Cell, Vol. 87, 565–576, November 1, 1996, Copyright ©1996 by Cell Press

Dissection of TNF Receptor 1 Effector Functions: JNK Activation Is Not Linked to Apoptosis While NF-κB Activation Prevents Cell Death

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Summary

Through its type 1 receptor (TNFR1), the cytokine TNF elicits an unusually wide range of biological responses, including inflammation, tumor necrosis, cell proliferation, differentiation, and apoptosis. We investigated how TNFR1 activates different effector functions; the protein kinase JNK, transcription factor NF-κB, and apoptosis. We found that the three responses are mediated through separate pathways. Recruitment of the signal transducer FADD to the TNFR1 complex mediates apoptosis but not NF-κB or JNK activation. Two other signal transducers, RIP and TRAF2, mediate both JNK and NF-κB activation. These two responses, however, diverge downstream to TRAF2. Most importantly, JNK activation is not involved in induction of apoptosis, while activation of NF-κB protects against TNF-induced apoptosis.

Introduction

Tumor necrosis factor (TNF) is a cytokine produced by many cell types, including macrophages, monocytes, lymphoid cells, and fibroblasts, in response to inflammation, infection, and other environmental challenges (Tracey and Cerami, 1993). TNF elicits a wide spectrum of organismal and cellular responses, including fever, shock, tissue injury, tumor necrosis, anorexia, induction of other cytokines and immunoregulatory molecules, cell proliferation, differentiation, and apoptosis (Tracey and Cerami, 1993; Vandenabeele et al., 1995). These responses are elicited by TNF-induced trimerization of two distinct cell surface receptors, TNFR1 (p55) and TNFR2 (p75), at least one of which is present in almost every cell type (Tartaglia and Goeddel, 1992; Smith et al., 1994; Vandenabeele et al., 1995). The structural similarity between the two TNF receptors is limited to their extracellular domains (Tartaglia and Goeddel, 1992). The TNFR ectodomains are related to those of CD30, CD40, CD27, and Fas, all of which belong to the TNF receptor superfamily (Smith et al., 1994). The ligands for these receptors are structurally related to TNF, and many of them are cell-surface anchored (Smith et al., 1994). Although most of the biological activities of TNF appear to be transduced by TNFR1, many can also be mediated by TNFR2 (Tartaglia and Goeddel, 1992; Smith et al., 1994; Vandenabeele et al., 1995). TNFR2, however, is a poor inducer of apoptosis.

Exposure to TNF results in activation of two transcription factors, AP-1 (Brenner et al., 1989) and NF-κB (Osborn et al., 1989). These transcription factors mediate induction of other cytokine and immunoregulatory genes, as well as metalloproteinases. Several second messengers have been proposed to mediate the biological effects of TNFR ligation, including various phospholipid breakdown products, arachidonic acid metabolites, free radicals, and increased intracellular Ca^{2+} (reviewed by Beyaert and Fiers, 1994). However, as discussed by Beyaert and Fiers, (1994), it is not clear whether these are true second messengers or secondary effects of TNFR activation. Several protein kinases were found to be activated rapidly in response to TNF, including yet-to-be-identified ceramide-activated kinase (Weigmann et al., 1994), IκB kinase (DiDonato et al., 1996), and a TNFR1-associated serine/threonine kinase (VanArsdale and Ware, 1994), as well as the molecularly identified Raf-1 (Belka et al., 1995), Jun N-terminal kinases (JNKs; Minden et al., 1994), and p38/Mpk2 (Raingeaud et al., 1995). Activation of the IκB kinase results in NF-κB activation (Verma et al., 1995; DiDonato et al., 1996), while Raf-1, JNK, and p38/Mpk2 activation contribute to induction of AP-1 activity (Karin, 1995). The pathways by which TNFR ligation causes activation of these protein kinases are not clear. Recently, much emphasis has been placed on the potential role of ceramide as a mediator of TNF signaling. TNF-induced phospholipid hydrolysis can result in ceramide production (Kolesnick and Golde, 1994), and exogenous ceramide can lead to activation of NF-κB (Weigmann et al., 1994) and JNK (Verheij et al., 1996) and apoptosis (Obeid et al., 1993). However, it is also possible that ceramide is produced as a result of TNF-induced cell death (Beyaert and Fiers, 1994).

A major advance in understanding early events in TNF signaling was the identification of protein molecules that are recruited to TNFR1 and TNFR2, following ligand induced trimerization (Rothe et al., 1994, 1995a; Hsu et al., 1995). While activation of TNFR1 results in recruitment of the TNFR1-associated death domain protein (TRADD; Hsu et al., 1995, 1996a), occupancy of TNFR2 leads to recruitment of TNFR-associated proteins 1 and 2 (TRAF1 and TRAF2; Rothe et al., 1994), as well as c-IAP1 and c-IAP2 (Rothe et al., 1995a). Recently TRADD was shown to interact directly with two other proteins, TRAF2 and Fas-associated protein with death domain (FADD; Hsu et al., 1996a). The recruitment of TRAF2 to both TNFR1 and TNFR2 explains why both receptors can elicit certain overlapping responses, despite having different cytoplasmic domains. Indeed, TRAF2 appears to mediate NF-κB activation by both TNFR1 and TNFR2, as well as by the related CD40 receptor (Rothe et al., 1995b; Hsu et al., 1996a). FADD (also known as MORT1) was originally identified as a protein that interacts with the cytoplasmic domain of Fas (Boldin et al., 1995; Chinnaiyan et al., 1995), which is structurally related to the

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cytoplasmic domain of TNFR1 (Itoh and Nagata, 1993; Tartaglia et al., 1993a). The death domain present in the cytoplasmic portions of both receptors mediates protein–protein interactions with other death domain–containing proteins, such as TRADD (Hsu et al., 1995) and FADD (Boldin et al., 1995; Chinnaiyan et al., 1995). Another death domain protein is the serine/threonine kinase RIP (for receptor-interacting protein; Stanger et al., 1995; Hsu et al., 1996b). Originally identified by its interaction with Fas (Stanger et al., 1995), RIP was recently shown to be recruited to the TNFR1 signaling complex via TRADD and to participate in NF-κB activation (Hsu et al., 1996b). The use of dominant-negative mutants suggests that TRADD is required for induction of apoptosis by TNFR1 (Hsu et al., 1996a, 1996b) and expression of both TRADD and RIP death domains is sufficient to activate this process (Hsu et al., 1995, 1996b; Stanger et al., 1995). Apoptosis induction by TNFR1 also appears to require FADD, but unlike TRADD and RIP, this activity is mediated by the FADD N-terminal domain rather than its death domain (Chinnaiyan et al., 1995; Hsu et al., 1996a). In fact, the FADD death domain blocks TNF-induced apoptosis (Hsu et al., 1996a; Chinnaiyan et al., 1996). The N-terminal death effector domain of FADD interacts with the ICE-like protease MACH (for MORT1-associating CED homolog) or FLICE (for FADD-like interleukin-1β-converting enzyme [ICE]), which appears to be a direct activator of the apoptotic protease cascade (Boldin et al., 1996; Muzio et al., 1996). These results suggest the protein-recruitment model for TNFR1 signaling that is illustrated in Figure 1. TNF-induced trimerization of TNFR1 results in recruitment of TRADD and RIP via death domain interactions. The TNFR1–TRADD complex then recruits at least three other signaling proteins: RIP, FADD, and TRAF2. While FADD activates the apoptotic machinery, TRAF2 and RIP mediate NF-κB activation.

