The Drosophila nuclear factor-κB (NF-κB)–like transcription factor Relish is activated by an endoproteolytic cleavage step mediated by the Drosophila caspase Dredd. We have examined the contribution of the caspase cascade to NF-κB activation via TRAIL, a mammalian tumor necrosis factor family ligand that is a potent activator of caspases. Our results demonstrate that TRAIL activates NF-κB in two phases as follows: an early caspase independent phase and a late caspase dependent phase. The late phase of the TRAIL-induced NF-κB is critically dependent on caspase 8 and can be blocked by pharmacological and genetic inhibitors of caspase 8 activation, such as benzylloxycarbonyl-Val-Asp-fluoromethyl ketone, benzylloxycarbonyl-IETD-fluoromethyl ketone, and small interfering RNA targeting caspase 8 and FADD. Whereas caspase 3 is required for TRAIL-induced apoptosis, it is not involved in TRAIL-induced NF-κB activation. The late phase of TRAIL-induced NF-κB activation involves caspase mediated cleavage of IκBa between Asp31 and Ser32 residues to generate an N-terminal truncated fragment that is degraded by the proteasome via the N-end rule pathway. Our results demonstrate that caspases play an evolutionarily conserved role as regulated entry points to the N-end rule pathway and in NF-κB activation in mammalian cells.

Nuclear factor-κB (NF-κB) is a critical transcription factor involved in the regulated expression of several genes involved in the inflammatory and immune response (1–5). Five known members of this family have been characterized to date and include c-Rel, NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p105), p65 (RelA), and RelB (4, 5). Although many dimeric forms of NF-κB have been described, the classical NF-κB complex is a heterodimer of the p65/RelA and p50 subunits and is found in most cells in association with a family of inhibitory proteins, called IκBs, of which the most common is IκBa (2, 4). The IκB proteins retain NF-κB in the cytoplasm by masking its nuclear localization signal. Stimulation by a number of cytokines, such as TNFα and IL-1, results in the activation of a multisubunit IκB kinase (IKK) complex, which leads to the inducible phosphorylation of IκB proteins at two conserved serine residues located within their N-terminal region (2, 4). Phospho-IκB proteins are ubiquitinated and subsequently degraded by the 26 S proteasome, thereby releasing NF-κB from their inhibitory influence (2). Once released, NF-κB is free to migrate to the nucleus and bind to the promoter of specific genes possessing its cognate binding site.

The members of the tumor necrosis factor receptor (TNFR) superfamily and their ligands have been recognized to play a crucial role in normal development and in the regulation of immune and inflammatory response (6, 7). In addition, a subgroup of these receptors, such as TNFR1, Fas/CD95, death receptor 3 (DR3/Trigeminal, DR5), death receptor 4 (DR4/TRAIL-R1), death receptor 5 (DR5/TLR-2), and death receptor 6 (DR6) are capable of inducing programmed cell death or apoptosis and are collectively referred to as death receptors (6, 8). The apoptosis inducing ability of these receptors has been mapped to a conserved cytoplasmic domain of 60–80 amino acids, called the death domain (DD) (6). TNFR1 is the prototypical and perhaps the best characterized death receptor (9). Ligand-induced trimerization of TNFR1 leads to the recruitment of the DD-containing adaptor protein TRADD, which helps in the formation of a plasma membrane-bound complex (complex I) via the recruitment of RIP1 and TRAF2 (10–12). Assembly of complex I occurs in lipid rafts and leads to NF-κB activation via RIP1-mediated recruitment of the IKK complex, whereas the c-Jun N-terminal kinase is activated via TRAF2-mediated activation of the mitogen-activated protein 3-kinase (10, 11). Subsequently, TRADD and RIP1 dissociate from complex I and associate with a cytoplasmic complex (complex II) consisting of DD-containing protein FADD and procaspase 8, the apical caspase of the caspase cascade (10). Under the conditions favoring TNFR1-induced apoptosis, procaspase 8 is activated upon recruitment to complex II and subsequently results in the activation of downstream caspases, such as caspase 3, 6, and 7, and eventual cell death (10). However, in most cell types, activation of the caspase cascade by TNFR1 is blocked by the concurrent activation of the NF-κB pathway, which is believed to protect cells against apoptosis by up-regulating the expression of anti-apoptotic molecules, such as cIAP1, and c-IAP2 (10).

