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The Distinct Roles of TRAF2 and RIP in IKK Activation by TNF-R1: TRAF2 Recruits IKK to TNF-R1 while RIP Mediates IKK Activation

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Summary

The death domain kinase RIP and the TNF receptor-associated factor 2 (TRAF2) are essential effectors in TNF signaling. To understand the mechanism by which RIP and TRAF2 regulate TNF-induced activation of the transcription factor NF-κB, we investigated their respective roles in TNF-R1-mediated IKK activation using both RIP$^{-/-}$ and TRAF2$^{-/-}$ fibroblasts. We found that TNF-R1-mediated IKK activation requires both RIP and TRAF2 proteins. Although TRAF2 or RIP can be independently recruited to the TNF-R1 complex, neither one of them alone is capable of transducing the TNF signal that leads to IKK activation. Moreover, we demonstrated that IKK is recruited to the TNF-R1 complex through TRAF2 upon TNF treatment and that IKK activation requires the presence of RIP in the same complex.

Introduction

The proinflammatory cytokine tumor necrosis factor (TNF) plays an important role in diverse cellular events such as septic shock, induction of other cytokines, cell proliferation, differentiation, and apoptosis (Tartaglia and Goeddel, 1992; Rothe et al., 1992; Tracey and Cerami, 1993). Many of these TNF-induced processes can be mediated by either one of the two TNF receptors, TNF-R1 and TNF-R2, both of which belong to the TNF receptor superfamily (Smith et al., 1994; Nagata and Golstein, 1995). In response to TNF treatment, the transcription factor NF-κB and c-Jun N-terminal kinase (JNK) are activated in most types of cells and, in some cases, apoptosis can also be induced (Brenner et al., 1989; Dérijard et al., 1994). However, induction of apoptosis is achieved mainly through TNF-R1, which is also known as a death receptor (Vandenabeele et al., 1995; Nagata, 1997; Ashkenazi and Dixit, 1998). Activation of the NF-κB and JNK pathways plays an important role in the induction of many cytokines and immunoregulatory proteins and is pivotal for many inflammatory responses (Siebenlist et al., 1994; Baeuerle and Baltimore, 1996; Karin et al., 1997).

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The molecular mechanisms that regulate TNF-mediated responses have been intensively studied in recent years. For TNF-R1 signaling, it is known that the binding of TNF to TNF-R1 leads to the recruitment of TRADD (TNF-R1-associated death domain protein) into the receptor complex (Hsu et al., 1995). TRADD subsequently recruits other effector proteins into the complex. FADD/MORT1 (FAS-associated death domain protein), TRAF2 (TNFR-associated factor 2), and the death domain kinase RIP (receptor interacting protein) not only are recruited but also have been shown to interact directly with TRADD (Rothe et al., 1994, 1995; Boldin et al., 1995; Chinnaiyan et al., 1995; Hsu et al., 1995, 1996a, 1996b; Stanger et al., 1995). While FADD/MORT1 is essential for TNF-induced apoptosis, RIP and TRAF2 seem to be involved in both NF-κB and JNK activation (Liu et al., 1996; Ting et al., 1996; Lee, S.Y. et al., 1997; Natoli et al., 1997; Reinhard et al., 1997; Yeh et al., 1997; Kelliher et al., 1998; Zhang et al., 1998). There is also cross-regulation between these distinct pathways. For instance, RIP is cleaved by Caspase-8 during apoptosis and this cleavage plays a role in regulating the balance between life and death in response to TNF (Lin et al., 1999). On the other hand, the occupancy of TNF-R2 by TNF leads to the recruitment of TRAF1 and TRAF2 as well as cIAP1 and cIAP2 (Rothe et al., 1994, 1995). However, it is less clear how these molecules correlate to transduce the diverse TNF signals through TNF-R2. Recently, the indispensable role of RIP in TNF-induced NF-κB activation was suggested by generating RIP-deficient Jurkat cells and RIP$^{-/-}$ mice (Ting et al., 1996; Kelliher et al., 1998). In contrast, the study with TRAF2$^{-/-}$ cells indicated that TRAF2 is essential for TNF-induced JNK activation, and TNF-induced NF-κB activation is just delayed and slightly reduced in those cells (Yeh et al., 1997).

Downstream to these effector molecules, the activation of NF-κB and JNK in response to TNF is regulated by distinct pathways. While NF-κB is activated through IKK and MAP3K, NIK, or MEKK1 (Chen et al., 1996; Lee, S.Y. et al., 1997; Malinin et al., 1997), JNK activity is regulated by JNKK1/MKK4 and MEKK1 (Dérijard et al., 1995; Karin, 1995; Lin et al., 1995; Liu et al., 1996; Natoli et al., 1997). Inactive NF-κB is sequestered in the cytoplasm through its interaction with the inhibitory proteins, known as IκBs (Siebenlist et al., 1994; Baeuerle and Baltimore, 1996). In response to various stimuli, IκBs are phosphorylated by IKK at serines 32 and 36 and are rapidly degraded by the proteasome after polyubiquitination. The degradation of IκBs results in the release of NF-κB and allows its translocation into the nucleus and the subsequent activation of its target genes (Baeuerle and Baltimore, 1996). IKK is composed of three subunits: IKKα, IKKβ, and IKKγ/NEMO (DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997; Rothwarf et al., 1998; Yamaoka et al., 1998; Hu et al., 1999; Li et al., 1999). Both IKKα and IKKβ are catalytic subunits while IKKγ is a regulatory subunit.

