Immunity, Vol. 12, 301–311, March, 2000, Copyright ©2000 by Cell Press

# Recruitment of the IKK Signalosome to the p55 TNF Receptor: RIP and A20 Bind to NEMO (IKKγ) upon Receptor Stimulation

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## Summary

**The adapter protein RIP plays a crucial role in NF-κB activation by TNF. Here we show that triggering of the p55 TNF receptor induces binding of RIP to NEMO (IKKγ), a component of the I-κ-B-kinase (IKK) "signalosome" complex, as well as recruitment of RIP to the receptor together with the three major signalosome components, NEMO, IKK1 and IKK2, and some kind of covalent modification of the recruited RIP molecules. It also induces binding of NEMO to the signaling inhibitor A20, and recruitment of A20 to the receptor. Enforced expression of NEMO in cells revealed that NEMO can both promote and block NF-κB activation and dramatically augments the phosphorylation of c-Jun. The findings suggest that the signaling activities of the IKK signalosome are regulated through binding of NEMO to RIP and A20 within the p55 TNF receptor complex.**

## Introduction

The function of NF-κB, a transcription factor controlling various immune defense mechanisms, is subject to intricate regulation through a number of different mechanisms (Baeuerle and Baltimore, 1996; Ghosh et al., 1998). The most widely encountered and thoroughly studied is its association with inhibitory proteins of the IκB family. The inhibitory effect of these proteins is alleviated upon their phosphorylation in response to NF-κB-inducing agents, which in turn target these proteins for ubiquitination and proteasomal degradation. A macromolecular complex, the "signalosome," plays a central role in this phosphorylation. This complex is comprised of three major proteins: two IκB kinases, IKK1 and IKK2, which can directly phosphorylate IκBα and β, and a protein called NEMO (or IKKγ), which lacks enzymatic activity and whose exact role in the function of the signalosome has up to now been unknown (Chen et al., 1996; DiDonato et al., 1997; Mercurio et al., 1997, 1999; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997; Cohen et al., 1998; Rothwarf et al., 1998; Yamaoka et al., 1998; Li et al., 1999).

One of the inducing agents employing the signalosome in NF-κB activation is tumor necrosis factor (TNF). The mechanisms by which this cytokine activates NF-κB have attracted particular attention in view of findings indicating that, in addition to its role as a mediator of TNF-induced activation of genes that contribute to inflammation and immune regulation, NF-κB also acts as a negative regulator of the cytotoxic function of TNF through induction of some antiapoptotic proteins (reviewed in Wallach et al., 1999). Stimulation of the p55 receptor, which mediates both NF-κB and cell death induction by TNF, incites the recruitment of a group of signaling proteins to the receptor. Some, like the adapter proteins TRADD (Hsu et al., 1996b), RIP (Hsu et al., 1996a), and TRAF2 (Shu et al., 1996) as well as the protein kinases NIK (Malinin et al., 1997) and MEKK1 (Baud et al., 1999), which bind to TRAF2 and are probably also recruited, have been suggested as participants in NF-κB activation. Others, like A20, a zinc finger protein that is itself induced by NF-κB and also binds to TRAF2, act as inhibitors of NF-κB activation (Opipari et al., 1990; Song et al., 1996).

Compelling evidence points to a crucial role for the adapter protein RIP (Stanger et al., 1995) in the activation of NF-κB by the p55 TNF receptor. Targeted disruption of the *RIP* gene (Kelliher et al., 1998) and its mutation in cultured cells (Ting et al., 1996), abolish this activation. Of the three distinct domains in RIP—a C-terminal death domain (DD), an intermediate region, and an N-terminal serine/threonine protein kinase domain—only the DD and the intermediate region seem to contribute to the activation (Hsu et al., 1996a; Ting et al., 1996). The RIP DD mediates the recruitment of RIP to the p55 TNF receptor (Hsu et al., 1996a). It binds to the DD of the adapter protein TRADD, which upon stimulation binds to the DD in the p55 TNF receptor (Hsu et al., 1996b). However, this domain alone does not suffice for NF-κB activation. In fact, overexpression of a RIP deletion mutant corresponding to just the DD blocks NF-κB induction (Hsu et al., 1996a). In contrast, expression of a RIP mutant consisting of only its intermediate domain suffices to activate NF-κB (Hsu et al., 1996a; Ting et al., 1996), suggesting that this domain transmits the signaling for downstream events in the activation.