These results, however, provide no clue to the mechanism by which TNFR1 can activate the JNK and p38 mitogen-activated protein kinase (MAPK) cascades or stimulate AP-1 activity. Furthermore, it was recently suggested that JNK and its target c-Jun are critical mediators of apoptosis induced by TNF or ceramide (Verheij et al., 1996). It is not clear how these results relate to the protein-recruitment model for TNFR1 and Fas signaling. In addition, Fas ligation results in only modest and delayed JNK activation (Z. G. L., unpublished data; Lenczowski et al., 1996), while CD40 ligation, which protects B cells against apoptosis (Liu et al., 1989; Tsubata et al., 1993), causes potent JNK activation (Berberich et al., 1996). There is also a considerable degree in overlap between the type of signals that activate NF-κB (Verma et al., 1995) and those that activate JNK and p38 (Raingeaud et al., 1995).

To determine the relationship between JNK activation in response to TNFR1 ligation, activation of NF-κB, and induction of apoptosis, we examined the ability of individual components of the TNFR1 signaling complex to activate these responses. In addition, we tested the ability of various dominant-negative mutants to interfere with JNK and NF-κB activation and induction of apoptosis. We found that TRAF2 and RIP but not FADD are the critical intermediates in JNK activation. Furthermore, we show that dominant negative TRAF2 and FADD mutants clearly dissociate JNK activation from induction

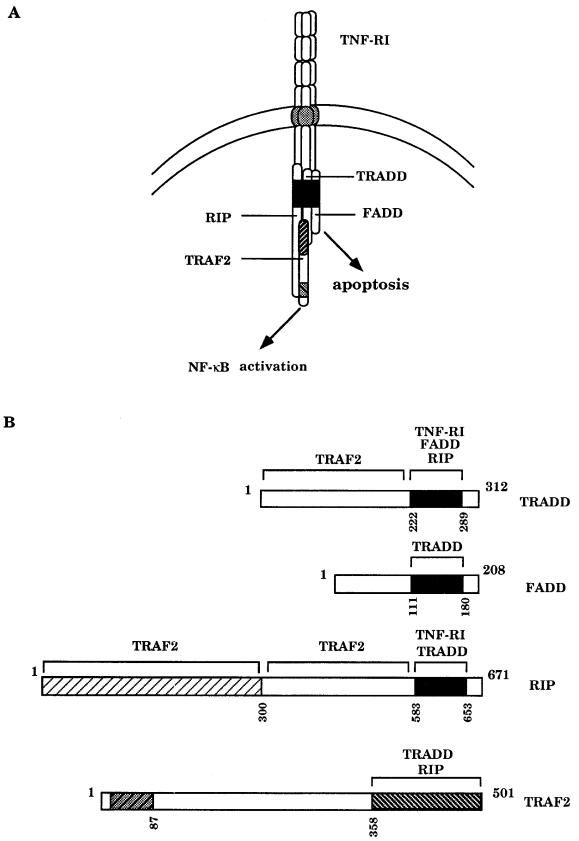


Figure 1. The Protein Recruitment Model for TNFR1 Signaling

(A) Ligand-induced trimerization of TNFR1 results in recruitment of TRADD to the cytoplasmic domain of the receptor via death domain (closed boxes) interactions. TRADD serves as a platform for recruitment of two additional death domain proteins, RIP and FADD, and for recruitment of TRAF2, which does not contain a death domain. The latter interaction is mediated by the N-terminal domain of TRADD and the C-terminal TRAF-C domain of TRAF2 (hatched box). While FADD signals to the apoptotic protease cascade, TRAF2 and RIP mediate NF-κB activation.

(B) The salient features of TRADD, FADD, RIP, and TRAF2. The protein domains and the targets with which they interact are indicated. Closed boxes, death domain. The different hatched boxes denote the RIP N-terminal kinase domain, the N-terminal ring finger of TRAF2, and its C-terminal TRAF domain.

of apoptosis. Although both JNK and NF-κB are activated via TRAF2 and RIP, we were able to dissociate these responses as well. We also found that inhibition of NF-κB activation, but not JNK, greatly increases the sensitivity of HeLa cells to TNF- induced apoptosis. By contrast, deliberate NF-κB activation protects against TNF-induced apoptosis. Thus, ligation of TNFR1 results in activation of at least three distinct effector functions, which can explain its ability to induce a plethora of diverse biological responses. These results can also explain why other members of the TNF receptor family can only affect subsets of these responses.

Results

Activation of JNK by Components of TNFR1 Signaling Complex

The protein recruitment model, for TNF signaling is illustrated in Figure 1A. According to this model, TNF-

induced trimerization of TNFR1 results in the recruitment of TRADD, which serves as a platform for recruitments of at least three additional proteins, FADD, RIP, and TRAF2 (Hsu et al., 1996a, 1996b). While both FADD and RIP contain death domains, which interact with the death domain of TRADD, TRAF2 uses its TRAF domain to interact with the N-terminal region of TRADD and the intermediate domain of RIP (Hsu et al., 1996b). Owing to the presence of a common TRAF domain, TRAF1 (Rothe et al., 1994) and TRAF3 (Hu et al., 1994) may also be recruited to TRADD and RIP signaling complexes (Hsu et al., 1996a, 1996b). The salient features of the different components of the TNFR1 signaling complex are shown in Figure 1B.

We examined the ability of these proteins to activate JNK in a cotransfection assay (Minden et al., 1994, 1995). Different expression vectors were cotransfected with a hemagglutinin (HA) epitope–tagged JNK1 vector into MCF7 cells, and their respective effects on HA–JNK1 expression and activity were determined 24 hr later by immunoblots and immune complex kinase assays. Owing to the ability of the death domain–containing proteins TRADD, FADD, and RIP to induce apoptosis (Hsu et al., 1996a, 1996b; Stanger et al., 1995; Chinnaiyan et al., 1995), their expression resulted in a considerable decrease in the amount of HA–JNK1 expression (Figures 2A and 2C). Therefore, we examined their effect on HA–JNK1 activity in the presence of the cowpox virus apoptosis inhibitor CrmA (Ray et al., 1992; Enari et al., 1995; Tewari and Dixit, 1995). The only components of the TNFR1 complex capable of stimulating JNK activity as efficiently as TNF were TRAF2 and RIP. Interestingly, TRADD, which activates apoptosis or NF-κB very efficiently (Hsu et al., 1996a; see below), had only a marginal effect on JNK activity (Figures 2A and 2C). None of the TNFR1 components was capable of stimulating ERK2 (for extracellular signal–regulated protein kinase) activity in MCF7 cells (Figure 2B). Similar results were obtained with all of the TNFR1 components in HeLa and 293 cells (data not shown). Both HeLa and MCF7 cells express TNFR1 and no or very low amounts of TNFR2 (Tartaglia et al., 1993b; H. H. and D. V. G., unpublished data). We also found that TRAF2 and RIP, but not the other proteins, were efficient p38 activators (data not shown).