In order to understand the mechanism of TNF-induced

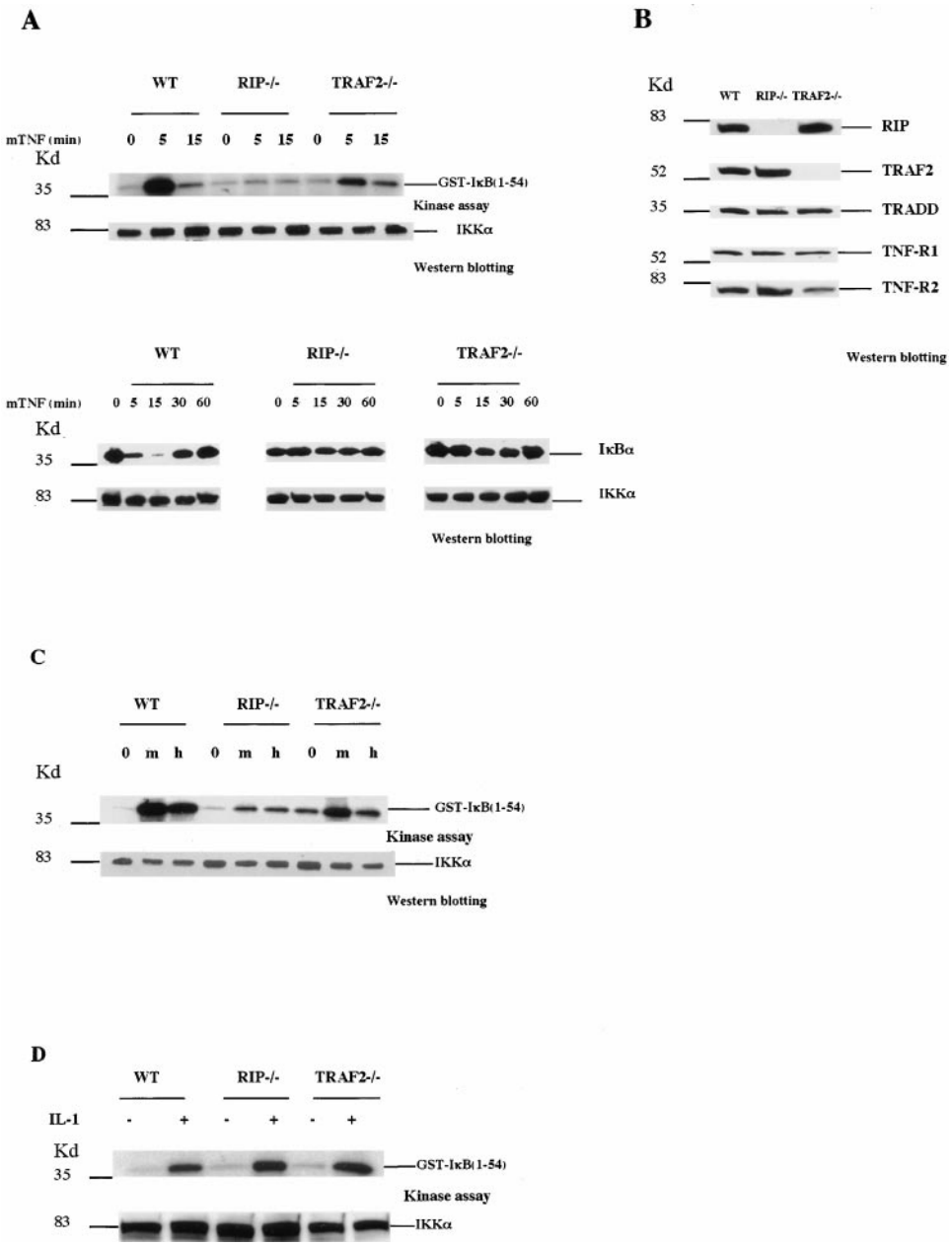

Figure 1. TNF-R1-Mediated IKK Activation Requires both TRAF2 and RIP

(A) Mouse TNF-induced IKK activation in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts. Mouse fibroblasts were treated with mouse TNF (40 ng/ml) for various times as indicated on the figure or left untreated as a control. Cell extracts were either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IκB or anti-IKKα.

(B) Protein expression levels of RIP, TRAF2, TRADD, TNF-R1, and TNF-R2 in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ cells. The same amount of cell extract from each cell line was applied to SDS-PAGE for Western blottings with anti-TRAF2, anti-RIP, anti-TRADD, anti-TNF-R1, and anti-TNF-R2 antibodies.

(C) Human TNF-induced IKK activation in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts. Wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts were treated with mouse TNF (40 ng/ml) or with human TNF (40 ng/ml) for 5 min. Nontreated cells were used as controls. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα antibody.

(D) IL-1-induced IKK activation. Wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts were either left untreated or treated with IL-1 (4 ng/ml) for 5 min for IKK kinase assay. Cell extracts were normalized according to protein assay and then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα antibody.

IKK activation, we investigated the role of RIP and TRAF2 in TNF-R1-mediated IKK activation in wild-type, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblast cells. We found that both RIP and TRAF2 are required for IKK activation through TNF-R1. Although RIP or TRAF2 can be recruited to the TNF-R1 complex independently of one

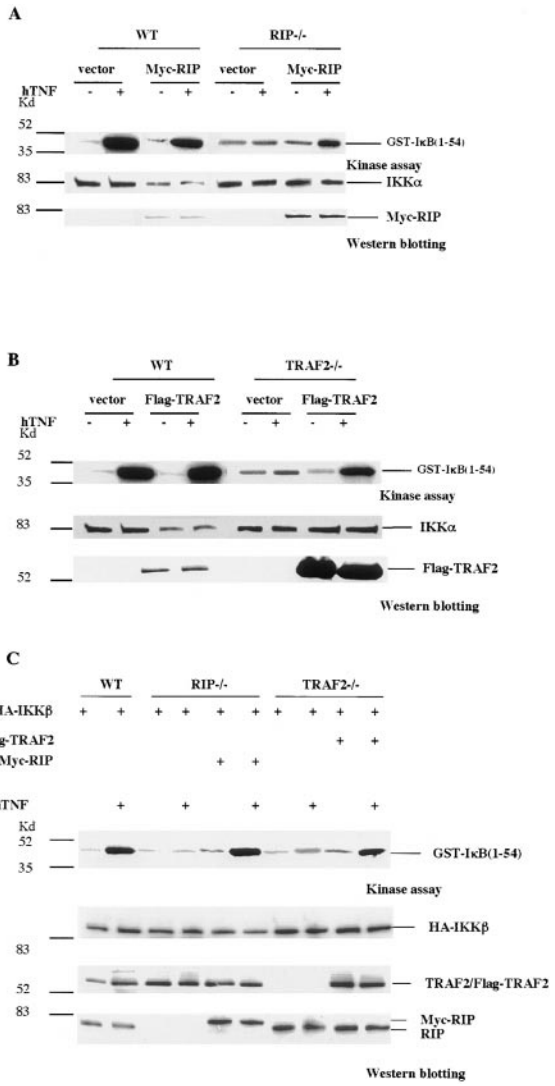

Figure 2. Reconstitution of TNF-Induced IKK Activation in RIP$^{-/-}$ and TRAF2$^{-/-}$ Fibroblasts

(A) Reconstitution of TNF-induced IKK activation in RIP$^{-/-}$ fibroblasts. Wt or RIP$^{-/-}$ cells were transfected with 2 μg of either the Myc-RIP expression plasmid or an empty vector in 100 mm dishes. Twenty-four hours after transfection, half of the transfected cells were treated with 40 ng human TNF for 5 min. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα or anti-Myc antibody.

(B) Reconstitution of TNF-induced IKK activation in TRAF2$^{-/-}$ fibroblasts. Wt or TRAF2$^{-/-}$ cells were transfected with 2 μg of either the Flag-TRAF2 expression plasmid or an empty vector in 100 mm dishes. Twenty-four hours after transfection, half of the transfected cells were treated with 40 ng human TNF for 5 min. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα or anti-Flag.

(C) Reconstitution of TNF-induced IKK activation in RIP$^{-/-}$ and TRAF2$^{-/-}$ fibroblasts by transient cotransfection. Wt, RIP$^{-/-}$, or TRAF2$^{-/-}$ cells were transfected with 1 μg of the HA-IKKβ expression plasmid and 0.5 μg of either the Flag-TRAF2 expression plasmid, the Myc-RIP expression plasmid or an empty vector in 60 mm dishes. Twenty-four hours after transfection, half of the transfected cells were treated with 40 ng mouse TNF for 5 min. HA-IKKβ content was quantified by Western blotting with anti-HA antibody, and cell extracts were then immunoprecipitated with anti-HA antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-RIP or anti-TRAF2 antibody.

another, the recruitment of either one of them alone is not sufficient to activate IKK. Importantly, we demonstrated that IKK is recruited to the TNF-R1 complex in response to TNF treatment and that TRAF2 plays an essential role in IKK recruitment. Furthermore, RIP, which is not necessary for IKK recruitment, mediates IKK activation.

