μM SB203580 (SB; black bars) or the carrier Me₂SO (DMSO; hatched bars) were performed as described in the legend to Fig. 3. Relative expression levels were calculated as described under “Experimental Procedures.” GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
checked whether this mutant also affects TRAIL- and anti-APO-1-induced NF-κB activation. In fact, overexpression of TRAF2-(87–501) blocked TRAIL- and anti-APO-1-mediated activation of an NF-κB-driven reporter gene construct in a dose-dependent manner (Fig. 4A). In addition, TNF- and TRAIL-induced NF-κB activation was abrogated by transient overexpression of a kinase-inactive mutant of the NF-κB-inducing kinase NIK, but not by dominant-negative MEKK1 (data not shown). Some recent reports suggest that TNF-induced expression of NF-κB-dependent genes is the result of a cooperative mechanism comprising translocation into the nucleus and DNA binding of NF-κB as well as modulation of the transcription machinery via the p38 kinase pathway (29–31). Therefore, we analyzed the effect of the p38 kinase inhibitor SB20580 on TRAIL- and anti-APO-1-induced NF-κB activation. As shown in Fig. 4B, in HeLa cells, treatment with 66 μM SB20580 inhibited 93, 83, and 72% of anti-APO-1-, TRAIL-, and TNF-induced IL-6 production, respectively. Moreover, treatment with SB20580 inhibited >70% of the TRAIL-induced up-regulation of TRAF1 mRNA (Fig. 4C). These data indicate that TRAIL receptors and Apo1/Fas utilize a cooperative mechanism for the induction of NF-κB-regulated genes, as has been already shown for TNF-R1. Taken together, our results suggest that TRAIL-, FasL-, and TRINF-induced gene inductions converge upstream of or at the level of TRAF2. Hence, the CHX-sensitive factor(s) that prevent NF-κB activation and gene induction by TRAIL-LZ and anti-APO-1 should be located upstream or parallel to TRAF2.

Overexpression of the FADD Death Domain Is Sufficient to Induce NF-κB Activation and Up-regulation of IL-6, TRAF1, and cIAP2 in a CHX-dependent Manner—The adaptor protein FADD has been implicated in the apoptotic signaling of Apo1/
Fas, TNF-R1, and the death domain-containing TRAIL receptors (14–20). Therefore, we next analyzed whether FADD and a deletion mutant of FADD (FADD-(80–208)) that lacks the amino-terminal death effector domain of the molecule and is therefore dominant-negative with respect to apoptosis induction are able to mediate gene induction. Transient overexpression of FADD as well as of GFP-tagged and untagged FADD-(80–208), but not the death domain of RAIDD, activated NF-κB in HeLa cells (Fig. 5A) and HEK293 cells (Fig. 5B), suggesting that the death domain of FADD is sufficient for activation of the NF-κB pathway. Interestingly, addition of CHX for 6 h significantly increased the synthesis of an NF-κB-driven reporter gene product in both cell lines. Next, we analyzed HeLa transfectants stably overexpressing GFP-tagged FADD-(80–208). Although GFP-FADD-(80–208) robustly activated NF-κB upon transient overexpression (Fig. 5, A and B), we found no evidence for a constitutive NF-κB activation in the GFP-FADD-(80–208)-expressing HeLa cells. However, low doses of CHX induced reporter gene activity (Fig. 5C) and IL-6 production (Fig. 5D) as well as up-regulation of TRAF1 and cIAP2 mRNAs (Fig. 5E) in these cells. In contrast, in various control cells, including GFP-RAIDD-(80–200) transfectants, addition of CHX failed to induce these NF-κB-dependent genes. This differential CHX dependence of FADD-(80–208)-mediated NF-κB activation observed between transiently and stably expressing cells most likely reflects differences in the expression levels of GFP-FADD-(80–208). If one assumes that CHX treatment reduces the concentration of a yet unknown inhibitor of FADD-dependent NF-κB activation, it is obvious that exceeding FADD expression would result in CHX-independent NF-κB activation. In fact, expression of GFP-FADD-(80–208) was found to be several times higher upon transient transfection than the level observed in the stable cell line (data not shown). CHX-induced IL-6 production in GFP-FADD-(80–208)-expressing cells was significantly reduced by the p38 kinase inhibitor SB203580, similar to TNF-, TRAIL-LZ-, and anti-APO-1-mediated IL-6 production (Fig. 5F). Moreover, dominant-negative TRAF2 interfered with FADD-(80–208)-mediated NF-κB activation (data not shown).