The only protein so far found to bind to the RIP intermediate domain is the adapter protein TRAF2 (Hsu et al., 1996a). Although this protein contributes to the activation of NF-κB by some other receptors of the TNF/NGF family, its involvement in NF-κB activation by the p55 TNF receptor seems to be limited, since neither the knockout of the *TRAF2* gene (Lee et al., 1997; Yeh et al., 1997) nor of the gene for TRAF5, a related adapter protein (Nakano et al., 1999), could abolish NF-κB activation by TNF.

In searching for additional interactions of RIP that are required for its function, we found that the intermediate domain in this protein also binds to the signalosome component NEMO and that NEMO, together with RIP and the two other signalosome components IKK1 and IKK2, is recruited to the p55 TNF receptor upon stimulation of the receptor. On further probing the function of NEMO, we found that this protein also binds A20, an

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Table 1. Binding of NEMO to RIP and A20 in Transformed Yeast

| DNA Binding Domain Construct | Activation Domain Construct | | | | | |
|---|---|---|---|---|---|---|
| | NEMO | RIP | TRAF-2 | TRADD | FADD | A20 |
| Full-length NEMO | + | + | − | − | − | + |
| Full-length RIP | + | + | + | + | + | − |
| RIP $KD_{(1-285)}$ | − | − | − | − | − | − |
| RIP $ID_{(286-579)}$ | + | − | + | − | − | − |
| RIP $DD_{(580-671)}$ | − | + | − | + | + | − |
| Full-length A20 | + | − | + | − | − | + |
| $A20_{(1-386)}$ | +/− | − | + | − | − | − |
| $A20_{(387-790)}$ | + | − | − | − | − | + |
| $NEMO_{(95-416)}$ | + | + | − | − | − | + |
| $NEMO_{(218-416)}$ | + | − | − | − | − | − |
| Lamin | − | − | − | − | − | − |

Yeast SFY526 cells were cotransformed with expression vectors encoding the indicated GAL4 activation domain and GAL4 binding domain fusion proteins. Interactions between the fusion proteins were assessed by filter assays for β-galactosidase activity. The plus sign indicates development of blue color within 2 hr of the assay, the plus/minus indicates color development in 6 hr, and the minus sign indicates no color development within 24 hr. In further specificity assessment of the binding observed in the two-hybrid test, NEMO and A20 were found not to bind to any of the following proteins involved in TNF signaling: the intracellular domains of the p55 and p75 TNF receptors; the adapter proteins TRADD, MORT1/FADD, and RAIDD; the protein kinase NIK; caspase-8; caspase-1; and Bcl-2. RIP displayed binding only to TRADD, MORT1/FADD, and RAIDD and to the intracellular domain of the p55 TNF receptor. KD, kinase domain; ID, intermediate domain; DD, death domain.

inhibitor of NF-κB activation. We also found that modulation of the cellular levels of NEMO may result in either augmentation or inhibition of NF-κB activation. It may also dramatically enhance the phosphorylation of the transcription factor c-Jun.

These findings indicate that regulation of the IKK signalosome by TNF involves interactions of NEMO with RIP and A20 within the TNF receptor complex.

## Results

### Identification of NEMO as a RIP- and A20-Binding Protein

To identify potential downstream components of the p55 receptor/RIP signaling pathway, we employed the yeast two-hybrid system (Fields and Song, 1989) to screen a human B cell cDNA library (Clontech) for RIP-interacting proteins. Several specifically interacting clones were recovered. One of these was identified as a partial clone of NEMO (IKKγ), a protein shown previously to be present in the signalosome and to associate with the IκB kinase IKK2 (Rothwarf et al., 1998; Yamaoka et al., 1998; Mercurio et al., 1999). Deletion analysis indicated that NEMO binds to the intermediate domain in RIP (Table 1), i.e., the region that links its DD to the kinase domain, which has been shown to mediate NF-κB activation (Hsu et al., 1996a; Ting et al., 1996).