Results

Both RIP and TRAF2 Are Required for TNF-R1-Mediated IKK Activation

Previous studies have shown that TNF-induced NF-κB activation is abolished in RIP-deficient Jurkat cells and RIP$^{-/-}$ cells (Ting et al., 1996; Kelliher et al., 1998) and is just delayed and slightly reduced in TRAF2$^{-/-}$ cells (Yeh et al., 1997). Because IKK activation is essential for TNF-induced NF-κB activation, we measured IKK activation following TNF treatment in wild-type (wt), RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblast cell lines by performing an in vitro kinase assay with GST-IκB (1-54) as a substrate (DiDonato et al., 1997). In these experiments, mouse TNFα (mTNFα) was used to treat the cells because it binds to both TNF-R1 and TNF-R2 (Lewis et al., 1991). As shown in Figure 1A, top panel, IKK is quickly activated in wt fibroblast cells in response to TNF treatment and its activation is decreased after 15 min of treatment. In contrast, IKK activation is almost completely abolished in RIP$^{-/-}$ cells and dramatically reduced in TRAF2$^{-/-}$ cells, although the IKK expression levels in these two cell lines are similar to that of wt cells (Figure 1A). As IKK is responsible for IκB phosphorylation, which is a crucial step for TNF-induced IκB degradation, we tested IκB degradation following TNF treatment in these cells by Western blotting. Consistent with the levels of IKK activation in those cell lines, TNF-induced IκB degradation is also altered: while IκB is rapidly degraded in wt cells after TNF treatment, no or only partial IκB degradation was detected in RIP$^{-/-}$ and TRAF2$^{-/-}$ cells, respectively (Figure 1A, bottom panel). Since IκB expression is regulated by NF-κB (Baeuerle and Baltimore, 1996) and the reduced IκB level following TNF engagement returned to the one of nontreated cells by 60 min after treatment (Figure 1A, bottom panel), the partial IκB degradation seemed to be sufficient to activate NF-κB in TRAF2$^{-/-}$ cells. IKK protein levels were also determined by Western blotting in these experiments as a control. The protein expression levels of RIP, TRAF2, TRADD, TNF-R1, and TNF-R2 in wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ cells were measured by Western blotting. As shown in Figure 1B, the expression level of each protein, when present, is similar in each of the three cell lines.

Mouse TNF binds to both TNF-R1 and TNF-R2, and these two receptors elicit certain overlapping responses. In order to investigate the requirement of RIP and TRAF2 in TNF-R1-mediated IKK activation, IKK activation was

determined in these fibroblast cell lines following human TNFα (hTNFα) treatment, which only binds to TNF-R1 (Lewis et al., 1991). As shown in Figure 1C, IKK activation is slightly decreased in wt cells treated with hTNFα when compared to mTNFα treatment, indicating a minor contribution of TNF-R2 in IKK activation. A similar observation was also made with TRAF2$^{-/-}$ cells although the mTNF-induced IKK activation is already impaired. As shown in Figure 1C, IKK activation is barely detected in TRAF2$^{-/-}$ cells after hTNF treatment. In RIP$^{-/-}$ cells, no difference in the marginal IKK activation was detected after either mTNF or hTNF treatment (Figure 1C). Moreover, IKK activation in response to IL-1 treatment is normal in both RIP$^{-/-}$ and TRAF2$^{-/-}$ cells compared to wt cells, as shown in Figure 1D, which indicates that this protein is activated normally in response to other stimuli in those cells. Taken together, these results suggested that both RIP and TRAF2 are essential for TNF-induced IKK activation via TNF-R1.

To rule out the possibility that some defects in the TNF-R1-mediated IKK activation pathway are present in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells, we tested whether TNF-R1-mediated IKK activation could be reconstituted in these cells. To do so, we ectopically expressed Myc-RIP and Flag-TRAF2 in RIP$^{-/-}$ and TRAF2$^{-/-}$ cells, respectively, and treated the transfected cells with hTNF. Transfected wt cells were used as a control. As shown in Figure 2A, top panel, RIP expression is able to restore the IKK activation in response to hTNF treatment in RIP$^{-/-}$ cells. The expression levels of IKK and Myc-RIP were also measured by Western blotting (Figure 2A, middle and bottom panels). In these experiments, the endogenous IKK was immunoprecipitated for in vitro kinase assays. Because only a certain percentage of cells was transfected with Myc-RIP, hTNF-induced IKK activation was partially reconstituted in those RIP$^{-/-}$ cells. Thus, lower IKK activation was detected in reconstituted RIP$^{-/-}$ cells compared to wt cells, which contain an endogenous RIP protein, although similar amounts of IKK were used in each kinase assay (Figure 2A, top and middle panels). Similarly, the ectopic-expression of Flag-TRAF2 also restored hTNF-induced IKK activation in TRAF2$^{-/-}$ cells (Figure 2B, top panel). The IKK and Flag-TRAF2 expression levels in these experiments were shown in Figure 2B, middle and bottom panels. To further confirm the results of these reconstitution experiments, either Myc-RIP or Flag-TRAF2 was cotransfected with HA-IKKβ into RIP$^{-/-}$ or TRAF2$^{-/-}$ cells, respectively. Then, the transfected HA-IKKβ was immunoprecipitated for in vitro kinase assay in order to examine the reconstitution of TNF-induced IKK activation. As shown in Figure 2C, these experiments with transfected IKKβ verified the results obtained from measuring the endogenous IKK activity. Taken together, these reconstitution experiments further supported that the failure of IKK activation in response to TNF treatment in RIP$^{-/-}$ or TRAF2$^{-/-}$ cells is due to the absence of RIP or TRAF2.

IKK Is Recruited to the TNF-R1 Complex through TRAF2

In order to transduce TNF signals, both RIP and TRAF2 need to be recruited to the TNF-R1 complex by TRADD (Hsu et al., 1996a, 1996b). Because RIP and TRAF2 can bind to each other (Hsu et al., 1996b), it is not clear whether the interaction between RIP and TRAF2 is required for their recruitment to the TNF-R1 complex. To address this question, we tested whether RIP and TRAF2 can be recruited to the TNF-R1 complex independently of one another. In these experiments, TNF-R1 from either untreated or TNF-treated wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts was immunoprecipitated, and Western blotting was performed sequentially with anti-RIP, anti-TRAF2, and anti-TRADD antibodies. Consistent with previous reports (Shu et al., 1996; Lin et al., 1999), TNF treatment induces TRADD, RIP, and TRAF2 recruitment to the TNF-R1 complex in wt cells (Figure 3A). Nontreated fibroblasts were used as a negative control, indicating none of these proteins were pulled down nonspecifically by the anti-TNF-R1 antibody. As shown in Figure 3A, TNF-induced recruitment of RIP and TRAF2 were also detected in TRAF2$^{-/-}$ and RIP$^{-/-}$ cells, respectively. These results suggested that RIP and TRAF2 are recruited independently of one another. Because IKK could not be activated through TNF-R1 in the absence of RIP or TRAF2 (Figure 1), these results demonstrated that RIP or TRAF2 recruitment to TNF-R1 is not sufficient to activate IKK. Unexpectedly, a significant increased amount of TRADD and TRAF2 was recruited to TNF-R1 upon TNF treatment in RIP$^{-/-}$ cells (Figure 3A). Since the expression levels of these two proteins and TNF-R1 in RIP$^{-/-}$ cells are similar to those in wt cells (Figures 1B and 3A), this observation indicated that RIP might compete with TRADD for binding to TNF-R1 in response to TNF treatment in wt cells. In TRAF2$^{-/-}$ cells, both RIP and TRADD are recruited to TNF-R1 upon TNF treatment in a fashion comparable to the one seen in wt cells.