According to the data shown above, the death domain of FADD is a candidate for coupling death domain-containing receptors to the activation of NF-κB and gene induction. To further substantiate this concept, we made use of a recently described deletion mutant of murine Fas (FasΔ) lacking amino acids 191–201 (32). This mutant fails to bind FADD, but is still able to activate JNK via DAXX (32). As shown in Fig. 5G, murine Fas, but not the FasΔ mutant derived thereof, activated NF-κB upon overexpression, which is in line with a role of FADD in Fas-mediated gene induction. Fluorescence-activated cell sorter analysis showed that both murine Fas proteins were present on the cell surface in comparable amounts (data not shown).

Death Receptor-induced NF-κB Activation Is Inhibited in FADD-deficient Jurkat Cells—The data described above point to FADD as the mediator of death receptor-induced NF-κB activation. To verify this concept experimentally, we analyzed a mutant Jurkat cell line that lacks FADD expression (33) with respect to TNF-, TRAIL-, and FasL-induced NF-κB activation. As shown in Fig. 6A, TNF induced an almost 20-fold up-regulation of an NF-κB-driven luciferase reporter gene in the parental cell line, whereas in the corresponding FADD-deficient Jurkat cell line, only an ~10-fold up-regulation was found. Moreover, TRAIL-induced NF-κB activation was 80% reduced in the FADD-deficient line compared with the parental cell line, and Fas-induced NF-κB activation was completely absent (Fig. 6B). Together, these data argue for a significant (TNF, TRAIL) or even essential (Fas) role of FADD in death receptor-induced NF-κB activation.

Dose Dependence of TNF-, TRAIL-, and Anti-APO-1-induced Cellular Responses—So far, our data indicate that the signaling systems initiated by TNF, TRAIL-LZ, and anti-APO-1 activate similar gene-inductive pathways. Nevertheless, TNF represents a pleiotropic cytokine with capabilities including the induction of apoptosis among many others, whereas Apo1/Fas and TRAIL may predominantly act as death inducers. This divergence cannot be exclusively explained by the fact that TNF-R1 is a potent inducer of anti-apoptotic NF-κB-dependent genes, as we have found in HeLa cells that TRAIL-LZ and anti-APO-1 also up-regulate NF-κB-dependent genes in an amount comparable to TNF. However, this became apparent only in the presence of CHX and when apoptosis was prevented. We therefore analyzed the dose dependence of gene induction and initiation of apoptosis by these reagents in more detail. Interestingly, we found that under the same conditions, namely the presence of CHX and Z-VAD-fmk, the ED50 of TNF-R1 for gene induction is ~500 times lower than its ED50 for the induction of apoptosis (Fig. 7A), whereas the dose-response analysis of TRAIL-LZ and anti-APO-1 revealed no differences for gene induction and apoptosis (Fig. 7, B and C). There was also no difference in the ED50 values when Fas and TRAIL receptors were triggered with cross-linked soluble ligands (data not shown). The dominance of the gene-inductive
anti-apoptotic pathway over the apoptosis-inducing pathway upon TNF triggering correlates well with the above-mentioned dominance of inflammatory and proliferative effects in TNF physiology.