On further characterizing the binding properties of NEMO, we found that this protein does not bind to any of the p55 TNF receptor–associated adapter proteins nor to the intracellular domains of the TNF receptors themselves. It does, however, bind to A20, a zinc finger protein that exerts inhibitory effects on TNF, and has also been shown to bind to the TNF receptor-associated protein TRAF2 (Song et al., 1996). Partial deletion analysis of A20 indicated that the latter binds to TRAF2 through its N-terminal part, a region that seems not to be essential for its inhibitory function (Song et al., 1996). Its binding to NEMO, however, occurs both through its N-terminal and, apparently more effectively, through its C-terminal (zinc finger) region. Partial deletion analysis of NEMO suggested that both RIP and A20 bind to a region in the middle of this protein (Table 1).

An assessment of the interaction of RIP, NEMO, and A20 within mammalian cells confirmed that NEMO binds to RIP as well as to A20. The bindings occurred constitutively in transfected cells that overexpressed these proteins (Figures 1A–1C). In cells that did not overexpress NEMO, however, both RIP and A20 bound to NEMO only after TNF application or in response to overexpression of the p55 TNF receptor (Figures 1D and 1E), which triggers signaling by this receptor (Boldin et al., 1995).

### p55 TNF-R Triggering Induces Recruitment of RIP and of the IKK Signalosome Components to the Receptor

RIP is recruited to the p55 TNF receptor upon stimulation of the receptor (Figure 2A; Hsu et al., 1996a). Interestingly, some of the recruited RIP molecules exhibit retarded migration on SDS-PAGE in a ladder-like pattern, a change that could not be observed in the RIP molecules recovered from the whole cell lysate (compare upper and lower panels in Figure 2A).

In view of the observed binding of RIP to NEMO and the reported association of NEMO with IKK2 and IKK1 (Rothwarf et al., 1998; Yamaoka et al., 1998; Mercurio et al., 1999), we examined whether the last three proteins also associate with the activated p55 TNF receptor. As shown in Figures 2B–2D, the three proteins were indeed found to coimmunoprecipitate with the p55 TNF receptor from extracts of TNF-treated HeLa cells, while showing little or no association with the receptor in the absence of TNF treatment.

To further confirm that the signalosome components associate with the p55 TNF receptor molecules that are located on the cell surface, we immunoprecipitated the receptor with antibodies that were applied not to the cell lysate but to the intact cells prior to their lysis, and then removed the antibody molecules that did not bind to the cell-surface receptors. As shown in Figure 2E, the coimmunoprecipitation of IKK1 with the receptor under these test conditions was as effective as when