However, the accuracy of these results relies upon the amount of TNF-R1 pulled down from each sample. Because the size of the TNF-R1 is about the same as the size of the antibody heavy chain, it is impossible to examine the precipitation of TNF-R1 by probing the same blot with anti-TNF-R1 antibody. To check the precipitation of TNF-R1, we crossed-linked the anti-TNF-R1 antibody to protein A-Sepharose beads with dimethyl pimelimidate (DMP) and then performed immunoprecipitation experiments again. As shown in Figure 3B, top panel, DMP efficiently linked the antibody to protein A beads, and no antibody was released from beads after boiling. Similar amount of TNF-R1, about 10% of input, was precipitated from each sample (Figure 3B, middle panel). The same blot was also probed with anti-TRADD antibody, and as shown in Figure 3B, bottom panel, the results of TRADD coprecipitation was similar to those in Figure 3A.

To be sure that the increased recruitment of TRADD and TRAF2 upon TNF treatment in RIP$^{-/-}$ cells is indeed due to RIP deletion and is not a peculiar feature of the RIP$^{-/-}$ fibroblasts, we performed the same experiment in wt and RIP-deficient Jurkat cells. As shown in Figure 3C, results similar to those described above were obtained in these Jurkat cells: an increased amount of TRADD and TRAF2 are present in the TNF-R1 complex in RIP-deficient Jurkat cells after TNF engagement. These results indicated that the presence of RIP minimized the recruitment of TRADD to the TNF-R1 complex.

In response to TNF, it is thought that the recruitment

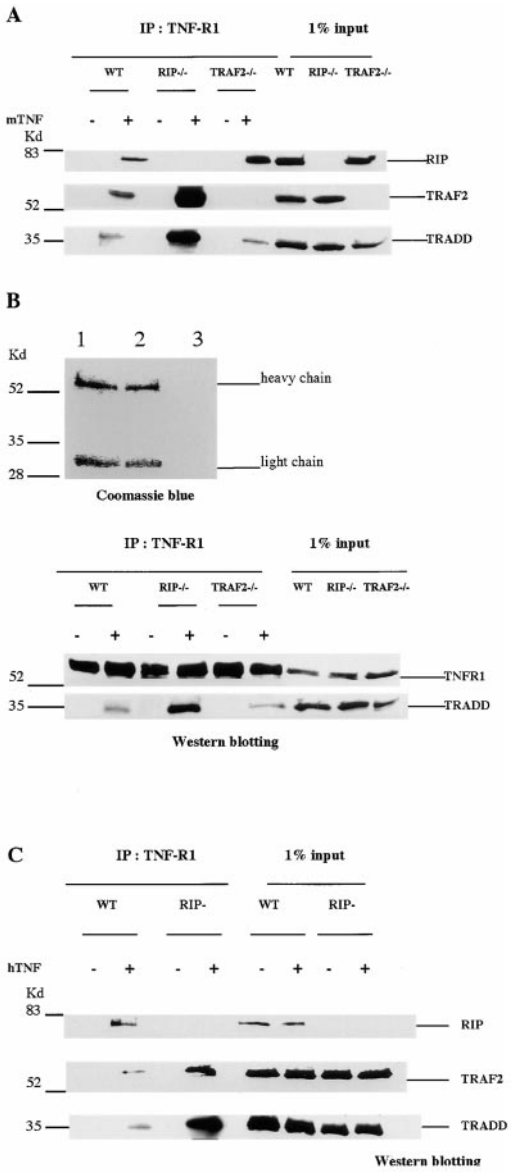

Figure 3. The Recruitment of RIP and TRAF2 to TNF-R1

(A) Recruitment of RIP and TRAF2 to TNF-R1 is independent of one another. Cell extracts were prepared from wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated with anti-TNF-R1 antibody overnight. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-RIP (top), anti-TRAF2 (middle), or anti-TRADD (bottom), respectively. One percent of cell extract from each treated sample was used as a control for protein content (input).

(B) Similar amounts of TNF-R1 was immunoprecipitated from each sample. The TNF-R1 antibody was coupled to the protein A-Sepharose beads with dimethyl pimelimidate (DMP) as described in the Experimental Procedures section. Coupling efficiency was examined by Coomassie blue staining. Top panel, all three samples were boiled for 3 min before loading: lane 1, 0.5 μg of TNF-R1 antibody was loaded as a control; lane 2, TNF-R1 antibody mixed with the beads before coupling; lane 3, TNF-R1 antibody mixed with the beads after coupling. Middle and bottom panels, cell extracts were prepared from wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ fibroblasts either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated overnight with anti-TNF-R1 antibody coupled with protein A beads. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-TNF-R1 (top) or anti-TRADD antibody (bottom). One percent of cell extract from each treated sample was used as a control for protein content (input).

(C) TRADD and TRAF2 are recruited to TNF-R1 more efficiently in the absence of RIP. Cell extracts were prepared from wt and RIP-deficient Jurkat cells either treated with 100 ng/ml hTNF or untreated. After normalization of the protein content according to the protein assay, cell extracts were immunoprecipitated with the anti-TNF-R1 antibody overnight. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-RIP (top), anti-TRAF2 (middle), or anti-TRADD (bottom), respectively. As a control for protein content, one percent of cell extract from each sample was loaded on the gel (input).

of TRAF2 and RIP would result in the activation of an IKK kinase such as NIK or MEKK1, which in turn would activate IKK (Malinin et al., 1997; Mercurio et al., 1997; Regnier et al., 1997). However, since IKK activation can be detected within a minute after TNF treatment (DiDonato et al., 1997), it is possible that such a quick response is achieved by the recruitment of IKK to the TNF-R1 complex. To test this hypothesis, using anti-IKKα and anti-IKKβ antibodies, we performed Western blotting with the same blots generated in the experiments shown in Figure 3. We found that IKKα and IKKβ were coimmunoprecipitated with TNF-R1 upon TNF treatment in wt cells (Figure 4A, lanes 1 and 2). IKKα and IKKβ were not detected in the TNF-R1 complex in TRAF2$^{-/-}$ cells (Figure 4A, lanes 5 and 6). Surprisingly, in RIP$^{-/-}$ cells, IKKα and IKKβ were also recruited to the TNF-R1 complex after TNF treatment (Figure 4A, lanes 3 and 4). The IKK expression levels in these three cell lines are similar (Figure 4A, lanes 7, 8, and 9). These results suggested that IKK is recruited to the TNF-R1 complex in response to TNF treatment, and its recruitment requires TRAF2. Identical results were obtained when probing the blot of Figure 3C: IKKα and IKKβ were detected in TNF-R1 complexes immunoprecipitated from wt and RIP-deficient Jurkat cells (Figure 4B and data not shown).