Cellular FLIP Is Rapidly Down-regulated by CHX and Inhibits Death Receptor-dependent NF-κB Activation in HeLa Cells—As already discussed above, our data suggest that TRAIL-, FasL-, and TNF-engaged gene inductions converge at the level of TRAF2 or upstream of this molecule. As TNF-, but not TRAIL-LZ- and anti-APO-1-induced NF-κB activation in HeLa cells occurred in the absence of CHX, the postulated CHX-sensitive factor involved in inhibition of gene induction by the latter two ligands should also act upstream or parallel to TRAF2. A known receptor-proximal regulator of TRAIL-, FasL-, and TNF-induced apoptosis is the caspase-8 homolog FLIP, which lacks protease activity. The level of FLIP expression regulates the apoptotic potential of TRAIL and FasL in FLIP transfectants (34). We therefore investigated cellular FLIP expression levels in untreated and CHX-treated HeLa cells. As shown in Fig. 8, FLIP protein was significantly down-regulated after CHX treatment for 3 or 7 h in HeLa cells, whereas the expression level of caspase-8 and -2 remained unaffected. This is in good accordance with the recently reported CHX sensitivity of FLIP expression in primary keratinocytes (35). In addition, the recently reported reduction of FLIP expression after treatment of melanoma cells with actinomycin D also argues for a high turnover of the FLIP protein (36). Moreover, Kym-1 cells, in which TRAIL-induced NF-κB activation can be induced in the absence of CHX/Z-VAD-fmk, expressed significantly lower levels of cellular FLIP compared with HeLa cells, but expressed similar amounts of caspase-8 and -2 (Fig. 8). Next, we checked whether FLIP can have an impact on death receptor-induced NF-κB activation using an NF-κB-driven reporter construct. As shown in Fig. 9, expression of FLIP led to a slight but significant activation of NF-κB in HeLa cells that was in the range of 5–10% of the activation that was achieved by stimulation with TNF, TRAIL-LZ, or anti-APO-1. However, more important, we found that TNF-induced (Fig. 9, A, B, and E), TRAIL-induced (Fig. 9, C and F), and anti-APO-1-induced (Fig. 9, D) NF-κB activation was inhibited in a dose-dependent manner by overexpression of FLIP. Some recent reports have shown that NF-κB activation is inhibited by caspase-generated cleavage products of components of the NF-κB signaling pathway that act as dominant-negative versions of their uncleaved forms (37–41). As the reporter plasmid analyses with TRAIL (Fig. 9C) and anti-APO-1 (Fig. 9D) were performed in the presence of Z-VAD-fmk (and CHX), FLIP-, induced apoptosis can be ruled out as the reason for FLIP-, dependent inhibition of TRAIL- and anti-APO-1-induced NF-κB activation. Moreover, in HeLa-CrmA cells, TRAIL-induced NF-κB activation was also inhibited by FLIP (Fig. 9F). To rule out that the inhibitory effect of FLIP on TNF-induced NF-κB activation is an epiphenomenon of FLIP-, induced apoptosis, we performed reporter plasmid analysis also in the presence of Z-VAD-fmk (Fig. 9B) or in HeLa-CrmA cells (Fig. 9E). Again, there was no impact on the NF-κB inhibitory effect of FLIP.