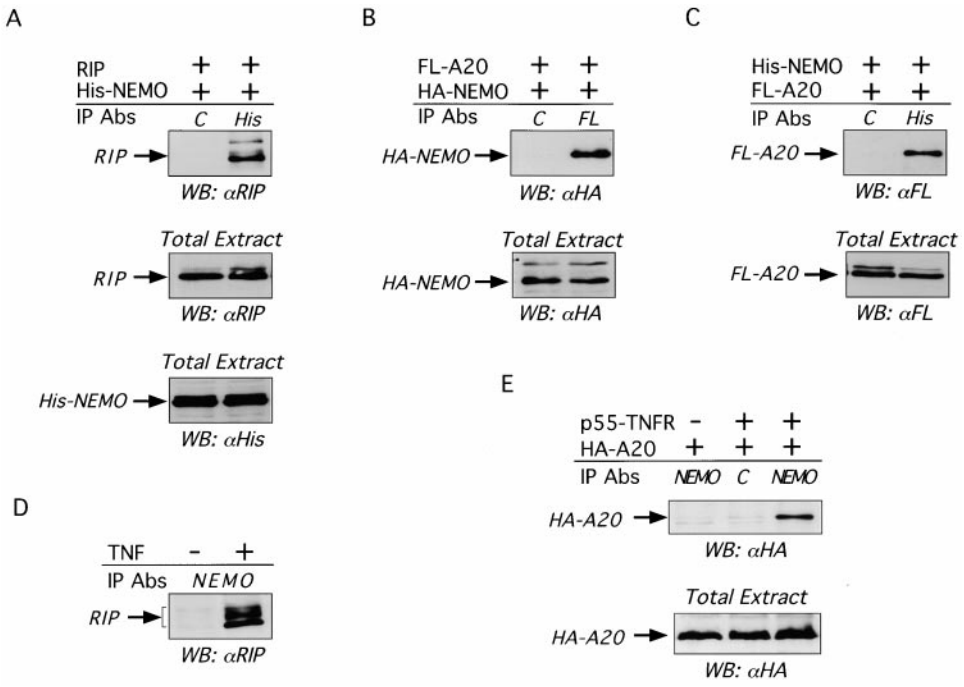


Figure 1. Interaction of NEMO with RIP and A20 in Mammalian Cells

(A) Coimmunoprecipitation of RIP with NEMO in cotransfection assays. HEK 293T cells were transfected with expression vectors for RIP and His epitope-tagged NEMO (5 μg each). Extracts were prepared and immunoprecipitated 24 hr later. In all figures, the antibodies applied for immunoprecipitation (IP Abs) are indicated on top of the autoradiogram (C, control mouse antibody; His, anti-His mAb, etc.) and the antibody applied for immunoblot analysis (WB) are indicated at the bottom.

(B and C) Coimmunoprecipitation of NEMO with A20 in cotransfection assays. HEK 293T cells were transfected with the indicated combinations of expression vectors for FLAG-A20 and HA-NEMO or His-NEMO (5 μg each).

(D) Association of the endogenous RIP and NEMO is TNF dependent. HeLa S3 cells were treated with TNF (100 ng/ml) for 10 min (second lane) or left untreated (first lane) before immunoprecipitation.

(E) Interaction of endogenous NEMO and transfected A20 is dependent on p55 TNF receptor triggering. HEK 293T cells were transfected with the indicated combinations of expression vectors for the p55 TNF receptor and HA-A20. To protect cells against apoptosis induced by the p55 TNF receptor, the receptor was transfected together with a construct expressing the p35 apoptosis inhibitor.

Samples (10 μl) of the cell lysates were applied for immunoblot analyses of the total cell extracts.

receptor molecules were immunoprecipitated from the cell lysate and showed just as strict dependence on TNF.

A kinetic follow-up showed that the recruitment of RIP and IKK1 to the receptor reaches its maximal extent within minutes of TNF application, and then gradually declines, though it is still detectable 30 minutes after TNF application (Figures 2F and 2G).

To evaluate the contribution of the TNF-induced association of RIP and NEMO to the recruitment of the signalosome components by the receptor, an antisense NEMO cDNA and an N-terminal deletion mutant of RIP, corresponding to its DD, were applied to cells. Both of these reagents are known to interfere with TNF-induced NF-κB activation (Hsu et al., 1996a; Rothwarf et al., 1998). As shown in Figure 2H, expression of either construct also reduced the recruitment of IKK1 to the p55 TNF receptor.

### Recruitment of the Signalosome to the p55 TNF Receptor Seems to Be Insufficient for Its Full Activation

To further explore the causal relationship between the recruitment and the activation of the signalosome by the receptor, we assessed the phosphorylation of IκB by the receptor-associated signalosome and compared it to the activity of signalosome isolated from the whole cell lysate by immunoprecipitation of NEMO. As shown in Figure 3, IκB phosphorylation by the receptor-associated signalosome preparation was significantly less effective than that of the signalosome isolated from the whole cell lysate (normalized on the basis of IKK1 levels in the two preparations). The signalosome components themselves displayed a differential pattern of phosphorylation. A protein with the molecular size of IKK1 underwent significantly more phosphorylation in the receptor-associated preparation. Conversely, phosphorylation of proteins whose sizes corresponded to those of IKK2 and NEMO could be discerned only in the cytoplasmic signalosome preparation.