To further confirm this observation, we performed Western blotting with the anti-IKKα and anti-IKKβ antibodies after immunoprecipitation experiments with an anti-TRAF2 antibody in wt and RIP-deficient Jurkat cells. As shown in Figure 4C, left side, top and middle panels, both IKKα and IKKβ were coimmunoprecipitated by the anti-TRAF2 antibody after TNF treatment in both wt and RIP-deficient Jurkat cells. Small amounts of IKKα and IKKβ were also detected in the immunoprecipitants from nontreated wt and RIP-deficient Jurkat cells (Figure 4C, left side, top and middle panels). This interaction is not due to a cross-reaction of the anti-TRAF2 antibody with IKK, since no IKK was detected in the immunoprecipitant generated with the same anti-TRAF2 antibody from TRAF2$^{-/-}$ cells (data not shown). RIP was also coimmunoprecipitated with TRAF2 in TNF-treated Jurkat cells but not in nontreated wt Jurkat cells (Figure 4C, left side, bottom panel). As stressed for the TNF-R1 immunoprecipitation, the accuracy of these results relies upon the amount of TRAF2 pulled down in the different conditions. Because the size of TRAF2 is close to the size of the antibody heavy chain, it is difficult to detect TRAF2

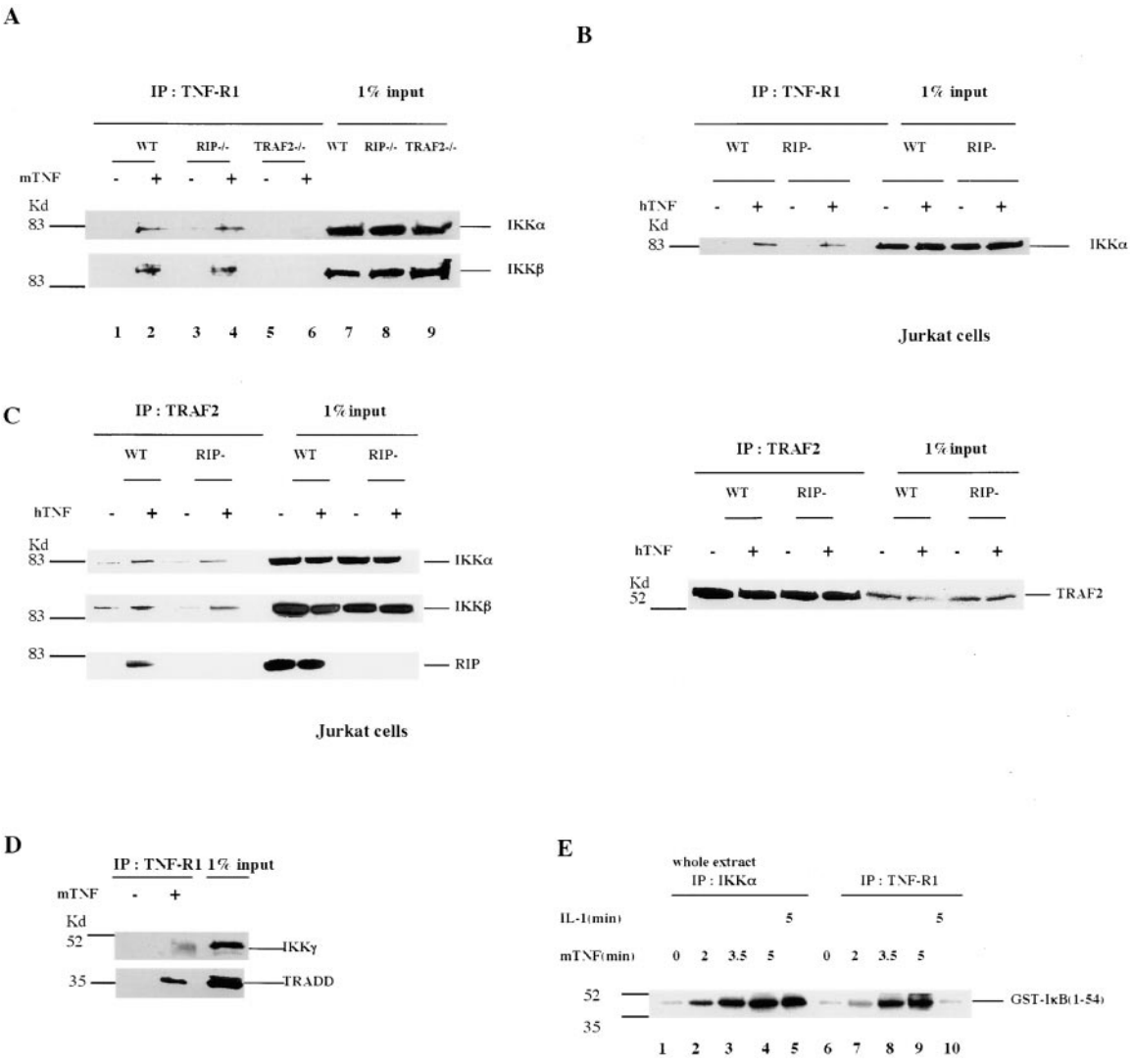

Figure 4. IKK Is Recruited to TNF-R1 by TRAF2 upon TNF Treatment

(A) IKK recruitment in wt, RIP$^{-/-}$, and TRAF2$^{-/-}$ cells. The Western blot from Figure 3A was probed with anti-IKKα and anti-IKKβ antibodies sequentially.

(B) IKK recruitment in wt and RIP-deficient Jurkat cells. The Western blot from Figure 3C was probed with anti-IKKα antibody.

(C) IKK is coimmunoprecipitated with TRAF2. Cell extracts were prepared from wt and RIP-deficient Jurkat cells either treated (100 ng/ml human TNF) or untreated. After normalization of the protein content according to the protein assay, cell extracts were immunoprecipitated with anti-TRAF2 antibody overnight. Left panel shows immunoprecipitates as well as 1% of the cell extracts were resolved by SDS-PAGE, and Western blotting was performed with anti-IKKα (top), anti-IKKβ (middle), and anti-RIP (bottom) antibodies sequentially. Right panel, in order to determine the relative amount of TRAF2 pulled down, TRAF2 antibody was coupled to the protein A beads as described in the Experimental Procedures section, and the immunoprecipitation was performed as described for the left panel. Again, immunoprecipitates as well as 1% of the cell extracts were resolved by SDS-PAGE, and Western blotting was performed with anti-TRAF2.

(D) IKKγ/NEMO is recruited to the TNF-R1 complex. Cell extracts were prepared from wt fibroblasts either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated overnight with anti-TNF-R1 antibody coupled with protein A beads. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-IKKγ (top) or anti-TRADD (bottom). One percent of untreated cell extract was used as a control for protein content (input).