We examined whether the death domain or the kinase domain of RIP are required for JNK activation. We found that RIP(1–558), which lacks the death domain and therefore is incapable of triggering apoptosis (Hsu et al., 1996b), was even more efficient than wild-type RIP in activating JNK (Figure 3). A kinase-defective RIP (K45A) mutant was somewhat less active but was still capable of JNK activation. The kinase-defective RIP was expressed as efficiently as wild-type RIP, while the truncated RIP(1–558) was expressed more efficiently. These results dissociate the apoptotic activity of RIP, which requires its death domain, from its ability to activate JNK.

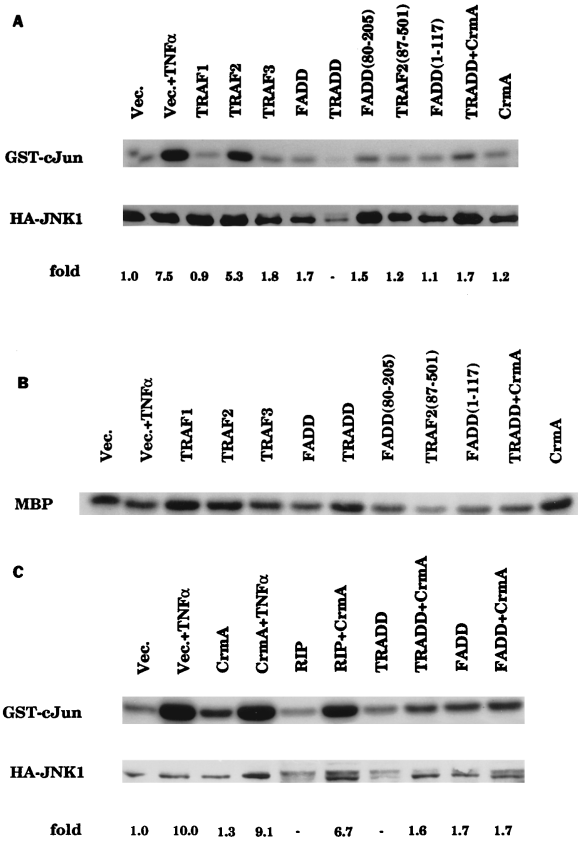


Figure 2. Activation of JNK by Components of the TNFR1 Signaling Complex

(A) MCF7 cells were cotransfected with an HA-JNK1 vector (0.5 μg per plate) and either an empty expression vector or expression vectors encoding the indicated components of the TNFR1 signaling complex, or fragments thereof (1 μg of each plasmid). When indicated, a CrmA expression vector (0.5 μg per plate) was also included. Total amount of DNA was kept constant (2.0 μg per plate) by using empty expression vector. After 24 hr some of the transfected cultures were treated with TNF (15 ng/ml) for 30 min, as indicated, and the rest left untreated. The cells were collected and lysed, and HA–JNK1 activity was determined by immunecomplex kinase assay with GST–c-Jun(1–79) as a substrate. The level of HA–JNK1 expression was determined by immunoblotting with a JNK1 monoclonal antibody. The fold increase in HA–JNK1 activity above the basal level in cells cotransfected with empty expression vector was determined by phosphoimaging and normalized to the level of HA–JNK1 expression. Similar results were obtained in at least three different experiments.

(B) MCF7 cells were transfected as described above except that an HA–ERK2 vector (0.5 μg) was used instead of the HA–JNK1 vector. ERK2 activity was determined by immunecomplex kinase assay with myelin basic protein (MBP) as a substrate.

(C) MCF7 cells were cotransfected as described in (A) with HA–JNK1 and either an empty expression vector (1.0 or 1.5 μg per plate) or RIP, TRADD, or FADD vectors (1.0 μg per plate). A CrmA vector (0.5 μg per plate) was included where indicated. After 24 hr, the cells were treated with TNF, when indicated, or left untreated. After 30 min, the cells were collected and HA–JNK1 activity and expression were determined as described above.

TNF-Induced JNK Activation Requires TRAF2 and RIP

To examine whether TRAF2 and RIP are required for JNK activation, we tested whether TRAF2(87–501), a mutant lacking the ring finger, or RIP(559–671), which consists only of the death domain, can prevent JNK activation by TNF. As both of these mutants are capable of binding to TRADD and inhibition of NF-κB activation, they probably compete for the endogenous components

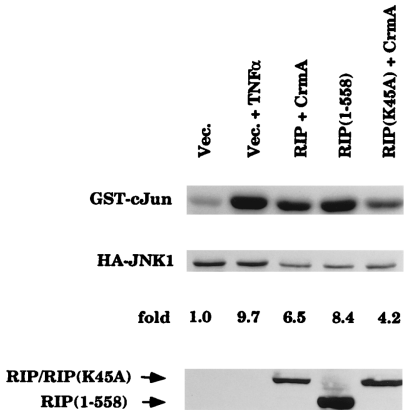


Figure 3. The Death Domain and the Kinase Activity of RIP Are Not Essential for JNK Activation

MCF7 cells were transfected with HA–JNK1 vector (0.5 μg per plate) and either empty expression vector or myc–RIP, myc–RIP(1–558), or myc–RIP(K45A) expression vectors (1 μg each). CrmA expression vector was included where indicated. After 24 hr, some of the cells were treated with TNF for 30 min and the rest left untreated. HA–JNK1 activity and expression were determined as described above. A sample of the transfected cell lysates was also used to determine the expression of RIP proteins by immunoblotting with Myc antibody (9E10, Pharmingen).

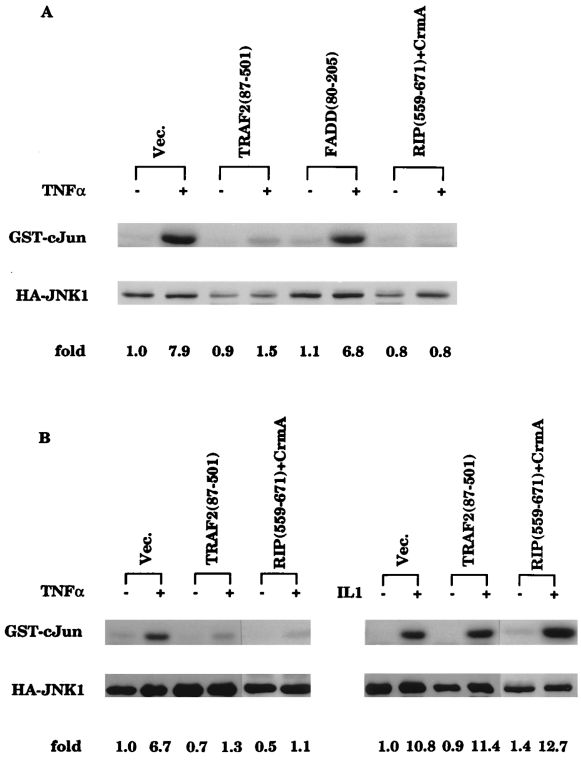


Figure 4. JNK Activation by TNF Is Blocked by the TRAF2(87–501) and RIP(559–671) Dominant Negative Mutants