### A20, although Blocking the Activation of NF-κB by TNF through Inhibition of IκB Phosphorylation, Augments Recruitment of the Signalosome to the p55 TNF Receptor

A20 inhibits the activation of NF-κB by TNF as well as by overexpression of the p55 TNF receptor or of its adapter proteins TRAF2 or RIP, although it does not

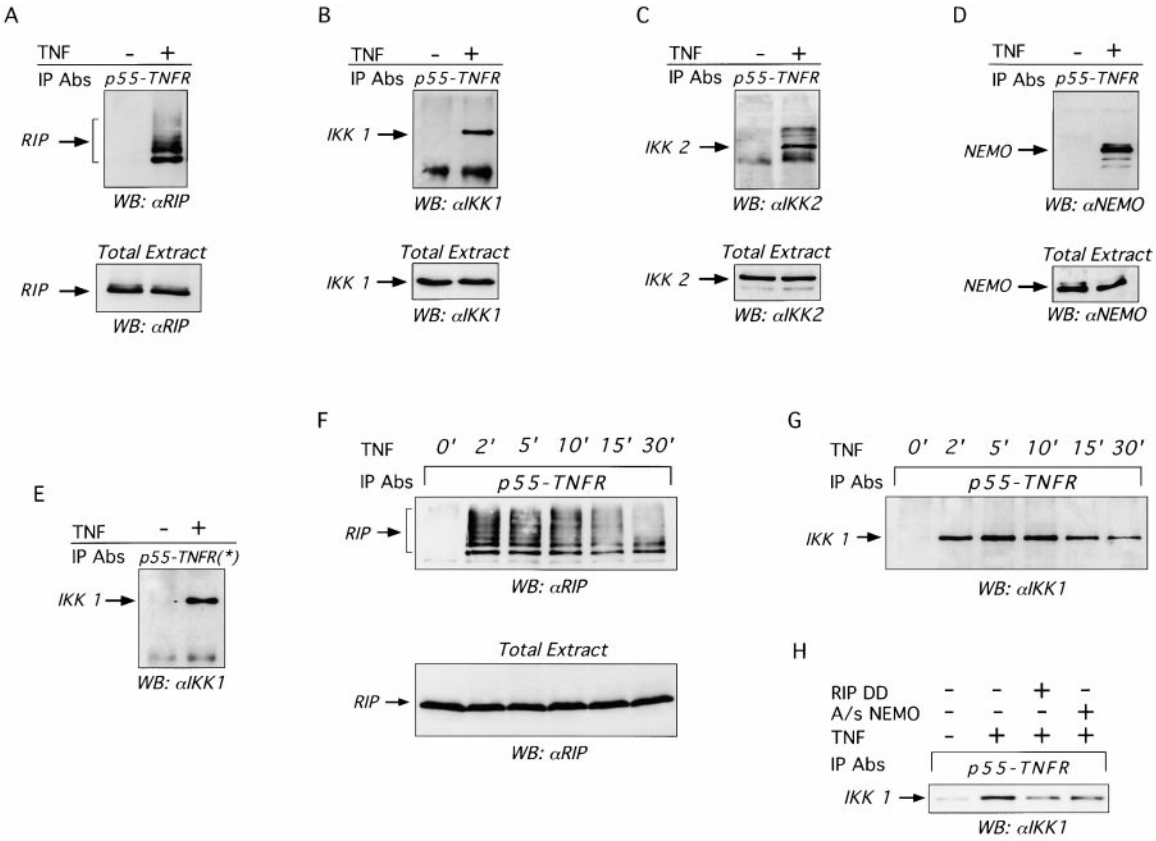


Figure 2. TNF Induces Recruitment of the IKK Signalosome to the p55 TNF Receptor Complex

(A–E) HeLa S3 cells were stimulated with TNF (100 ng/ml) for 10 min (right lane) or left untreated (left lane). Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibodies (A, B, and E) or with mouse monoclonal anti-p55 TNF receptor antibodies (C and D). Coprecipitating RIP, IKK1, IKK2, and NEMO were detected by immunoblot analysis using the indicated antibodies. In experiment (E), the anti-p55 TNF receptor polyclonal antibodies (1 μg/ml) were applied in a way that allowed them to interact only with the receptors expressed on the cell surface. They were added to the tissue culture dishes before cell lysis, just after TNF application. The dishes were gently shaken for 2 hr at 4°C, and the unbound antibody was then rinsed off twice with PBS. The cells were then lysed and 40 μl of protein G-Sepharose beads were added to the lysates to precipitate the TNF receptor complex.