(E) IKK is activated in the TNF-R1 complex. Wt fibroblasts were treated with mTNF (40 ng/ml) or IL-1 (4 ng/ml) for various times as indicated in the figure or left untreated as a control (lanes 1 and 6). After normalization of the protein content according to the protein assay, cell extracts were immunoprecipitated with either anti-IKKα antibody or anti-TNF-R1 antibody to perform a kinase assay.

with the same antibody when it was also used in immunoprecipitation. To check the precipitation of TRAF2, we cross-linked the anti-TRAF2 antibody to protein A-Sepharose beads with DMP and then performed immunoprecipitation experiments. As shown in Figure 4C, right panel, similar amount of TRAF2 was precipitated from each sample. These results indicated that there might be some weak interaction between TRAF2 and IKK before TNF treatment, and this interaction is strengthened by TNF treatment. Importantly, because it is observed in RIP deficient cells, this interaction is independent of RIP.

Since IKKγ/NEMO is essential for IKK activation (Yamaoka et al., 1998), it is important to know whether it

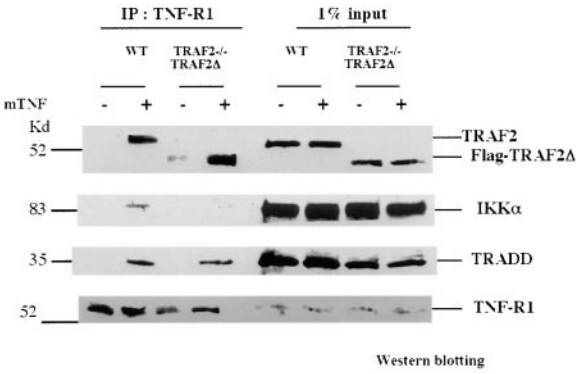

Figure 5. The Dominant-Negative Mutant TRAF2, TRAF2(87-501), Is Defective in IKK Recruitment

Cell extracts were prepared from wt and the TRAF2Δ-TRAF2$^{-/-}$ (the Flag-TRAF2(87-501) stable cell line) cells either treated with 40 ng/ml mTNF or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated overnight with the anti-TNF-R1 antibody coupled to protein A beads. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-TRAF2 and anti-Flag sequentially (top), anti-IKKα (second panel), anti-TRADD (third panel), or anti-TNF-R1 antibody (bottom). One percent of cell extract from each sample was used as a control for protein content (input).

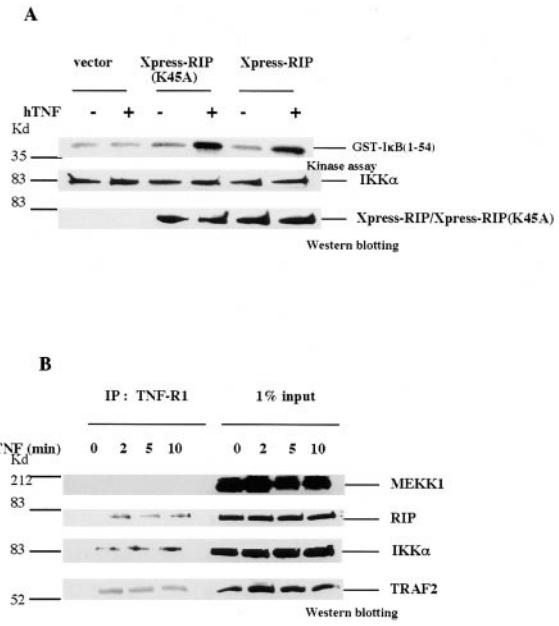

Figure 6. TNF-Induced IKK Activation Does Not Requir the Kinase Activity of RIP

(A) The kinase activity of RIP is not essential for TNF-induced IKK activation. RIP$^{-/-}$ cells were transfected with 2 μg of an empty vector, the expression plasmid for Xpress-RIP (K45A), or the expression vector for Xpress-RIP in 100 mm dishes. Twenty-four hours after transfection, half of transfected cells were treated with 40 ng hTNF for 5 min. Cell extracts were quantified by protein assay and were then either immunoprecipitated with anti-IKKα antibody to perform a kinase assay or resolved on SDS-PAGE for Western blotting with anti-IKKα or anti-Xpress antibodies.

(B) MEKK1 is not detected in the TNF-R1 complex. Cell extracts were prepared from wt fibroblasts either treated with 40 ng/ml mTNF for 2, 5, and 10 min or untreated. After normalization of protein content according to the protein assay, cell extracts were immunoprecipitated with anti-TNF-R1 antibody overnight. Immunoprecipitants were resolved by SDS-PAGE, and Western blotting was performed with anti-MEKK1 (top), anti-RIP (second panel), anti-IKKα (third panel), or anti-TRAF2 antibody (bottom). As a control for protein content, one percent of cell extract from each sample was also loaded on the gel (input).

is also recruited to the TNF-R1 complex. Because the size of IKKγ is close to the size of the antibody heavy chain, we cross-linked TNF-R1 antibody to protein A-Sepharose beads and then performed immunoprecipitation experiments with extracts from TNF-treated and untreated wt fibroblast cells. As shown in Figure 4D, IKKγ was also recruited to the TNF-R1 complex upon TNF treatment. The same blot was also probed with anti-TRADD, as shown in the bottom panel of Figure 4D.

Next, we investigated whether IKK is activated after its recruitment to the TNF-R1 complex. To do so, we performed in vitro kinase assays with the immunoprecipitants generated with the anti-TNF-R1 antibody from wt fibroblasts. In these experiments, cells were treated with TNF for 2, 3.5, and 5 min or IL-1 for 5 min. One percent of the total cell extract of each sample, which was used for TNF-R1 immunoprecipitation, was used for the regular kinase assay as described in Figure 1. As shown in Figure 4E, the treatment of TNF or IL-1 induced IKK activation (lanes 2–5). But with the immunoprecipitants, IKK activation was only detected in those samples from TNF-treated cells (Figure 4D, lanes 6, 7, and 8). The IKK activity in the immunoprecipitants from cells treated with TNF for 3.5 and 5 min was significantly higher than that from cells treated for 2 min. No IKK activity was observed in the immunoprecipitants from nontreated or IL-1-treated cells (Figure 4D, lanes 6 and 10). Because the amount of IKK recruited to the TNF-R1 complex after 2, 3.5, or 5 min TNF treatment is similar (data not shown but see Figure 6B), these results demonstrated that the recruited IKK was activated in the TNF-R1 complex.

It is known that the TRAF2 mutant TRAF2(87-501), whose ring finger domain is deleted, functions as a dominant-negative mutant on TNF-induced NF-κB activation when it is overexpressed (Rothe et al., 1995). Therefore, it is important to know whether TRAF2(87-501) is defective in IKK recruitment. To address this question, we stably introduced Flag-TRAF2(87-501) into TRAF2$^{-/-}$ cells and performed immunoprecipitation experiments with this stable cell line and the wt fibroblasts. In these experiments, the anti-TNF-R1 antibody was cross-linked to protein A-Sepharose beads. As shown in Figure 5, top two panels, both wt and Flag-TRAF(87-501) were recruited to the TNF-R1 upon TNF treatment, but the Flag-TRAF2(87-501) failed to recruit any IKKα. TRADD was recruited to the TNF-R1 complex normally in this Flag-TRAF2(87-501) stable cell line (Figure 5, the second bottom panel). Similar amounts of TNF-R1 were precipitated from each sample (Figure 5, bottom panel). These results suggested that the ring finger domain of TRAF2 was critical for recruiting IKK.