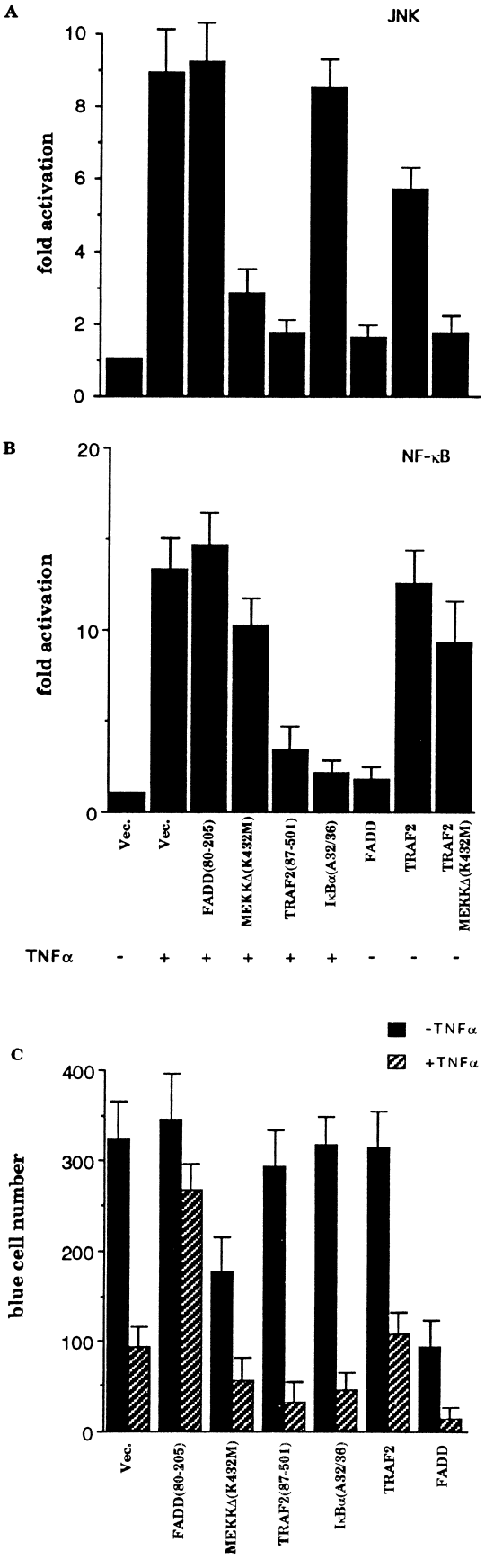
(A) MCF7 cells were cotransfected with HA–JNK1, TRAF3(87–501), FADD(80–205), RIP(559–671), and CrmA expression vectors, as indicated. After 24 hr, half of the transfected cultures were treated with TNF for 30 min and the other half left untreated. The cells were collected, and HA–JNK1 expression and activity were determined, as described in the legend to Figure 2.

(B) MCF7 cells were cotransfected with HA–JNK1 together with either empty expression vector, TRAF2(87–501), or RIP(559–671) expression vectors. A portion of the transfected cultures were treated with TNF (15 ng/ml) or IL-1 (4 ng/ml) and a portion left untreated. After 30 min, the cells were collected, and HA–JNK1 activity and expression were determined.

of the TNFR1 signaling complex (Hsu et al., 1996a, 1996b). We found that both TRAF2(87–501) and RIP(559–671) also inhibited JNK activation by TNF (Figure 4A). The RIP(559–671) mutant had to be coexpressed with CrmA because it is a potent inducer of apoptosis, thereby preventing the recovery of transiently expressed HA–JNK. We also found that a FADD(80–205) mutant, which completely blocks TNF-induced apoptosis (Chinnaiyan et al., 1996; Hsu et al., 1996a), had no effect on JNK activation (Figure 4A). These results, the ability of RIP(559–671) to induce apoptosis, and the inability of TRAF2(87–501) to block TNF-induced apoptosis (Hsu et al., 1996a, 1996b) strongly argue that the different dominant-negative mutants act specifically by replacing the endogenous wild-type proteins rather than by disrupting the entire TNFR1 signaling complex. Further evidence for the specificity of the inhibitory activity of these mutants is provided by the inability of TRAF2(87–501) or RIP(559–671) to inhibit JNK activation by interleukin-1 (IL-1) (Figure 4B).

Activation of JNK, NF-κB, and Apoptosis Are Separate Signaling Responses

We compared the effects of the different components of the TNFR1 complex as well as the other regulatory proteins (MEKK1 [for MAPK/ERK kinase kinase 1] and IκBα) on activation of JNK, NF-κB, and apoptosis. As shown in Figure 5, all three responses were activated by TNF in MCF7 cells. While TNF-induced cell death was blocked by transfection of FADD(80–205), this mutant did not inhibit either JNK or NF-κB activation. Both JNK and NF-κB activities were stimulated by TRAF2 expression in the absence of TNF, although TRAF2 by itself did not induce the apoptotic response. Correspondingly, both JNK and NF-κB activation by TNF were inhibited by TRAF2(87–501), which did not inhibit TNF-mediated apoptosis. Instead, TRAF2(87–501) enhanced the apoptotic response to TNF. Expression of FADD induced apoptosis even in the absence of TNF, but was insufficient for JNK or NF-κB activation. JNK activation by either TNF or TRAF2 was inhibited by coexpression of catalytically inactive MEKK1(K432M), which did not prevent NF-κB activation or TNF-induced apoptosis. The approximate 2-fold decrease in the number of β-galactosidase expressing cells in response to MEKK1 (K432M) expression was due to either a reduction in transfection efficiency or, more likely, a reduction in *lacZ* expression, but was not due to induction of apoptosis. In the absence of TNF, none of the *lacZr*- and MEKK1 (K432M)-cotransfected cells had an apoptotic morphology (data not shown, but see Figure 7). Induction of NF-κB activity was specifically blocked by coexpression of the phosphorylation-defective IκBα(A32/36) mutant (DiDonato et al., 1996), which had no effect on JNK activation (Figure 5). The dominant negative IκBα(A32/36) did not block the apoptotic response to TNF. On the



contrary, it seemed to enhance the induction of cell death. These results indicate that activation of JNK, NF-κB, and apoptosis are three separate responses to TNF.

NF-κB Activation Inhibits TNF-Induced Apoptosis

Similar results were obtained in HeLa cells (Figure 6). In HeLa cells, however, as in many other tumor cells, induction of apoptosis by TNF requires inhibition of ongoing protein synthesis (Beyaert and Fiers, 1994). Neither TNF nor cycloheximide alone induced considerable cell death, but when the were combined, an intensive apoptotic response was observed (Figure 6C). Cycloheximide and several other protein synthesis inhibitors can stimulate JNK activity (Kallunki et al., 1994; Kyriakis et al., 1994). Indeed, cycloheximide treatment resulted in modest JNK activation and potentiated its activation by TNF (Figure 6A). As cycloheximide was added simultaneously with TNF, we could not assess its effect on NF-κB activation, because it presumably prevented the translation of the newly induced luciferase mRNA produced by the NF-κB reporter (Figure 6B). However, NF-κB activation is independent of new protein synthesis (Auphan et al., 1995).