Unlike TNFR1, signaling via Fas, DR4/TRL-R1, and DR5/...
TRAIL-R2 delivers a strong and rapid pro-apoptotic signal (8, 13). Ligand binding to these receptors leads to DD-mediated recruitment of FADD directly without the involvement of TRADD (13, 14). FADD subsequently leads to the recruitment and activation of procaspase 8 (8, 13). DR4/DR5-TRAIL-R1 and DR5-TRAIL-R2 are also known to activate the NF-κB pathway, although activation of this pathway via these receptors is of lower magnitude and slower kinetics as compared with TNF-R1 (15–18). Nevertheless, RIP1 and IKK complex-mediated phosphorylation and degradation of IκBα has been implicated in NF-κB activation via the TRAIL receptors as well (18), suggesting that they share at least some components of NF-κB signaling with TNF-R1.

The mammalian tumor necrosis factor receptor signaling pathway shares several similarities with the Drosophila immune deficiency (imd) pathway, which controls the induction of antimicrobial peptide genes after Gram-negative bacterial infection (see Fig. 7) (1). The central transcription factor in the imd signaling pathway is Relish, a Drosophila NF-κB homolog. Relish resembles the mammalian p100 and p105 proteins in overall structure and is composed of a DNA-binding Rel homology domain and an inhibitory IκB-like ankyrin-repeat domain (1). However, in contrast to its mammalian counterparts, activation of Relish does not require proteasome-mediated degradation of its IκB-like region. Instead, Relish is activated by endoproteolytic cleavage in the linker region between the Rel and IκB-like domains (1). The cleavage of Relish requires the activities of two arms of the imd pathway (Fig. 7). The first arm includes IMD- (Drosophila RIP1 homolog) and dTAK1 (Drosophila transforming growth factor-β activating kinase)-mediated activation of Drosophila IκB kinase (DmIKK) complex (19–21). The DmIKK complex consists of Drosophila IKKβ/immunoreponse-deficient 5 (Ird5) and Drosophila IKKγ/Kenny and phosphorylates Relish, triggering its cleavage (21–23). Actual cleavage of Relish is believed to be mediated by the second arm of the imd pathway that involves Drosophila FADD (dFADD)-mediated activation of Dredd, a Drosophila caspase (Fig. 7) (24–29). Dredd resembles caspase 8 in structure and is believed to participate in Relish activation by inducing its cleavage between the Rel and IκB homology domains (25).

As discussed above, the first arm of imd pathway is conserved in mammalian cells and has been shown to play a critical role in NF-κB activation via the TNFR family members. However, a role for caspase activation in NF-κB activation in mammalian cells has been lacking so far (1). In this report we have analyzed NF-κB activation via TRAIL, a TNF family ligand that is a powerful activator of the caspase cascade and is also known to induce NF-κB activation. We present evidence demonstrating a conserved role for the second, caspase-dependent, arm of the imd pathway in NF-κB activation via the TRAIL receptors.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—293s, 293T, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Rabbit polyclonal antibodies against IκBα and NEMO were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase 8 and cleaved PARP were from Cell Signaling, and those against FADD and RIP were from Transduction Laboratories. Human recombinant TRAIL, TNFα, and IL-1β were obtained from R&D Systems (Minneapolis, MN) and were used at concentrations of 100, 10, and 10 ng/ml, respectively. Caspase inhibitors were purchased from Calbiochem and dissolved in MeSO4.

Expression Constructs—A retroviral construct encoding FLAG-DR4 was made by amplifying a DNA fragment encoding DR4-FLAG by using Pfu polymerase and subsequently subcloning into the MSCV-neo vector (Clontech). Constructs encoding IκBα and an NF-κB-luciferase reporter have been described previously (30, 31). The cDNA encoding IκBα was subcloned into the pCMVTag-1 vector (Stratagene, La Jolla, CA) to make the construct FLAG-IκBα-Myc that carries an N-terminal FLAG tag and a C-terminal Myc tag. The DS1A mutant of IκBα was made by using site-directed mutagenesis kit (Stratagene). Adeno-associated viral vectors encoding C-terminal HA-tagged IκBα and its mutants were made by using a commercially available kit (Stratagene).

siRNA Oligonucleotides—siRNA oligonucleotides with two thymidine residues (dTdT) at the 5'-end of the sequence were designed to caspase 8 (sense, 5′-GCCAAGCCCCAUAGGACTT-3′), caspase 3 (sense, 5′-GCCAUAAGGGCUCAGTATT-3′), caspase 6 (sense, 5′-CAUGACAGAGACAGAGCTTAT-3′), caspase 7 (sense, 5′-GAUUC-GUGGAGGUAAGCTTGTAT-3′), FADD (sense, 5′-GCUCAUAGUGGUAUAUGGdTdT-3′), and Lamin A/C (sense, 5′-CUGGACCUCCAGAAGACATdtdt-3′), along with their corresponding antisense oligonucleotides. The RNA oligonucleotides were synthesized at DHarmaco Research Inc. (Lafayette, CO) or Oligonucleotide Synthesis Core Facility, University of Texas Southwestern Medical Center.