DISCUSSION

The death domain-containing receptor TNF-R1 is capable of initiating apoptosis, but also has pronounced gene-inductive properties. In contrast, Apo1/Fas and the death domain-containing TRAIL receptors are predominantly characterized as inducers of apoptosis. However, some recent reports demonstrate the principal capability of these receptors to activate NF-κB at least in some cell lines (16, 17, 21–23), leading to the question of whether TRAIL receptors and Apo1/Fas engage similar gene regulatory pathways as TNF. We have found that in Kym-1 cells, both TNF (data not shown) and TRAIL (Fig. 1A) activate NF-κB and concomitantly induce cell death without further treatment of the cells. In contrast, in HeLa cells, neither TRAIL nor the agonistic mAb anti-APO-1 are capable of activating NF-κB in otherwise untreated cells, whereas TNF does (Fig. 1, C and D). However, in the presence of the metabolic inhibitor CHX, all three receptor systems mediate cell death and, in parallel, activation of NF-κB (Fig. 1, B–D). Hence, for TRAIL receptors and Apo1/Fas, but not for TNF-R1, the activation of NF-κB correlates with the induction of apoptosis. Nevertheless, NF-κB activation by TRAIL-LZ and anti-APO-1 appears not to be an epiphenomenon of ongoing cell death, as NF-κB activation is not blocked (rather than enhanced) in the presence of the caspase inhibitor Z-VAD-fmk, which completely prevents death domain receptor-induced apoptosis (Fig. 1). Moreover, in cells that are resistant to death receptor-induced apoptosis by overexpression of the cowpox serpin CrmA, TRAIL-LZ still strongly up-regulates NF-κB (data not shown) and IL-6 production (Fig. 2B) in the presence of CHX alone. Together, these data argue for the existence of a CHX-sensitive
inhibitor that blocks TRAIL receptor- and Fas- but not TNF-R1-mediated NF-κB activation.

Enhancement of NF-κB activation by inhibition of concomitantly induced cell death is in good accordance with recent data demonstrating that NF-κB inhibitory molecules can be generated by caspase-dependent cleavage of components of the NF-κB pathway. In fact, cleavage of IκB, p65, TRAF1, and receptor interacting protein at conserved caspase cleavage sites generates fragments that act as potent inhibitors of the NF-κB pathway (37–41).

TNF-R1-mediated NF-κB activation and TNF-R1-induced cell death bifurcate at TRADD (12). As in HeLa cells, TNF-induced apoptosis, but not TNF-mediated NF-κB activation, requires the presence of CHX, it is postulated that a CHX-sensitive negative regulator of apoptosis interferes with TNF signaling downstream of TRADD. However, in HeLa cells, both NF-κB activation and death induction by TRAIL and anti-APO-1 were dependent on the presence of CHX (Fig. 1). These results suggest that besides more complicated models, either a first CHX-sensitive factor blocks death domain receptor-induced apoptosis in HeLa cells and a second CHX-sensitive factor selectively interferes with TRAIL-LZ- and Apo1/Fas- but not TNF-mediated NF-κB activation, or alternatively, a common CHX-sensitive factor blocks TNF-, TRAIL-, and Apo1/Fas-mediated cell death as well as TRAIL- and Apo1/Fas-induced NF-κB activation. Accordingly, the postulated CHX-sensitive factor has to interfere with Apo1/Fas and TRAIL receptor signaling upstream of the bifurcation point of apoptosis and NF-κB activation of the respective receptors, i.e. at the level of FADD (Fig. 10). As TNF can engage FADD-dependent apoptosis in the presence of CHX, it should activate in parallel the FADD-dependent NF-κB pathway under these conditions, in addition to the CHX-independent pathway (Fig. 10). This concept is in good accordance with the fact that TNF-R1- but not IL-1-induced NF-κB activation was enhanced by treatment with CHX (Fig. 3), as IL-1 does not utilize FADD-dependent pathways.