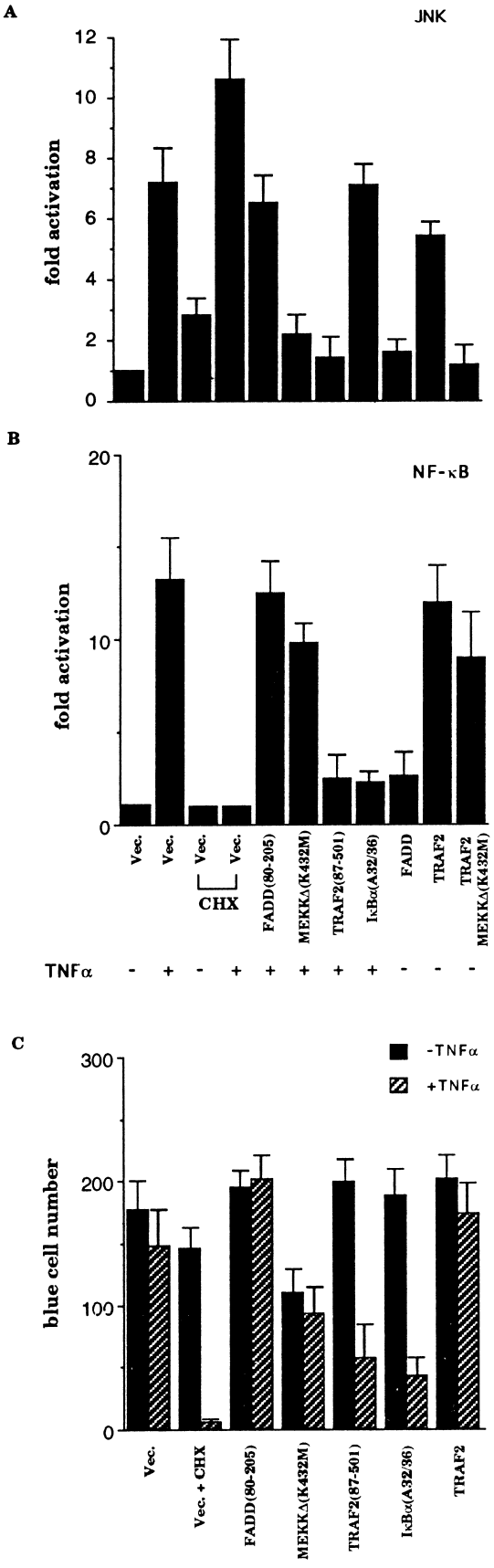
As described above, coexpression of either TRAF2 (87–501) or IκBα(A32/36) potentiated the apoptotic response to TNF in MCF7 cells. This effect was even more dramatic in HeLa cells, where both mutants enabled TNF to induce apoptosis in the absence of cycloheximide (Figure 6C). Some of the raw results of the β-galactosidase expression assay are shown in Figure 7A. Whereas cells cotransfected with pCMV*lacZ* and either TRAF2 (87–501) (shown in [e]) or IκBα(A32/36) (shown in [g]) had normal appearance when incubated in standard growth medium, the majority of these cotransfected cells assumed a characteristic apoptotic appearance, being round and condensed, when incubated in the

Figure 5. JNK and NF-κB Activation and Induction of Apoptosis by TNFα Are Regulated through Distinct Pathways in MCF7 Cells

(A) JNK activation. MCF7 cells were cotransfected with HA–JNK1 and either an empty expression vector or the different expression vectors indicated in the figure. Where indicated, the cells were treated with 15 ng/ml TNFα for 30 min at 24 hr, after transfection. HA–JNK1 activity and its expression level were determined as described above. The fold increase in JNK activity was normalized to its expression level. The data presented are averages of three individual experiments.

(B) NF-κB activation. MCF7 cells were cotransfected with 2xNF-κB LUC reporter (1 μg per plate), pCMV*lacZ* reporter (0.2 μg per plate), and either empty expression vector or the expression vectors indicated in the figure (1 μg each, total amount of DNA per plate 2.2 μg). Where indicated, cells were incubated for 12 hr with TNF at 12 hr posttransfection. Luciferase activity was measured and normalized to the level of β-galactosidase activity. The results shown are averages of three separate experiments done in duplicates.

(C) Induction of apoptosis. MCF7 cells were cotransfected with pCMV*lacZ* vector (0.5 μg per plate) and either empty expression vector or the different expression vectors indicated in the figures (1.5 μg per plate each). Half of the transfected cultures were treated with TNFα (15 ng/ml) at 12 hr after transfection and the other half left untreated. All the cells were fixed and stained at 24 hr after transfection. The number of blue cells in each transfection experiment was determined by counting five different randomly chosen fields. The results presented are averages of three individual transfection experiments.



presence of TNF (shown in [f] and [h]). Cells transfected with empty expression vector and treated with TNF alone (shown in [b]) did not exhibit this morphology. In addition to morphological changes, the apoptotic response was associated with a marked decrease in total β-galactosidase-positive cell number, because most of the rounded and condensed cells have detached from the plate. These results strongly suggest that NF-κB activation protects HeLa cells against TNF-induced apoptosis.

To examine whether deliberate NF-κB activation does indeed protect against TNF-induced apoptosis, we cotransfected HeLa cells with the pCMV*lacZ* reporter and expression vectors for the NF-κB subunits p50, p65 (RelA), or c-Rel. After 24 hr, the cells were incubated with cycloheximide with or without TNF for 12 hr, and then the number of blue cells and their morphology were determined. As shown in Figure 7B, transfection of the c-Rel expression vector resulted in almost complete inhibition of TNF-induced apoptosis, while p65 expression resulted only in partial protection. Expression of p50 did not produce a considerable protective effect. In reporter activation assays, the c-Rel and p65 expression vectors led to similar degrees of transcriptional activation (17- and 13-fold, respectively), whereas by itself, the p50 vector did not produce considerable activation (data not shown). Microscopic examination of the cells clearly indicated that the c-Rel- and *lacZ*-cotransfected cells were resistant to TNF-induced apoptosis, while the surrounding nontransfected cells were all dead (Figure 7C).

Discussion

JNK Activation Is Not Involved in TNF-Induced Apoptosis

Two different mechanisms have been proposed to explain how occupancy of TNFR1 and Fas results in apoptosis and other downstream responses. TNFR1 and Fas are unique among the TNF receptor family in

Figure 6. Activation of JNK, NF-κB, and Apoptosis by TNF Is Mediated by Separate Signaling Pathways in HeLa Cells

(A) JNK activation. HeLa cells were cotransfected with HA–JNK1 and the different expression vectors, as described above for MCF7 cells. When indicated, the cells were treated with TNF (15 ng/ml), cycloheximide (CHX, 10 μg/ml), or a combination of the two for 30 min at 24 hr posttransfection. HA–JNK1 expression and activity were determined as described above. The results are averages of three separate experiments.

(B) NF-κB activation. HeLa cells were cotransfected with 2xNF-κB-LUC reporter and the different expression vectors indicated in the figure. After 12 hr, some of the cultures were incubated for an additional 12 hr with TNF or cycloheximide, as indicated, before collection and determination of luciferase activity. The results shown are averages of three separate experiments done in duplicate.

(C) Induction of apoptosis. HeLa cells were cotransfected with pCMV*lacZ* reporter and the different expression vectors indicated in the figure. After an additional 12 hr, half of the cultures were treated with TNF, while the other half were left untreated. Where indicated, cycloheximide was added also at 12 hr posttransfection. At 24 hr posttransfection, the cells were stained with X-Gal, and the numbers of blue cells in five randomly chosen fields were determined. The data presented represent averages of three separate transfection experiments.