Transfection of siRNA Oligonucleotides—293NF-κB FLAG-DR4 cells were seeded into 24-well plates and transfected with various siRNA oligonucleotides (40 nm) using calcium phosphate or LipofectAMINE 2000 (Invitrogen). Forty eight hours after transfection, cells were treated with PBS, TRAIL (100 ng/ml), TNFα (10 ng/ml), and IL-1β (10 ng/ml) for 10 h and subsequently lysed for reporter assay and Western blotting.

Generation of 293NF-κB-DR4 Cells—Generation of 293NF-κB-Luc cells has been described previously (32). 293NF-κB-Luc cells were infected with an empty retroviral vector or one encoding FLAG-tagged DR4 and selected with G418. One of the clones that responded to TRAIL-signaling was chosen for further studies.

Luciferase Reporter Assays—The NF-κB reporter assay was performed essentially as described previously (33). 293NF-κB vector cells and 293NF-κB-DR4 cells were seeded in 24-well plates. Thirty-six hours later cells were treated in duplicates with MeSO4 or Z-VAD-fmk for 30 min prior to treatment with the ligands. The cells were lysed 10 h following treatment, and lysates were used for determining the luciferase activity and protein concentration (Bradford-assay; Bio-Rad). Luciferase activity was normalized relative to protein concentration. All experiments were in duplicate and repeated at least two times. The values shown are average (mean ± S.E.) of a representative of at least two independent experiments.

RESULTS

Generation and Characterization of 293NF-κB-DR4 Cell Line—In order to facilitate a comparative analysis of NF-κB activation by ligands of the TNF family, we generated subclones of 293 cells (human embryonic kidney) cell line with stably expressed an NF-κB-driven luciferase reporter construct. One of the subclones, designated 293NFκB-Luc, demonstrated low level basal luciferase activity but a robust increase (~100-fold) upon stimulation with TNFα, a known activator of the NF-κB pathway. However, unlike TNFα, treatment with TRAIL led to only a minimal increase in luciferase activity in 293NFκB-Luc cells, which was probably reflective of the weak expression of TRAIL receptors in these cells. In order to make 293NFκB-Luc cells responsive to TRAIL-induced NF-κB activation, we used retrovirally mediated gene transfer to generate 293NFκB-Luc cells stably expressing an empty vector or an N-terminal FLAG epitope-tagged DR4 (also called TRAIL-R1), one of the TRAIL receptors capable of activating the NF-κB pathway. As shown in Fig. 1, A and B, 293NFκB-DR4 cells demonstrated modest expression of DR4 as measured by cell surface staining with a FLAG antibody. Consistent with the expression of DR4, 293NFκB-DR4 cells demonstrated an increase in the NF-κB reporter activity upon stimulation with TRAIL as compared with the empty vector-expressing cells (Fig. 1, C and D). However, despite the presence of the exogenously expressed DR4, NF-κB activation by TRAIL was relatively modest (~25-fold increase) as compared with that induced by treatment with TNFα (~120-fold increase) (Fig. 1, C and D). Treatment of 293NFκB-DR4 cells with TRAIL also led to apoptosis in ~15–25% of cells, whereas treatment with TNFα failed to do so (not shown). TRAIL-induced apoptosis in 293NFκB-DR4 cells could be completely blocked by a broad

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failed to block either TNF
and 5-fold reduction in TRAIL-induced NF-
erase activity which was only minimally influenced by Z-VAD-
cells. For this purpose, cells were treated with 20
range caspase inhibitor, Z-VAD-fmk, thereby indicating the
involvement of caspases in this process (data not shown).