In FADD-deficient embryonic fibroblasts, TNF-R1-, Apo1/Fas-, and DR3- but not TRAIL-R1-induced apoptosis is impaired (14, 15). Overexpression of a FADD deletion mutant lacking the amino-terminal death effector domain interferes with TRAIL-R1- and TRAIL-R2-mediated cell death (16–19);
and in FADD-deficient Jurkat cells, TRAIL-R2-induced cell death is abrogated (20). Thus, FADD and/or FADD-related molecules could also play a role in TRAIL-induced signaling processes. Interestingly, activation-induced proliferation in T cells (15) and development of the ventricular myocardium (14) are impaired in FADD−/− mice, arguing for additional non-apoptotic functions of FADD. In accordance with the non-apoptotic signaling capacities of FADD, we found that treatment with low doses of CHX was sufficient to induce NF-κB and NF-κB-regulated genes in HeLa cells stably overexpressing an amino-terminal GFP-tagged deletion mutant of FADD lacking the death effector domain, thus being resistant to death domain receptor-induced cell death (Fig. 5). Hence, it seems that upon down-regulation of a CHX-sensitive factor in HeLa cells, the death domain of FADD is sufficient to initiate gene-inductive pathways reminiscent of that engaged by TRAIL and anti-APO-1. A role of FADD in NF-κB-inducing pathways via its death domain is also in good accordance with the strong NF-κB activation found with the FADD death domain in transient reporter assays (Fig. 5, A and B) (12). According to the above-mentioned concept, FADD is the most likely target of the postulated CHX-sensitive factor, as (i) FADD death domain-mediated gene-inductive effects are blocked in untreated cells; and (ii) in the TNF-R1 signaling complex, FADD is immediately downstream of TRADD, a molecule that allows the propagation of TNF-R1-induced gene induction in the absence of CHX. Additional evidence for FADD as an important intermediate in death receptor-induced NF-κB activation comes from studies with FADD-deficient Jurkat cells (Fig. 6). In this cell line compared with the parental cell line, TNF-induced NF-κB activation was reduced by 50%, and NF-κB activation by TRAIL and FasL was reduced completely (FasL) or by ~80% (TRAIL). The rather modest inhibition of TNF-induced NF-κB activation in FADD-deficient Jurkat cells nicely correlates with our finding that TNF can activate NF-κB by two distinct pathways: one CHX-dependent in some cells and one CHX-independent. Consequently, FasL- and TRAIL-induced NF-κB activation, being elicited according to our data only via the CHX/FADD-dependent pathway, is more drastically affected in the FADD-deficient cells. The residual NF-κB activation of ~20% found upon TRAIL stimulation could be caused by TRAIL-R1, which signals apoptosis independent of FADD (14, 15). In fact, we have evidence from agonistic and antagonistic TRAIL-R1-specific antisera that there is a small amount of TRAIL-R1 expression in Jurkat cells not detectable by fluorescence-activated cell sorter analyses.2

Possible candidates for the postulated CHX-sensitive factor are some recently cloned molecules (FLASH, F1a, and cellular E10) that can be part of the receptor signaling complexes of death receptors (42–44). However, FLIP is certainly the prime candidate for this unknown CHX-sensitive factor, as it interacts with FADD as well as with TRAF2 (34, 45). In accordance with this hypothesis, we have found that the expression level of FLIP, in HeLa cells was significantly reduced upon CHX treatment, whereas the expression level of the related protein caspase-8 remained unaffected (Fig. 7). Moreover, in another cell line (SV80), we made the observation that the short splice form of FLIP (FLIPs) is also CHX-sensitive (data not shown). Last but not least, we found a pronounced inhibitory effect of FLIP overexpression on death receptor-mediated NF-κB activation in reporter gene assays (Fig. 8). In this respect, Chaudhary et al. (46) have recently shown that some (but not all) viral homologs of FLIP inhibit NF-κB activation engaged by cotransfected death receptors.

In the cellular models investigated in this study, stimulation of Fas or the TRAIL receptors leads to the concomitant activation of NF-κB and the NF-κB inhibitory apoptotic pathway with the consequence that the gene-inductive properties of these receptors become apparent provided induction of apoptosis is artificially blocked. Hence, the question of the physiological relevance of Fas- and TRAIL receptor-mediated gene induction arises. In this regard, as our data argue for a bifurcation of Fas-induced apoptosis and Fas-mediated NF-κB activation at the level of FADD, a physiological role of Fas- and TRAIL receptor-mediated gene induction is conceivable in a situation in which apoptosis induction is counteracted downstream of FADD/caspase-8 in the absence of the CHX-sensitive factor. Such a situation can be readily envisaged in cells in which induction of apoptosis is dependent on mitochondrial factors and in which inhibition of cell death is achieved by members of the Bcl2 family. In addition, the gene-inductive properties of Fas and the TRAIL receptors could become relevant in cells that are sensitive to FasL- and TRAIL-induced apoptosis under pathophysiological conditions in which the apoptotic pathway is selectively abrogated, e.g. in the course of virus infection by specific viral genes such as ccrmA (see also Fig. 2).