(F) and (G) present the time course of the recruitment of RIP and IKK1, respectively, to the p55 TNF receptor complex. For each lane, $5 \times 10^7$ HeLa S3 cells were treated with TNF (100 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibody. The amounts of coprecipitating RIP and IKK1 and of RIP in aliquots of the cell lysates (10 μl) were assessed by immunoblotting with the indicated antibodies.

(H) HeLa HtTA-1 cells ($1 \times 10^7$) were transfected with expression vectors for the RIP DD (559–671) or NEMO antisense. To prevent cell death, p35 expression vector was added to the RIP DD transfection. After 24 hr, cells were treated with TNF (100 ng/ml) for 10 min or left untreated. Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibodies, and coprecipitating IKK1 was detected by immunoblot analysis with anti-IKK1 mAb. Immunoblotting of aliquots of the total cell extracts (10 μl) with anti-NEMO and anti-RIP or with anti-IKK1 mAbs demonstrated specific reduction of NEMO expression in the cells expressing the NEMO antisense (data not shown).

interfere with NF-κB activation by overexpression of NIK and has only a partial inhibitory effect on its activation by MEKK1 (Figure 4A; Song et al., 1996; Heyninck et al., 1999). The inhibition of NF-κB activation correlated with a marked decrease in the degradation of IκB (Figure 4B). Indeed, the IκB-phosphorylating ability of signalosome complexes isolated from extracts of A20-expressing HeLa cells treated with TNF was substantially lower than that of signalosome isolated from cells that did not express A20. Interestingly, however, the effectiveness of self-phosphorylation of the IKKs in these signalosome preparations seemed not to decrease but rather to be increased compared to cells that did not express A20 (Figure 4C).

As expected from the reported binding of A20 to TRAF2 (Song et al., 1996), which is recruited to the p55 TNF receptor following stimulation (Shu et al., 1996), transfected A20 was found to associate with the p55 TNF receptor in cells that overexpress this receptor (Figure 4D). In view of the indicated requirement of the NEMO-RIP interaction for activation of the signalosome, it was of interest to determine whether the inhibition of NF-κB activation by A20 reflects inhibition of the recruitment of the signalosome to the receptor.

As shown in Figures 4E and 4F, HEK 293T cells expressing transfected A20 did not manifest a decrease but rather a significant enhancement of IKK1 association with the p55 TNF receptor.

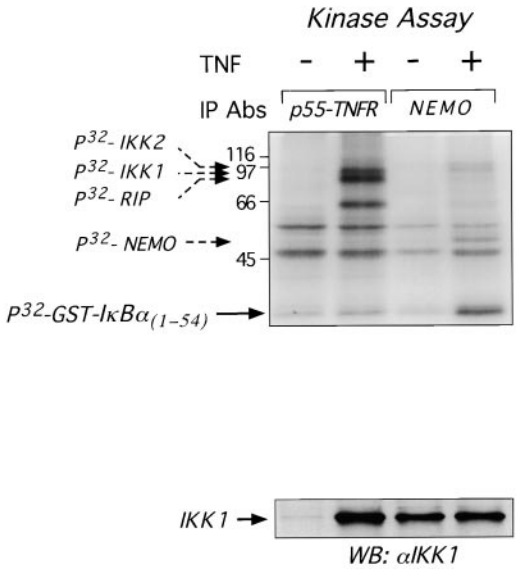


Figure 3. Kinase Activities of p55 TNF Receptor-Recruited Signalosome Compared with Those of Cytoplasmic IKK Signalosome

HeLa S3 cells ($5 \times 10^7$) were treated with TNF (20 ng/ml) for 10 min (second and fourth lanes) or left untreated (first and third lanes). The IKK signalosome was immunoprecipitated with anti-p55 TNF receptor (first and second lanes) or anti-NEMO (third and fourth lanes) polyclonal antibodies, and the associated kinase activities were determined with GST-IκBα (1–54) as a substrate. The amounts of IKK1 in the two immune complexes were assessed by immunoblotting with anti-IKK1 mAb (bottom panel).