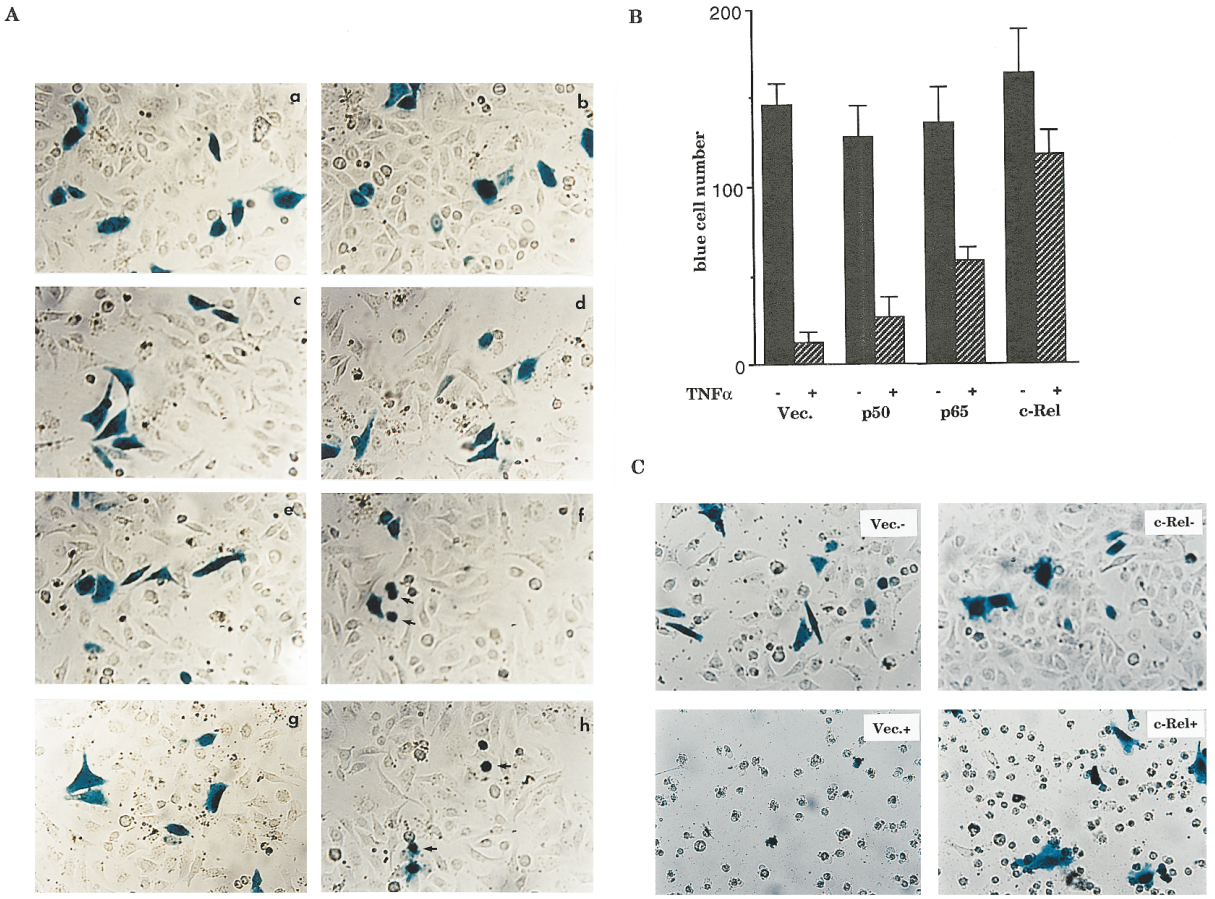


Figure 7. Activation of NF-κB Protects against TNF-Induced Apoptosis

(A) Inhibition of NF-κB sensitizes HeLa cells to TNF. HeLa cells were transfected with pCMV*lacZ* reporter and either empty (a and b), MEKK1(K432M) (c and d), TRAF2 (87–501) (e and f) or IκBα(A32/36) (g and h) expression vectors. After 12 hr, half of the cultures were treated with TNFα (15 ng/ml) (b, d, f, and h), and the other half were left untreated (a, c, e, and g). After 12 more hr, the cells were fixed and stained with X-Gal. The arrows indicate cells with apoptotic morphology. These results are representative of three separate experiments summarized above.

(B) Deliberate NF-κB activation protects against TNF-induced apoptosis. HeLa cells were transfected with pCMV*lacZ* and either an empty expression vector or expression vectors for the p50, p65, or c-Rel subunits of NF-κB (1 μg of DNA per plate). After 24 hr, the cells were incubated with cycloheximide (10 μg/ml) in the absence or presence of TNFα (15 ng/ml). After 12 hr, the cells were stained with X-Gal and counted. The number of blue cells in five randomly chosen fields was determined, and the data shown are averages of three separate experiments.

(C) Results of a representative NF-κB protection experiment. HeLa cells were transfected with either an empty or a c-Rel expression vector, incubated with cycloheximide (10 μg/ml) in the absence (−) or presence (+) of TNFα (15 ng/ml), and stained with X-Gal. While all of the β-galactosidase-expressing cells transfected with empty expression vector are apoptotic, most of the β-galactosidase expressing cells cotransfected with c-Rel expression vector are viable, but the surrounding cells are dead.

having cytoplasmic death domains (Tartaglia et al., 1993a; Itoh and Nagata, 1993). Correspondingly, most other family members function in costimulation of immune responses rather than induction of apoptosis (Smith et al., 1994). Some of them, such as CD40, even enhance cell survival and protect against apoptosis (Liu et al., 1989; Tsubata et al., 1993). Being protein–protein interaction domains, the death domains of TNFR1 and Fas recruit the other death domain–containing proteins TRADD, FADD, and RIP to the trimerized receptors (Hsu et al., 1995, 1996a, 1996b; Chinnaiyan et al., 1995; Boldin et al., 1995; Stanger et al., 1995). In addition to its death domain, FADD has a death-effector N-terminal domain, which recruits the ICE-like protease MACH/FLICE to activated receptor complexes (Boldin et al., 1996; Muzio et al., 1996). This appears to be the critical step in activation of the proapoptotic protease cascade (Chinnaiyan et al., 1995). We refer to this mechanism as the protein-recruitment model.

Other mechanisms proposed to explain TNFR- and Fas-mediated signaling are based on second messenger production (reviewed by Beyaert and Fiers, 1994; Nagata and Golstein, 1995). Currently the most favored candidate for such a role is ceramide (Kolesnick and Golde, 1994). Occupancy of either TNFR1 or Fas was reported to result in activation of sphingomyelinases, phospholipases that act on sphingomyelin to release the lipid mediator ceramide (Weigmann et al., 1994; Gulbins et al., 1995). When applied exogenously, ceramide can cause apoptosis (Obeid et al., 1993) and NF-κB

(Wiegmann et al., 1994) and JNK (Verheij et al., 1996) activation by yet-to-be-identified mechanisms. JNK activation was, in fact, proposed to mediate apoptosis in response to either TNF or exogenous ceramide via a c-Jun dependent mechanism (Verheij et al., 1996). However, c-Jun is a nuclear protein and a transcription factor (Angel and Karin, 1991), while TNF and Fas ligand induce apoptosis without new protein or RNA synthesis and can do so even in the absence of a nucleus (Nagata and Golstein, 1995). It is therefore difficult to understand how c-Jun induction and phosphorylation can mediate apoptosis by these factors. To solve this problem, we investigated which components of the TNFR1 signaling complex lead to JNK activation.