Two recent studies have shown that caspase activation (in particular, activation of caspase-8) is necessary for CD3-induced T cell proliferation and that FasL can enhance this process (47, 48). Moreover, it has been shown that the FasFc fragment can partially inhibit CD3-induced T cell proliferation (48). As already discussed, it is evident from the FADD knockout mice that FADD is also necessary for T cell proliferation. If one assumes that caspase activation in T cell proliferation is mediated by death receptors (e.g. Fas), it is obvious that in this case, FLIP is absent; hence, NF-κB should be active in these cells in the way described in our study. In fact, Kennedy et al. (48) observed that Fas/CD3 co-stimulation augments the NF-κB pathway.

Acknowledgments—We thank Marcus Peter for the anti-APO-1 antibody, Prof. Klaus Schulze-Osthoff for the anti-caspase-8 antibody, and Peter Juo and John Blenis for the FADD-deficient Jurkat cell line.

REFERENCES
1. Armitage, R. J. (1994) Curr. Opin. Immunol. 6, 407–413
2. Smith, C. A., Farrah, T., and Goodwin, R. G. (1994) Cell 76, 859–962
3. Schulze-Osthoff, K., Ferrari, D., Lor, M., Wesselborg, S., and Peter, M. E. (1998) Eur. J. Biochem. 254, 439–459
4. Pan, G., Bauer, J. H., Haridas, V., Wang, S., Liu, D., Yu, G., Vincenz, C., Aggarwal, B. B., Ni, J., and Dixit, V. M. (1998) FEBS Lett. 431, 351–356
5. Boldin, M. P., Goncharov, T. M., Golovnev, Y. V., and Wallach, D. (1996) Cell 83, 803–815
6. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O’Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827

2 H. Wajant, E. Haas, R. Schwenzer, F. Mühlenbeck, S. Kreuz, G. Schubert, M. Grell, C. Smith, and P. Scheurich, unpublished data.
Gene Induction by TRAIL Receptors and Fas