TRAIL-induced NF-κB Activation Is Inhibited by Broad Range Caspase Inhibitor Z-VAD-fmk—As the 293NF-κB-DR4
cells activated NF-κB in response to both TNF and TRAIL but
induced caspase-dependent apoptosis only in response to TRAIL,
they provided a convenient system to investigate the
contribution of caspases to NF-κB activation. We began by
studying the effect of the broad range caspase inhibitor Z-VAD-
fmk on TNFα- and TRAIL-induced NF-κB activation in these
cells. For this purpose, cells were treated with 20 μM Z-VAD-
fmk for 30 min before and during the treatment with the
ligands, and luciferase activity was measured on cellular ex-
tracts obtained after 10 h of treatment. As shown in Fig. 2A,
treatment with TNFα led to strong activation of NF-κB lucif-
erase activity which was only minimally influenced by Z-VAD-
fmk. In contrast, treatment with Z-VAD-fmk led to an almost
5-fold reduction in TRAIL-induced NF-κB luciferase activity as
comparing with Me₂SO-treated cells (Fig. 2B). To confirm that
the observed effect is specific to the inhibition of caspases and
not cathepsin B, which is also inhibited by Z-VAD-fmk (34), we
used the cathepsin B inhibitor E64. Treatment with 20 μM E64
failed to block either TNFα- or TRAIL-induced NF-κB (Fig. 2, A
and B). We also ruled out the possibility that NF-κB activation
observed in 293NF-κB-DR4 cells is a nonspecific effect of
caspase activation and resultant cell death by using several
agents known to induce caspase activation via receptor-inde-
dependent mechanisms. Consistent with our results published
previously (35), transient transfection of plasmids encoding
several pro-apoptotic molecules, such as Bax, Bid, human
caspase 6, 9, and 10, led to varying levels of cell death but failed
to stimulate NF-κB luciferase activity (Fig. 2C). Collectively,
the above results indicate that NF-κB activation observed fol-
lowing treatment with TRAIL is not a nonspecific consequence of
caspase activation or cell death.

TRAIL-induced NF-κB Activation Is Inhibited by Preferential Inhibitors of Caspase 8—Caspase 8 plays a critical role in
the activation of the caspase cascade via the TRAIL receptors
(14). Z-IETD-fmk, a cell-permeable caspase inhibitor that pre-
ferentially blocks caspase 8, had no significant effect on TNFα-
induced NF-κB activation but was as effective as Z-VAD-fmk in
blocking NF-κB activation by TRAIL (Fig. 3, A and B). In con-
trast, Z-YDVAD-fmk, a preferential inhibitor of caspase 2,
was only partially effective in blocking TRAIL-induced NF-κB
(Fig. 3, C and D). The data using peptidyl-based caspase inhib-
itors, such as Z-IETD-fmk and Z-YDVAD-fmk, should be inter-
preted with caution as they lack absolute specificity. Therefore,
in order to provide genetic proof of the involvement of caspase 8
in TRAIL-induced NF-κB activation, we used the RNA inter-
ference approach to down-regulate its expression. As shown in
Fig. 3E, effective suppression of caspase 8 protein in 293NF-
κB-DR4 cells was achieved following a single round of trans-
fection with a caspase 8-specific siRNA duplex. Similarly, an
siRNA duplex targeted against FADD, the adaptor that re-
cruits caspase 8 to the TRAIL DISC, led to near complete loss of
its expression (Fig. 3F). The caspase 8 and FADD siRNAs
also completely blocked TRAIL-induced NF-κB activation
observed in 293NF-κB-DR4 cells treated with TNFα, PBS, TNFα, or TRAIL. We used
siRNAs directed against lamin A/C as a control for these ex-
periments. As shown in Fig. 3, G and H, siRNAs against
caspase 8 and FADD had no significant effect on TNFα- and
IL-1β-induced NF-κB but effectively blocked TRAIL-induced
NF-κB activation. Taken together with the results using
Z-IETD-fmk, the above studies confirm the involvement of
caspase 8 in TRAIL-induced NF-κB activation.

Caspase 3 Is Required for TRAIL-induced Apoptosis but Not TRAIL-induced NF-κB—Caspase 3 is an executioner caspase
that is known to play a key role in death receptor-induced
apoptosis. We used the siRNA approach to specifically down-
regulate expression of caspase 3 and to test its involvement in TRAIL-induced NF-κB and apoptosis. As shown in Fig. 4, A and B, caspase 3 siRNA effectively silenced its expression and blocked TRAIL-induced apoptosis in 293NF-κB DR4 cells. Most interestingly, unlike the case with caspase 8, silencing of caspase 3 had no significant effect on either TNFα- or TRAIL-induced NF-κB (Fig. 4, C and D). Furthermore, Z-VA D-fmk could effectively block TRAIL-induced NF-κB activation even in cells in which TRAIL-induced apoptosis was blocked due to silencing of caspase 3 expression (Fig. 4, E and F). In addition to caspase 3, caspases 6 and 7 are also known to act as executioner caspases. However, siRNA-mediated down-regulation of caspases 6 and 7 failed to block TRAIL-induced NF-κB (Fig. 4, G and H). Taken together, the inhibitory effect of Z-VA D-fmk on TRAIL-induced NF-κB is independent of its effect on TRAIL-induced apoptosis and activation of caspases 3, 6, or 7.