RIP but Not Its Kinase Activity Is Required for IKK Activation

It has been suggested that the kinase activity of RIP is not essential for RIP to mediate TNF-induced NF-κB activation (Hsu et al., 1996b; Ting et al., 1996). To be sure that this is also true for IKK activation, we investigated

whether the kinase dead RIP(K45A) is able to restore TNF-induced IKK activation in RIP$^{-/-}$ fibroblasts. We ectopically expressed wt RIP or RIP(K45A) in RIP$^{-/-}$ cells and treated half of the transfected cells with hTNF. Cells transfected with an empty vector were used as a control. As shown in Figure 5A, top panel, the kinase-deficient RIP(K45A) restored the TNF-induced IKK activation as efficiently as the wt RIP did. The expression levels of IKK, RIP(K45A), and RIP were measured by Western blotting (Figure 6A, middle and bottom panels). These results suggested that the kinase activity of RIP is not required for TNF-induced IKK activation. Since IKK is activated in the TNF-R1 complex (Figure 4D), the role of RIP in IKK activation is most likely the recruitment of an IKK kinase to the TNF-R1 complex.

Several kinases including NIK, MEKK1, and AKT have been suggested to play an important role in TNF-induced NF-κB activation (Lee, F.S. et al., 1997; Lee, S.Y. et al., 1997; Malinin et al., 1997; Nemoto et al., 1998; Ozes et al., 1999). To examine whether any of these kinases are present in the same TNF-R1 complex as IKK is, we performed a time course study with a TNF-R1 immunoprecipitation experiment. Wt fibroblast cells were treated with TNF for 0, 2, 5, and 10 min before they were collected. As seen in Figure 6B, while RIP, IKKα, and TRAF2 were existent in the immunoprecipitants from all of the TNF-treated cells, no MEKK1 was detected. We also failed to observe the presence of NIK and AKT in the TNF-R1 complex when we probed the same blot with anti-NIK and anti-AKT antibodies (data not shown). Therefore, according to these results, it is not clear whether any of those kinases is the IKK kinase that activates IKK in response to TNF treatment.

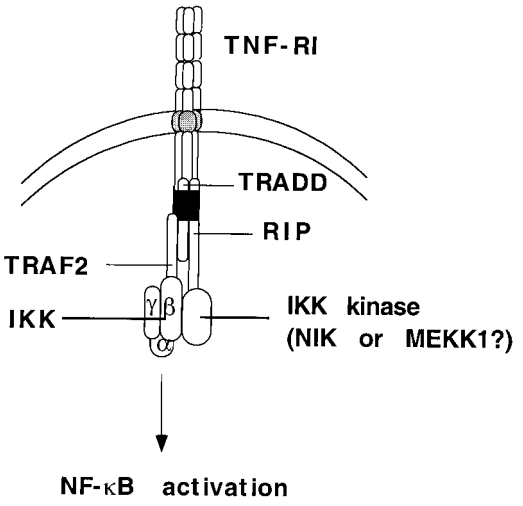

Figure 7. The Distinct Roles of TRAF2 and RIP in TNF-R1-Mediated IKK Activation

In response to TNF treatment, IKK is recruited to the TNF-R1 complex, and this recruitment is accomplished through TRAF2. The activation of IKK in the TNF-R1 complex requires the presence of RIP.

Discussion

Tremendous efforts have been made to study the molecular mechanisms of TNF-mediated cellular responses in recent years. It is known that the activation of the transcription factor NF-κB plays a critical role in many TNF-induced biological processes (Siebenlist et al., 1994; Baeuerle and Baltimore, 1996; Karin et al., 1997). The signal transduction pathway from TNF receptors to NF-κB activation has largely been worked out (Rothe et al., 1994, 1995; Hsu et al., 1995, 1996a, 1996b). For TNF-R1-mediated NF-κB activation, it is thought that the trimerization of TNF-R1 recruits TRADD to TNF-R1 and then TRADD recruits TRAF2 to the TNF-R1 complex. The aggregation of TRAF2 would then allow it to interact with NIK, which leads to the subsequent activation of NIK. In turn, NIK would activate IKK, and the activated IKK then phosphorylates IκB. Finally, the phosphorylated IκB will be rapidly degraded after ubiquitination, and NF-κB is translocated into the nucleus. However, this model does not address the importance of RIP in TNF-induced NF-κB activation, although RIP is essential for this process (Ting et al., 1996; Kelliher et al., 1998). In our study, we demonstrated that both TRAF2 and RIP play essential and distinct roles in IKK activation. As summarized in Figure 7, our data clearly indicated that IKK is recruited to the TNF-R1 complex and that TRAF2 is essential for this recruitment. Although RIP has no effect on IKK recruitment, its presence in the same receptor complex is crucial for TNF-induced IKK activation. Because IKK is activated in the TNF-R1 complex and the kinase activity of RIP is dispensable for IKK activation (Figures 5 and 6), the most likely function of RIP is to recruit the IKK kinase to the TNF-R1 complex.

Previous studies have suggested that TRAF2 is solely responsible for TNF-induced JNK activity; however, TNF-induced NF-κB activation is slightly delayed and decreased in TRAF2 null cells (Yeh et al., 1997). In our study, we found that mTNF-induced IKK activation is significantly reduced in TRAF2$^{-/-}$ cells compared to wt cells, but the remaining IKK activity is sufficient for NF-κB activation (Figure 1A). Our data suggested that the decrease of IKK activation is accountable for the delayed and slightly reduced NF-κB activation in TRAF2$^{-/-}$ cells. Importantly, unlike mTNF, hTNF induced little IKK activation in TRAF2$^{-/-}$ cells (Figure 1C). Since hTNF only binds to TNF-R1, it seems that TRAF2 plays a greater role in TNF-R1-mediated IKK activation than it does in the TNF-R2 pathway. Meanwhile, other TRAF proteins, such as TRAF5, may also partially substitute the function of TRAF2. In contrast, mTNF and hTNF showed no difference in activating IKK in RIP$^{-/-}$ cells. It is intriguing to speculate that RIP is involved in both TNF-R1 and -R2 signaling pathways. This possibility is supported by the fact that RIP interacts with TRAF1 and TRAF2, both of which are components of the TNF-R2 complex (Hsu et al., 1996b).

RIP was initially identified by its interaction with Fas, but it is not required for Fas-mediated apoptosis (Stanger et al., 1995). It has also been reported that RIP could weakly interact with TNF-R1 (Hsu et al., 1996b). Subsequently, RIP was found to be a key effector in the TNF-R1 complex (Liu et al., 1996; Ting et al., 1996; Kelliher et al., 1998). In this study, we found that the presence of RIP in the TNF-R1 complex is crucial for

IKK activation, although its kinase activity does not play a role in this process. Therefore, the essential role of RIP in TNF-R1-mediated IKK activation may be to bring the IKK kinase to the TNF-R1 complex. Our observation that TRADD was recruited to TNF-R1 much more efficiently in $RIP^{-/-}$ cells than it was in wt cells suggests that RIP may bind to TNF-R1 directly instead of being recruited to the TNF-R1 complex through TRADD as previously reported (Hsu et al., 1996b). Further study is necessary to verify this possibility. Interestingly, although much more TRAF2 was recruited to the TNF-R1 complex in $RIP^{-/-}$ cells, the recruitment of IKK was not increased (Figures 3A and 4A). These results imply that RIP may play a role in stabilizing the interaction between TRAF2 and IKK.