The only two components of the TNFR1 complex whose transient expression results in JNK activation are TRAF2 and RIP (Figure 2). While TRAF2 does not mediate apoptosis (Hsu et al., 1996a; Figures 5 and 6), RIP does (Stanger et al., 1995; Hsu et al., 1996b). However, a RIP deletion mutant that lacks the death domain and therefore is incapable of inducing apoptosis (Hsu et al., 1996b) is fully competent in activation JNK (Figure 3). Furthermore, expression of the RIP death domain (RIP(559–641)), which is sufficient for induction of apoptosis (Stanger et al., 1995; Hsu et al., 1996b), does not activate JNK. Rather, RIP(559–671) blocks JNK activation by TNF, but not by the nonrelated cytokine IL-1 (Figure 4). Thus, although RIP is capable of both JNK activation and induction of apoptosis, the two responses can be separated and involve different functional domains of this protein. Further evidence that JNK activation and induction of apoptosis are two separate downstream responses to TNF is provided by the findings that the FADD(80–205) death domain blocked TNF-induced apoptosis but not JNK activation, while wild-type FADD induced apoptosis but not JNK activity; that the catalytically inactive MEKK1(K432M) mutant blocked JNK activation but not apoptosis; and that TRAF2(87–501) inhibited JNK activation while potentiating the apoptotic response (Figures 5 and 6).

In addition to ruling out the involvement of JNK in TNF-induced apoptosis, these results are consistent with other observations. Like TNFR1, ligation of CD40 causes robust JNK activation (Berberich et al., 1996), but unlike TNFR1, CD40 is not a direct mediator of apoptosis, and its activation prevents apoptosis in B cells (Smith et al., 1994; Liu et al., 1989; Tsubata et al., 1993). Another potent JNK activator is IL-1, which, like TNF, is also a poor activator of the ERK group of MAPKs (Raingeaud et al., 1995). Unlike TNF or Fas ligand, IL-1 does not induce apoptosis (Tracey and Cerami, 1993). On the other hand, Fas ligation, which is even more effective than TNFR1 in inducing apoptosis (Nagata and Golstein, 1995), leads to modest and slow JNK activation, which appears to be secondary to activation of the apoptotic protease cascade (Lenczowski et al., 1996).

Our findings, however, are inconsistent with the proposal that JNK activation and c-Jun are critical mediators of apoptosis in response to TNF (Verheij et al., 1996; Cuvillier et al., 1996). These proposals are based on the ability of ceramide to activate JNK and catalytically inactive JNKK1 (SEK1) or N-terminally truncated c-Jun to block apoptosis (Verheij et al., 1996). As mentioned above, c-Jun is a nuclear protein, while induction of apoptosis by TNF does not depend on nuclear events. Therefore, the protection conferred by the N-terminally truncated c-Jun is likely to be mediated through a nonspecific side effect. Curiously, the N-terminally truncated c-Jun also protected cells against a very high dose of UV-C (Verheij et al., 1996), which is capable of reducing cell viability by 2–3 orders of magnitude (unpublished data). By contrast, previous work using both HeLa cells (Devary et al., 1992) and yeast (Engelberg et al., 1994), suggested that the eukaryotic UV response involving AP-1 activation is a protective response. This hypothesis is strongly supported by recent results obtained using c-*jun*-null mouse fibroblasts (F. Piu and M. K., unpublished data; E. Wagner, personal communication). It is likely that the correlation between ceramide production, JNK activation, and apoptosis is caused by a secondary and delayed effect of TNFR1 or Fas activation. For example, the cell death process probably results in activation of sphingomyelinases and other phospholipases that mediate degradation of membranes. Indeed, it was recently shown that inhibitors of ICE proteases can block the slow and modest JNK activation caused by Fas ligation (Lenczowski et al., 1996). In addition, inhibition of ICE-like proteases was shown to block ceramide production by Drosophila melanogaster tissue culture cells expressing the death domain protein REAPER (Pronk et al., 1996).

The Relationship between JNK and NF-κB

Both JNK and p38 are activated by a spectrum of stimuli similar to those that cause NF-κB activation (Raingeaud et al., 1995; Verma et al., 1995). It was reported that transient MEKK1 expression can lead to NF-κB activation and that JNK can associate with c-Rel (Meyer et al., 1996). Our results, however, show that although there are certain similarities between NF-κB and JNK activation by TNF, these two responses are distinct. Both NF-κB (Hsu et al., 1996a; Rothe et al., 1995b) and JNK (Figure 2) are activated by transiently expressed TRAF2 or RIP. In addition, their activation by TNF is blocked by expression of TRAF2(87–501) or by the RIP death domain (residues 559–771). However, NF-κB activity is potently stimulated by transient TRADD expression (in the presence of CrmA; Hsu et al., 1995), which has only a marginal effect on JNK activity (Figure 2). Furthermore, TNF-mediated JNK activation is blocked by MEKK1 (K432M), which has no effect on NF-κB activation. Thus, the NF-κB and JNK pathways are two distinct effectors of TNF signaling. As JNK and NF-κB activation are both mediated by RIP and TRAF2, it is not obvious which of these two signaling proteins is the most downstream mediator. However, on the basis of the ability of CD40 to elicit potent NF-κB (Rothe et al., 1995b) and JNK (Berberich et al., 1996) activation, it is likely that TRAF2 is the more downstream and critical mediator of JNK and NF-κB activation (Figure 8). As CD40 lacks a death domain and is unable to trigger apoptosis (Smith et al., 1994), the CD40 signaling complex is unlikely to include proteins such as RIP.

The Relationship between NF-κB and Apoptosis

Induction of apoptosis by TNF is independent of new protein synthesis. On the contrary, protein synthesis

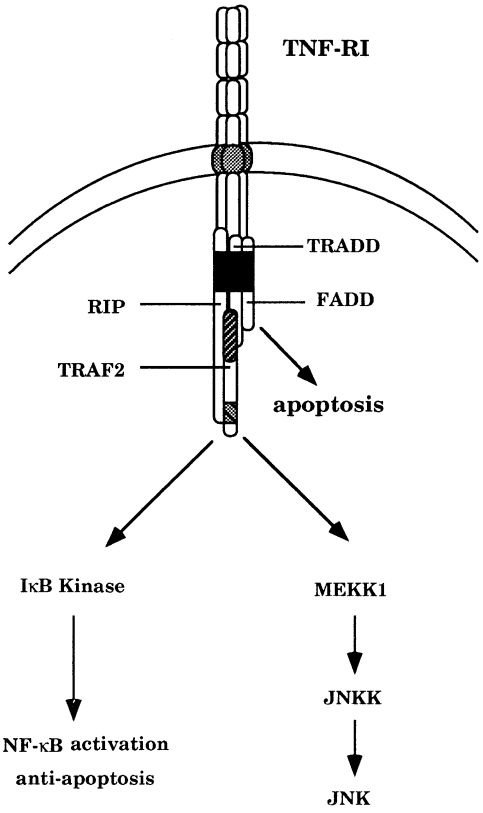


Figure 8. Summary of TNFR1-Mediated Signaling Pathways

TNF-induced JNK or NF-κB activation is mediated by TRAF2 and RIP, while FADD induces apoptosis. The activation of NF-κB via IκB kinase protects cells from TNF-induced apoptosis. JNK activation procedes via MEKK.