Caspase Activity Is Required for the Late Phase of NF-κB Activation by TRAIL—To understand the mechanism by which caspases promote TRAIL-induced NF-κB activation, we carried out an electrophoretic mobility shift assay. As shown in Fig. 5A, Z-VA D-fmk had no effect on the early phase (up to 6 h) of TRAIL-induced NF-κB DNA binding activity. However, while TRAIL-induced NF-κB DNA binding activity increased at the 8- and 10-h time points in Me2SO-treated cells, co-treatment with Z-VA D-fmk led to a gradual decline beginning at 8 h (Fig. 5B).
TRAIL treatment and its inhibition by Z-VAD-fmk.

Western analysis with an antibody against the cleaved form of PARP treatment has no effect on the expression of RIP, NEMO, or actin. Z-VAD-fmk blocks the decline in IκB, and the constitutive NF-κB activity (Fig. 5A). Caspase Inhibitors Block the Late Phase of TRAIL-induced NF-κB Activation—We next sought to determine whether Z-VAD-fmk blocks TRAIL-induced NF-κB by influencing the level of the IκB protein. In the absence of Z-VAD-fmk, IκBα almost completely disappeared following 2 h of TRAIL treatment (Fig. 5C, lane 4). The expression of IκBα reappeared at the 4- and 8-h time points (Fig. 5C, lanes 5 and 6), which probably reflected newly synthesized protein, and disappeared again at the 10- and 12-h time points (Fig. 5C, lanes 7 and 8). The decrease in expression of IκBα observed at the latter time points was not a nonspecific consequence of caspase activation as we observed no significant change in the expression of several control proteins, such as actin, NEMO, and RIP1 (Fig. 5C).

Most importantly, consistent with the gel-shift assay, cotreatment with Z-VAD-fmk had no effect on the early phase (2-h time point) of IκBα decline (Fig. 5C, lane 12) but effectively blocked the delayed (10- and 12-h time points) decrease in its expression (Fig. 5C, lanes 15 and 16).

We next studied the effect of siRNA-mediated caspase 8 silencing on the late phase down-regulation of IκBα expression following treatment with TRAIL and TNFα. For this purpose, cells were transfected with siRNAs against caspase 8 or Lamin A/C (negative control) and at 48 h post-transfection were treated with TNFα or TRAIL for 10 h. As expected, both TRAIL and TNFα treatments led to a significant decline in IκBα expression (Fig. 5D). However, while silencing of caspase 8 expression led to significant inhibition of IκBα down-regulation following treatment with TRAIL, it had only a marginal effect on TNFα-induced IκBα degradation (Fig. 5D). Taken together with our results using Z-VAD-fmk, the above studies demonstrate a key role for caspase 8 in the delayed phase of IκBα down-regulation following TRAIL treatment.

**Fig. 5. Caspase activation is required for the late phase of TRAIL-induced NF-κB activation and IκBα degradation.** A and B, electrophoretic mobility shift assays. Z-VAD-fmk has no major effect on the early phase but significantly inhibits the late phase of TRAIL-induced NF-κB DNA binding activities in 293NF-κB-DR4 and HeLa cells. The position of the induced NF-κB complex is marked with an arrow, and the constitutive NF-κB complexes are marked with asterisks. C, Z-VAD-fmk blocks the late phase of TRAIL-induced IκBα down-regulation in 293NF-κB-DR4 cells. Whole cell lysates containing equal amount of proteins were subjected to Western blot analysis using the indicated antibodies. Treatment with TRAIL leads to caspase-mediated down-regulation of IκBα expression at 10- and 12-h time points (lanes 7 and 8) which is blocked by Z-VAD-fmk (lanes 15 and 16). TRAIL treatment has no effect on the expression of RIP, NEMO, or actin. Western analysis with an antibody against the cleaved form of PARP (cl.PARP) is used to demonstrate activation of the caspase cascade upon TRAIL treatment and its inhibition by Z-VAD-fmk. D, caspase 8 siRNA blocks the decline in IκBα level in TRAIL-treated cells. 293NF-κB-DR4 cells were transfected with control and caspase 8 siRNA and 48 h later treated with PBS, TNFα, or TRAIL for 10 h. Whole cell lysates containing equal amounts of proteins were subjected to Western blot analysis using an IκBα antibody.

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Caspase-mediated NF-κB Activation—We next sought to determine whether Z-VAD-fmk blocks TRAIL-induced NF-κB by influencing the level of the IκBα protein. In the absence of Z-VAD-fmk, IκBα almost completely disappeared following 2 h of TRAIL treatment (Fig. 5C, lane 4). The expression of IκBα reappeared at the 4- and 8-h time points (Fig. 5C, lanes 5 and 6), which probably reflected newly synthesized protein, and disappeared again at the 10- and 12-h time points (Fig. 5C, lanes 7 and 8). The decrease in expression of IκBα observed at the latter time points was not a nonspecific consequence of caspase activation as we observed no significant change in the expression of several control proteins, such as actin, NEMO, and RIP1 (Fig. 5C).