TRAF2 has been suggested to play a critical role in recruiting downstream kinases; several kinases, including NIK and MEKK1, have been reported to interact with TRAF2 (Malinin et al., 1997; Song et al., 1997; Baud et al., 1999). Previous studies also reported that NIK and MEKK1 could interact with IKK (Woronicz et al., 1997; Nemoto et al., 1998). However, all of these observations were made by cotransfection experiments. More evidence is necessary to prove that these interactions indeed happen under physiological conditions. In our study, we failed to observe the presence of NIK or MEKK1 in the TNF-R1 complex in our coimmunoprecipitation experiments. One possibility is that the interaction between NIK or MEKK1 with the TNF-R1 complex is weak; therefore, it cannot be detected under our experimental conditions. Because the presence of RIP in the TNF-R1 complex is critical for IKK activation, we are currently investigating whether RIP interacts with NIK or MEKK1. Most recently, another kinase, AKT, has also been shown to play a role in TNF-induced NF-κB activation (Ozes et al., 1999). In contrast to what has been reported, we found that AKT is not linked to TNF-induced IKK and NF-κB activation. In our system (mouse fibroblasts and Jurkat cells), wortmannin had no effect on TNF-induced IKK and NF-κB activation although it completely abolished AKT activation (Y. R. and Z-G. L., unpublished data). While we were unable to detect the interaction between TRAF2 with these kinases, we found that IKK was recruited to the TNF-R1 complex in response to TNF treatment. The rapid recruitment of IKK to the TNF-R1 signaling complex allows IKK to be efficiently activated. The recruitment of IKK to the TNF-R1 complex relies on the presence of TRAF2; RIP has little effect on IKK recruitment. Although our preliminary data suggested that TRAF2 directly interacted with IKK, the biochemical basis for TRAF2-mediated IKK recruitment is under study. However, because the TRAF2 mutant TRAF2(87-501) was unable to recruit IKK (Figure 5), one of the functions of the ring domain of TRAF2 is to mediate IKK recruitment.

Recently, it was found that the oligomerization of the N-terminal effector domain of TRAF2 was sufficient to induce IKK activation (Baud et al., 1999). Because our data indicated a pivotal role of RIP in TNF-induced IKK activation, we tested whether RIP is required for IKK activation by the oligomerization of the TRAF2 effector domain. We found that the aggregation of the TRAF2 effector domain did not induce IKK activation in $RIP^{-/-}$ cells (data not shown). This result indicated that IKK activation by the oligomerization of the TRAF2 domain also requires the presence of RIP. It is possible that the oligomerization of the TRAF2 effector domain will result in RIP recruitment to the signaling complex.

TNF-induced NF-κB activation is achieved through multiple steps. While the signaling pathway that leads to NF-κB activation in response to TNF has been largely elucidated, the molecular mechanism for IKK activation is still not conclusive. In this report, we demonstrated the distinct roles of TRAF2 and RIP in TNF-R1-mediated IKK activation. In order to be activated, IKK needs to be recruited to the TNF-R1 complex. While TRAF2 is required for IKK recruitment, RIP mediates the activation of IKK. Therefore, our study provided a mechanism for TNF-R1-mediated IKK activation.

Experimental Procedures

Reagents

Anti-RIP antibody was purchased from Transduction Laboratories. Anti-TRAF2, anti-Xpress, anti-IKKα, anti-IKKβ, anti-TNF-R2, anti-TRADD, anti-HA, and anti-MEKK1 antibodies were purchased from Santa Cruz. Anti-JNK1, anti-IKKγ, and anti-Myc antibodies were from PharMingen. The anti-Flag antibody was purchased from Sigma. Anti-IκB and anti-NIK antibodies were from Dr. DiDonato. The anti-TNF-R1 antibodies were from R&D Systems. Human and mouse TNFα were purchased from R&D systems. [γ-^{32}P]ATP was from Amersham Pharmacia Biotech.

Cell Culture and Transfection

Wt fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum or 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. $RIP^{-/-}$ and $TRAF2^{-/-}$ cells were cultured in the calf serum medium as wt cells, except 0.3 mg/ml G418 was included. Cells were transfected with Lipofectamine PLUS reagent (GIBCO–BRL) following the instructions provided by the manufacturer. When cells were transfected with the RIP plasmid, 0.1 μg CrmA was added. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The stable TRAF2(87-501)-$TRAF2^{-/-}$ cell line was generated by cotransfecting Flag-TRAF2(87-501) and an expression plasmid for the hygromycin-resistant gene. Cell lines that are resistant to hygromycin were selected and examined for the presence of Flag-TRAF2(87-501) by Western blotting with an anti-Flag antibody.

Plasmids

The mammalian expression plasmids for Myc-RIP, Myc-RIP(K45A), and Flag-TRAF2 have been described previously (Hsu et al., 1996b; Liu et al., 1996). The mammalian expression plasmids for Xpress-RIP and Xpress-RIP(K45A) have been described previously (Lin et al., 1999). The expression plasmid for HA-IKKβ has been described previously (Zandi et al., 1997).

Western Blot Analysis and Coimmuneprecipitation

After treatment with TNFα for different times as described in the figure legend, cells were washed twice in phosphate-buffered saline (pH 7.2) and then collected and lysed in M2 buffer (20 mM Tris [pH 7], 0.5% NP40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 mM pNpp). Fifty micrograms of the cell lysates were fractionated by 4%–20% SDS-polyacrylamide gels, and Western blottings were performed with the desired antibodies. The proteins were visualized by enhanced chemiluminescence, according to the manufacturer's (Amersham) instructions. For immunoprecipitation assays, 3×10^7 of TNF- (40 ng/ml) treated or nontreated wt, $RIP^{-/-}$, and $TRAF2^{-/-}$ fibroblasts were lysed in lysis buffer (50 mM HEPES [pH 7.6], 250 mM NaCl, 0.1% NP40, 5 mM EDTA, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). The lysates

were mixed and precipitated with the relevant antibody and protein A-Sepharose beads by incubation at 4°C for 4 hr to overnight. The beads were washed four times with lysis buffer, and the bound proteins were resolved in 10% SDS-polyacrylamide gels and detected by Western blot analysis. For immunoprecipitations with antibodies that were cross-linked to protein A-Sepharose beads, antibodies (100 μg antibody/ml wet beads) were coupled to the beads with dimethyl pimelimidate (DMP) as described (Harlow and Lane, 1999).

Kinase Assay

Cells were collected in M2 buffer (see above). Endogenous IKK was immunoprecipitated with anti-IKKα antibody, and in vitro kinase assays were performed as described previously, using GST-IκB(1-54) as the substrate (DiDonato et al., 1997). HA-IKK was immunoprecipitated with anti-HA antibody. For the kinase assay with anti-TNF-R1 immunoprecipitants, GST-IκB(1-54) and [γ-^{32}P]ATP were mixed directly with them in the kinase buffer (20 mM HEPES [pH 7.5], 20 mM β-glycerol-phosphate, 10 mM pNpp, 10 mM $MgCl_2$, 1 mM DTT, 50 μM sodium vanadate, and 20 μM cold ATP).

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