inhibitors confer sensitivity to TNF in most types of TNF-resistant tumor cells (Beyaert and Fiers, 1994). Such observations raised the possibility that in resistant cell types, TNF may induce synthesis of antiapoptotic proteins. Our results shed light on the mechanism by which TNF may induce such apoptosis inhibitors. We observed that in HeLa cells, where TNF cannot induce apoptosis by itself, the apoptotic response can be activated when either dominant-negative TRAF2(87–501) or IκBα(A32/36) is expressed together with TNF treatment. While TRAF2(87- 501) inhibits both JNK and NF-κB activation, IκBα(A32/36) is a specific inhibitor of NF-κB. However, an inhibitor of JNK activation, MEKK1(K432M), which has no effect on NF-κB, does not enhance the apoptotic response. Furthermore, expression of the NF-κB component c-Rel and, to a lesser extent, expression of p65(RelA) protect HeLa cells against TNF-induced apoptosis (in the presence of cycloheximide). We therefore conclude that induction of antiapoptosis genes by TNF is most likely mediated by NF-κB (Figure 8). While the NF-κB-activated antiapoptotic genes remain to be identified, there are two likely candidates for such a role: manganese superoxide dismutase (Wong and Goeddel, 1988) and the zinc finger protein A20 (Opipari et al., 1992). Expression of the genes encoding these proteins is TNF inducible, and constitutive expression of each of them provides partial protection against apoptosis (Wong et al., 1989; Opipari et al., 1992).

These results are consistent with the demonstrated ability of IL-1, another potent NF-κB activator, to protect against TNF-induced apoptosis (Holtmann and Wallach, 1987). In addition, knockout mouse embryos deficient in the p65(RelA) subunit of NF-κB exhibit increased apoptosis in the liver (Beg et al., 1995). It will therefore be of interest to determine whether inhibition of NF-κB activation can confer sensitivity to TNF-induced apoptosis in normal, nontransformed cells. These results may also explain why Fas activation, which unlike that of TNFR1 does not lead to NF-κB activation, results in a more efficient and rapid apoptotic response than the one usually caused by TNFR1 activation (Nagata and Golstein, 1995).

Conclusions

The results described above indicate that the TNFR1 signaling complex leads to activation of at least three distinct effector functions, JNK and NF-κB activation and induction of apoptosis (Figure 8). This multiplicity of effector functions can explain why TNFR1 can transduce an unusually large number of diverse biological responses, while other members of the TNF reporter family elicit only subsets of these responses and even antagonize some of the TNFR1-mediated responses (Smith et al., 1994). While TNF-induced apoptosis is most likely mediated via FADD-induced MACH/FLICE recruitment and activation of the proapoptotic protease cascade (Boldin et al., 1996; Muzio et al., 1996), JNK and NF-κB activation are mediated via TRAF2 and RIP recruitment. Despite the reliance on common mediators, JNK and NF-κB activation are two separate responses. JNK activation is probably the critical step mediating induction of AP-1 activity by TNF (Brenner et al., 1989; Karin, 1995). Together with NF-κB, AP-1 is likely to mediate induction of other cytokines and immunoregulatory molecules by TNF, leading to a variety of inflammatory responses. Interestingly, both AP-1 and NF-κB activities are inhibited by glucocorticoids, which are potent anti-inflammatory agents (Auphan et al., 1995). As TRAF2 is also recruited to TNFR2 and CD40 (Rothe et al., 1994, 1995b), it is not surprising that these receptors also lead to efficient activation of NF-κB, JNK, and AP-1-mediated responses. On the other hand, these receptors do not recruit FADD or other death domain proteins and therefore are unable to affect apoptosis as TNFR1 and Fas do. By contrast, a receptor such as Fas, which recruits FADD but not TRAF2, is unable to cause direct and efficient JNK or NF-κB activation and therefore unable to elicit inflammatory responses. As NF-κB activation can protect cells against apoptosis, the inability of Fas to elicit this response can explain why it is a more effective apoptosis inducer than TNFR1. On the other hand, receptors such as CD40 that activate NF-κB but are unable to recruit death domain proteins can protect cells against apoptosis.

Experimental Procedures

Cell Culture and Cytokines

Human breast carcinoma MCF7 cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 1 mM glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Chinnaiyan et al., 1995). HeLa and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS,

1 mM glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human recombinant TNFα and IL-1α were purchased from R & D Systems.

Expression Vectors and Transfections

pSRα-HA-JNK1, pSRα-HA-ERK2, pSRα-MEKK(K432M), pSRα-IκBα(A32/36), and p2xNF-κB-LUC have been described (Dérijard et al., 1994; Minden et al., 1994; DiDonato et al., 1995). Expression vectors for TRAF1, TRAF2, TRAF3, FADD, TRADD, TRAF2(87–501), FADD(80–205), RIP, RIP(K45A), RIP(1–558), RIP(559–671), FADD(1–117), and CrmA have also been described (Hsu et al., 1995, 1996a, 1996b; Chinnaiyan et al., 1995). Expression vectors for p50, p65, and c-Rel have been described (Kieran et al., 1990; Ruben et al., 1992; Mercurio et al., 1993). For transfections, 2×10^5 cells in 35-mm dishes were transfected with 1.5–2.2 μg of DNA, by using lipofectamine (GIBCO BRL) as described (Minden et al., 1994). Luciferase activity was determined and normalized relative to β-galactosidase expression as described (Hsu et al., 1995).

Immunoblotting

Cell lysates (30 μg of protein) were resolved by 10% SDS–PAGE and transferred onto Immobilon P membrane (Millipore). After blocking with 5% milk in PBS-T (PBS with 0.05% Tween 20) for 1 hr, the membranes were probed with monoclonal antibodies anti-JNK1 333.8 (Pharmingen) or anti-c-MYC 9E10 (Pharmingen) and visualized with enhanced chemiluminescence detection (Amersham International) as described (Minden et al., 1994).

Kinase Assays

Transfected cells were collected in 300 μl of M_2 lysis buffer 24 hr after transfection as described (Minden et al., 1994). HA–JNK1 or HA–ERK2 was immunoprecipitated with HA antibody, and their kinase activities were determined by using 2 μg of GST–cJun(1–79) or MBP as substrates (Dérijard et al., 1994). Fold activation of HA–JNK1 was determined by phosphoimaging and normalized to its expression level.

Apoptosis Assays

Cells were cotransfected with a pCMV*lacZ* reporter plasmid and different expression vectors and stained with X-Gal 24 hr or 36 hr after transfection as described (Hsu et al., 1995). The number of blue cells per plate was determined by counting five different randomly chosen fields.

Acknowledgments

Correspondence should be addressed to M. K. We thank Dr. V. M. Dixit for the MCF7 cell line and FADD plasmids, and Dr. J. DiDonato for the NF-κB expression vectors and advice. We thank Jianing Huang for providing RIP constructs. We also thank Ms. P. Alford for her excellent assistance in manuscript preparation. Z.-g. L. is supported by a postdoctoral fellowship from the Breast Cancer Research Program, University of California. This work was supported by grants from the National Institutes of Health and the Breast Cancer Research Program.

Received August 22, 1996; revised September 20, 1996.

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Note Added in Proof

After this paper was accepted for publication, we found that Wu et al. reported that inhibition of NF-κB/Rel activity can induce spontaneous apoptosis of B cells (Wu et al. [1996] EMBO J., *15*, 4682–4690.