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We next studied the effect of siRNA-mediated caspase 8 silencing on the late phase down-regulation of IκBα expression following treatment with TRAIL and TNFα. For this purpose, we generated a point mutant of IκBα containing an Asp to
Ala mutation at the amino acid residue 31, which abolished the caspase cleavage site. This construct carried an N-terminal FLAG epitope tag and a C-terminal Myc tag to help in the detection of the cleaved fragment (Fig. 6D). We transiently transfected the wild-type and D31A mutant IxBα constructs into 293NF-κB-DR4 cells and subsequently treated them with TRAIL in the absence or presence of MG132. An N-terminal cleaved from of IxBα was readily detected upon treatment with TRAIL and MG132 in cells transfected with the wild-type IxBα construct (lane 4) but not in those transfected with the D31A mutant (lane 8). The doublet of IxBα was detected by using an antibody against the C-terminal Myc epitope tag. Treatment with TRAIL and MG132 (10 μg/ml) leads to the generation of an N-terminal truncated fragment of IxBα (ΔIxBα) in cells transfected with the wild-type IxBα construct (lane 4) but not in those transfected with the D31A mutant (lane 8). The doublet of IxBα is probably due to translation initiation at the start codons located in the N-terminal FLAG tag and the IxBα cDNA, respectively. D, a schematic representation of IxBα D31A mutant construct with the sites of translation initiation shown by arrows. The site of caspase cleavage and an internal lysine residue (Lys38), which represents one of the potential sites of ubiquitination, is marked. E, 293NF-κB DR4 cells were infected in triplicate with adeno-associated viral vectors encoding the indicated HA-tagged IxBα mutant constructs. Approximately 48 h after infection, cells were treated with TRAIL for 10 h, and cell lysates were used for luciferase reporter assay. F, immunoblotting with the indicated antibodies on representative samples was performed to demonstrate expression of the various mutant constructs and equal protein loading. The values shown are averages (mean ± S.E.) of a representative of at least two independent experiments.

Ala mutation at the amino acid residue 31, which abolished the caspase cleavage site. This construct carried an N-terminal FLAG epitope tag and a C-terminal Myc tag to help in the detection of the cleaved fragment (Fig. 6D). We transiently transfected the wild-type and D31A mutant IxBα constructs into 293NF-κB-DR4 cells and subsequently treated them with TRAIL in the absence or presence of MG132. An N-terminal cleaved from of IxBα was readily detected upon treatment with TRAIL and MG132 in cells transfected with the wild-type construct and was completely absent in those transfected with the D31A mutant construct (Fig. 6C). Collectively, the above results support the hypothesis that caspase-mediated cleavage of IxBα between Asp31 and Ser32 residues targets it for proteasome-mediated degradation and contributes to the late phase of NF-κB activation by TRAIL.

We next sought to determine the effect of the caspase-resistant mutant of IxBα on TRAIL-induced NF-κB activation. For this purpose, we generated adeno-associated viral vectors expressing C-terminal HA-tagged D31A (cleavage-resistant), S32A/S36A (phosphorylation-resistant), and D31A/S32A/S36A mutants of IxBα. As shown in Fig. 6, E and F, TRAIL-induced NF-κB was partially blocked by D31A and S32A/S36A mutants, respectively. However, infection with the triple mutant (D31A/S32A/S36A) led to the most effective inhibition of TRAIL-induced NF-κB, presumably reflecting the inhibition of both the early and the late phase of NF-κB activation (Fig. 6, E and F).

**DISCUSSION**

Genetic and biochemical data support a key role for caspase activation in Relish processing in *Drosophila* (1, 40). In this report, we demonstrate that TRAIL, a TNF family ligand that is a powerful activator of the caspase cascade, activates NF-κB in two phases as follows: an early caspase-independent phase and a late caspase-dependent phase. Our results further demonstrate that the late caspase-dependent phase of TRAIL-induced NF-κB involves cleavage of the IxBα protein and its subsequent degradation by proteasome. Thus, like the situation with Relish processing, caspase-mediated cleavage of IxBα is also involved in NF-κB activation in mammalian cells, suggesting the existence of an evolutionary conserved signaling pathway (Fig. 7).