7. Vincenz, C., and Dixit, V. M. (1997) J. Biol. Chem. 272, 6578–6583
8. Martin, D. A., Siegel, R. M., Zheng, L. X., and Lenardo, M. J. (1998) J. Biol. Chem. 273, 4345–4349
9. Yang, X. L., Chang, H. Y., and Baltimore, D. (1998) Mol. Cell 1, 319–325
10. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
11. Boldin, M. P., Varfolomeev, E. E., Panzer, Z., Mett, I. L., Camonis, J. H., and Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
12. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495–504
13. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
14. Yeh, W. C., Pompa, J. L., McCurrach, M. E., Shu, H. B., Elia, E. J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Deiry, W. S., Lowe, S. W., Goeddel, D. V., and Mak, T. W. (1998) Science 279, 1954–1958
15. Zhang, J., Cado, D., Chen, A., Kabra, N. H., and Winoto, A. (1998) Nature 392, 296–299
16. Chaudhary, P. M., Eby, M., Jasmin, A., Bookwalter, A., Murray, J., and Hood, L. (1997) Immunity 7, 821–830
17. Schneider, P., Thome, M., Burns, K., Bodmer, J. L., Hofmann, K., Kataoka, K., Holler, N., and Tschopp, J. (1997) Immunity 7, 831–836
18. Walczak, H., Degli-Eposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour, M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T. (1997) EMBO J. 16, 5386–5397
19. Wajant, H., Johannes, F. J., Haas, E., Siemienski, K., Schubert, G., Weiss, T., Grell, M., and Scheurich, P. (1998) Curr. Biol. 8, 113–116
20. Bodmer, J. L., Hailer, N., Reynard, S., Vinciguerra, P., Schneider, P., Joo, P., Blenis, J., and Tschopp, J. (2000) Nat. Cell Biol. 2, 241–243
21. Ponton, A. M., Clement, V., and Stamenkovic, I. (1996) J. Biol. Chem. 271, 8991–8995
22. Malinin, N. I., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) Nature 385, 540–544
23. Rensing-Ehl, A., Hess, S., Ziegler-Heitbrock, H.-W., Riethmüller, G., and Engelmann, H. (1995) J. Inflamm. 45, 161–174
24. Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
25. Mühlenbeck, F., Haas, E., Schwenzer, R., Schubert, G., Grell, M., Smith, C., Scheurich, P., and Wajant, H. (1998) J. Biol. Chem. 273, 33981–33988
26. Schwenzer, R., Siemienski, K., Liptart, S., Schubert, G., Peter, N., Scheurich, P., Schmidt, R., and Wajant, H. (1999) J. Biol. Chem. 274, 19368–19374
27. Chu, Z.-L., McKinsey, T. A., Liu, L., Gentry, J. J., Malim, M. H., and Ballard, D. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10057–10062
28. Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995) Science 269, 1424–1427
29. Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W. (1996) EMBO J. 15, 1914–1923
30. Bergmann, M., Hart, L., Lindsay, M., Barnes, P. J., and Newton, R. (1998) J. Biol. Chem. 273, 6607–6610
31. Vanden Berghe, W., Plaisance, S., Boone, E., De Bosscher, K., Schmitz, M. L., Fiers, W., and Haegeman, G. (1998) J. Biol. Chem. 273, 3285–3290
32. Chang, H. Y., Yang, X., and Baltimore, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1252–1256
33. Joo, P., Woo, M. S., Kuo, C. J., Signorelli, P., Biemann, H. P., Hannun, Y. A., and Blenis, J. (1999) Cell Growth Differ. 12, 797–804
34. Irmler, M., Thome, M., Hahn, M., Schneider, P., Hofmann, B., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Nature 388, 190–195
35. Leverkus, M., Neumann, M., Mengling, T., Rauch, C. T., Brocker, E. B., Krammer, P. H., and Walczak, H. (2000) Cancer Res. 60, 555–559
36. Griffith, T. S., Chin, W. A., Jackson, G. C., Lynch, D. H., and Kubin, M. Z. (1998) J. Immunol. 161, 2833–2840
37. Barket, M., Xue, D., Horvitz, H. R., and Gilmore, T. D. (1997) J. Biol. Chem. 272, 29419–29422
38. Levkau, B., Scatena, M., Giachelli, C. M., Ross, R., and Raines, E. W. (1999) Nat. Cell Biol. 1, 227–233
39. Reuther, J. Y., and Baldwin, A. S. (1999) J. Biol. Chem. 274, 20664–20670
40. Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z.-G. (1999) Genes Dev. 13, 2514–2526
41. Irmler, M., Martinon, F., Hailer, N., Steiner, V., Ruegg, C., Wajant, H., and Tschopp, J. (2000) FEBS Lett. 468, 129–133
42. Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K., and Yonehara, S. (1999) Nature 396, 777–785
43. Chan, S.-L., Yang, X., and Baltimore, D. (1999) EMBO J. 18, 1161–1168
44. Costanzo, A., Guiet, C., and Vito, P. (1999) J. Biol. Chem. 274, 20127–20132
45. Shu, H. B., Halpin, D. R., and Goeddel, D. V. (1997) Immunity 6, 751–763
46. Chaudhary, P. M., Jasmin, A., Eby, M. T., and Hood, L. (1999) Oncogene 18, 5738–5746
47. Alam, A., Cohen, L. Y., Aouad, S., and Sekaly, R.-P. (1999) J. Exp. Med. 190, 1879–1890
48. Kennedy, N. J., Kataoka, T., Tschopp, J., and Budd, R. C. (1999) J. Exp. Med. 190, 1891–1895