We demonstrate that none of the major downstream caspases involved in death receptor-induced apoptosis, such as caspases 3, 6, and 7, are required for TRAIL-induced NF-κB. Thus, like the situation with Dredd-mediated cleavage of Relish in *Drosophila*, cleavage of IxBα in mammalian cells might be primarily mediated by caspase 8 (Fig. 7). Another interesting and unexpected conclusion of this study is that activation of caspase 8 following TRAIL signaling may have two major con-
Regulated by the NF-κB and cIAP-2, two known inhibitors of caspase 3, is positively activated by caspase 8, which results in the cleavage of IκBα by caspase 3. A subsequent study demonstrated that IκBα is cleaved in cells undergoing TNFα-induced apoptosis or those dying as a result of IL-3 withdrawal to generate an N-terminal truncated form (ΔN-IκBα), which could bind the p65 subunit of NF-κB and suppress TNFα-induced NF-κB activation (37). Although caspase-mediated cleavage of IκBα was also encountered in our study, we have found that the N-terminal truncated form of IκBα is rapidly degraded by the proteasome pathway resulting in NF-κB activation rather than suppression. The exact cause for the discrepancy between the two studies is not known at the present. It is conceivable that proteasome-mediated degradation of ΔN-IκBα is governed by its signal-dependent secondary modification(s) (e.g., phosphorylation and ubiquitination) which is different between cells undergoing IL-3 withdrawal or those treated with TRAIL. Alternative explanations, including the fact that the two studies involved different cell lines, may apply as well. The ubiquitin ligase that recognizes primary (type 3) destabilizing residues of the N-end rule pathway (i.e. Ser, Ala, and Thr) in mammalian cells has yet to be identified (39). A difference in the expression and/or activity of this or related enzymes of the N-end rule pathway in different cell types or in response to different stimuli may also be a key determinant of the fate of ΔN-IκBα and cellular NF-κB response following TRAIL stimulation.

Our results involving DR4 signaling are consistent with a recent report (41) demonstrating a role for caspase activation in the induction of IL-8 gene expression in human astroglialoma cells via DR5/TRAIL-R2, another TRAIL receptor that is capable of activating both caspase and NF-κB pathways. In addition to the TRAIL receptors DR4 and DR5, it is conceivable that caspase activation also contributes to NF-κB activation via other death receptors. However, we have observed only a marginal effect of caspase inhibitors on TNFα-induced NF-κB activation in the current study. There are several possible explanations for this difference in signaling via the various death ligands. First, unlike TRAIL, TNFα is a strong activator of the IKK complex and through this complex results in robust phosphorylation and near-complete degradation of IκBα. Second, unlike TRAIL, TNFα is a weak activator of the caspase cascade. Thus, the lack of a role for caspases in TNFα-induced NF-κB activation in this study may be a combined outcome of strong activation of the IKK complex-mediated IκBα degradation and failure to activate the caspase cascade. However, TNFα is known to activate caspases in some cell types and under certain circumstances (e.g. in the presence of protein synthesis inhibitors), and it is conceivable that caspase activation does contribute to TNFα-induced NF-κB activation in these situations.

The N-end rule pathway is present in all organisms examined and relates the in vivo half-life of proteins to the identity of their N-terminal residues (38, 39, 42). In this pathway, the N-terminal degradation signal (N-degron) is produced by a proteolytic cleavage that yields a destabilizing N-terminal residue (38, 39, 42). Processing proteases that have been demonstrated to participate in the N-end rule pathway include me-
shown to be degraded by the N-end rule pathway or to play a generated mammalian protein fragment has been actually (38, 39, 42). However, unlike generate fragments that are putative N-end rule substrates entry points to the N-end rule pathway as well. A large number dependant N-end rule pathway, thereby establishing caspases as Drosophila thionine aminopeptidases and separases (38, 39, 42). A recent separation of figures.

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\[ \text{REFERENCES} \]

1. Silverman, N., and Maniatis, T. (2001) Genes Dev. 15, 2321–2342
2. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663
3. Li, Q., and Verma, I. M. (2002) Nat. Rev. Immunol. 2, 725–734
4. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–269
5. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
6. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) Cell 104, 487–501
7. Baud, V., and Karin, M. (2001) Trends Cell Biol. 11, 374–377
8. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
9. Chen, G., and Goeddel, D. V. (2002) Science 296, 1634–1635
10. Micheau, O., and Tschopp, J. (2003) Cell 114, 181–190
11. Legler, D. F., Micheau, O., Doucey, M. A., Tschopp, J., and Bron, C. (2003) Immunity 18, 655–664
12. Harper, N., Hughes, M., MacFarlane, M., and Cohen, G. M. (2003) J. Biol. Chem. 278, 25534–25544
13. Peter, M. E., and Krammer, P. H. (2003) Cell Death Differ. 10, 26–35
14. Peter, M. E. (2000) Cell Death Differ. 7, 759–760
15. Chaudhary, P. M., Eby, M., Jasmin, A., Bookwalter, A., Murray, J., and Hood, L. (1997) Immunity 7, 821–830
16. Schneider, P., Thome, M., Burns, K., Bodner, J. L., Hofmann, K., Kataoka, T., Holler, N., and Tschopp, J. (1997) Immunity 7, 831–836
17. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, L. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. (1997) Science 277, 818–821
18. Lin, Y., Devin, A., Cook, A., Keane, M. M., Kellifer, M., Lapkowitz, S., and Liu, Z. G. (2000) Mol. Cell. Biol. 20, 6638–6645
19. Georganos, P., Naitza, S., Kappler, C., Ferrandon, D., Zachary, D., Swimmer, C., Kopczynski, C., Duyk, G., Reichhart, J. M., and Hoffmann, J. A. (2001) Dev. Cell 1, 503–514
20. Vidal, S., Khush, R. S., Leulier, F., Tsou, P., Nakamura, M., and Lemaitre, B. (2001) Genes Dev. 15, 1900–1912
21. Silverman, N., Zhou, R., Stoven, S., Pandey, N., Hultmark, D., and Maniatis, T. (2000) Genes Dev. 14, 2461–2471
22. Lu, Y., Wu, L. P., and Anderson, K. V. (2001) Genes Dev. 15, 104–110
23. Rutschmann, S., Jung, A. C., Zhou, R., Silverman, N., Hoffmann, J. A., and Ferrandon, D. (2000) Nat. Immunol. 1, 342–347
24. Stephen, S., Ando, I., Kadalayitl, L., Engstrom, Y., and Hultmark, D. (2000) EMBO Rep. 1, 347–352
25. Steven, S., Silverman, N., Junell, A., Hedengren-Olcott, M., Erturk, D., Engstrom, Y., Maniatis, T., and Hultmark, D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5991–5996
26. Eldred-Erickson, M., Mishra, S., and Schneider, D. (2000) Curr. Biol. 10, 781–784
27. Leulier, F., Rodriguez, A., Khush, R. S., Abrams, J. M., and Lemaitre, B. (2000) EMBO Rep. 1, 353–358
28. Leulier, F., Vidal, S., Saigo, K., Ueda, R., and Lemaitre, B. (2002) Curr. Biol. 12, 996–1000
29. Naitza, S., Rossie, C., Kappler, C., Georgel, P., Belvin, M., Gub, D., Camonis, J., Hoffmann, J. A., and Reichhart, J. M. (2002) Immunity 17, 575–581
30. Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y., and Ballard, D. W. (1995) Mol. Cell. Biol. 15, 2809–2818
31. Berberich, I., Shu, G. L., and Clark, E. A. (1994) J. Immunol. 153, 4357–4366
32. Matta, H., Sun, Q., Moses, G., and Chaudhary, P. M. (2003) J. Biol. Chem. 278, 52496–52511
33. Kumar, A., Eby, M. T., Sinha, S., Jasmin, A., and Chaudhary, P. M. (2001) J. Biol. Chem. 276, 2668–2677
34. Sikadis, P., Dedhar, S., Van Hulff, S., Vandenaheele, P., and Beyaert, R. (1999) FEBS Lett. 442, 117–121
35. Chaudhary, P. M., Eby, M. T., Jasmin, A., Kumar, A., Liu, L., and Hood, L. (2000) Oncogene 19, 4451–4460
36. Barkett, M., Xie, D., Horvitz, H. R., and Gilmore, T. D. (1997) J. Biol. Chem. 272, 29419–29422
37. Reutter, J. Y., and Bäumlein, A. S., Jr. (1999) J. Biol. Chem. 274, 20664–20670
38. Varshavsky, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12142–12149
39. Varshavsky, A. (2003) Nat. Cell Biol. 5, 373–376
40. Khush, R. S., Leulier, F., and Lemaitre, B. (2001) Trends Immunol. 22, 260–264
41. Choi, C., Kutsch, O., Park, J., Zhou, T., Seld, D. W., and Benveniste, E. N. (2002) Mol. Cell. Biol. 22, 724–736
42. Varshavsky, A. (2000) Harvey Lect. 96, 93–116
43. Ditzel, M., Wilson, R., Tenev, T., Zachariae, A., Paul, A., Deas, E., and Meier, P. (2003) Nat. Cell Biol. 5, 467–473

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