### A Mild Increase in the Cellular Levels of NEMO Potentiates NF-κB Activation, whereas a Greater Increase Interferes with NF-κB Activation and Augments c-Jun Phosphorylation

Assessment of the effect of enforced expression of NEMO in HEK 293T cells revealed marked inhibition of NF-κB activation in cells transfected with large amounts of a NEMO-expressing cDNA construct (Li et al., 1999), whereas transfection of low amounts of this construct resulted in significant potentiation of NF-κB activation as well as in increased basal activity of this transcription factor (Figure 5A and inset). The inhibition of NF-κB activation at high cellular concentrations of NEMO could be shown to reflect suppression of IκB degradation (Figure 5B). Moreover, consistent with the proposed role of the NEMO-RIP association in the transmission of signaling, the inhibition of NF-κB activation by NEMO overexpression was associated with decreased recruitment of the signalosome to the receptor (Figure 5C).

Interestingly, high cellular NEMO was also associated with a marked increase in phosphorylation of c-Jun at the same sites as those found to be phosphorylated in response to TNF ($Ser^{63}/Ser^{73}$) (Karin et al., 1997). Notably, the SDS-PAGE migration patterns of the phosphorylated c-Jun in NEMO-overexpressing cells differed somewhat from those in cells treated by TNF, suggesting that, along with the shared effects of TNF and NEMO on phosphorylation of $Ser^{63}$ and $Ser^{73}$, they also induced additional phosphorylation(s) of c-Jun, at specific sites. The effect of NEMO on c-Jun phosphorylation was synergistic with that of TNF (Figure 5D, upper panel). It was not associated with any change in the amounts of c-Jun (Figure 5D, middle panel) and could be fully blocked by expression of a dominant-negative mutant of SEK1 (MKK4), a MAP2K that acts upstream of JNK (data not shown). Yet, unlike the increase in c-Jun phosphorylation that occurs in response to TNF, the increase observed in NEMO-overexpressing cells was not associated with an enhanced phosphorylation of JNK at its activation sites ($Thr^{183}/Tyr^{185}$; Karin et al., 1997; Figure 5D, lower panel). Similar modulation of NF-κB function and c-Jun phosphorylation by NEMO could also be observed in HeLa cells (data not shown).

## Discussion

The choice of the term "signalosome" (signaling organelle) for the IκB phosphorylating complex implies that it was conceived as a distinct structural and functional entity. Up to now, in most fractionation studies aimed at characterizing the IκB phosphorylating kinases, these proteins have indeed appeared to be assembled into a distinct and highly stable macromolecular complex, containing about equal amounts of three unique components: IKK1, IKK2, and NEMO (DiDonato et al., 1997; Mercurio et al., 1997, 1999; Zandi et al., 1997; Rothwarf et al., 1998). Notably, however, the approaches taken in those studies were biased toward the detection of components that are most prevalent and that bind most avidly to each other. Use of high urea washes, for example, may well have eliminated some loosely bound yet functionally important components (Mercurio et al., 1997, 1999; Rothwarf et al., 1998). The findings of the present study indicate that, apart from the three "core" components already identified, the IκB phosphorylating kinases may at times associate with some other cellular proteins, which modulate their function. Specifically, in cells treated by TNF, a subpopulation of the IκB phosphorylating complexes is shown to become an integral part of the p55 TNF receptor signaling complex. NEMO, a major component of the core signalosome complex whose function has until now remained elusive, is shown here to link the IκB kinases (IKKs) to components of the p55 receptor complex. Being based on assessment of interactions of signaling molecules in their normal cellular amounts, the present data provide a more reliable notion of the signaling events than that obtained up to now through overexpression of these proteins. We cannot yet draw any definite conclusions as to the functional implications of the observed interactions. However, the findings are consistent with the idea that the binding of NEMO to components of the receptor complex constitutes an early though not necessarily sufficient step in the activation of the signalosome.

Two novel interactions of NEMO were revealed in this study. NEMO was found to bind to RIP, an adapter protein crucial for NF-κB activation by the p55 TNF receptor (Ting et al., 1996; Kelliher et al., 1998; also see Li et al., 1999), as well as to A20, a protein with inhibitory effects on several TNF functions (Opipari et al., 1990; Jaattela et al., 1996). The binding of NEMO to each of these proteins could be monitored at high fidelity in the yeast two-hybrid test. Within the mammalian cell, however, the binding was stimulus dependent, being

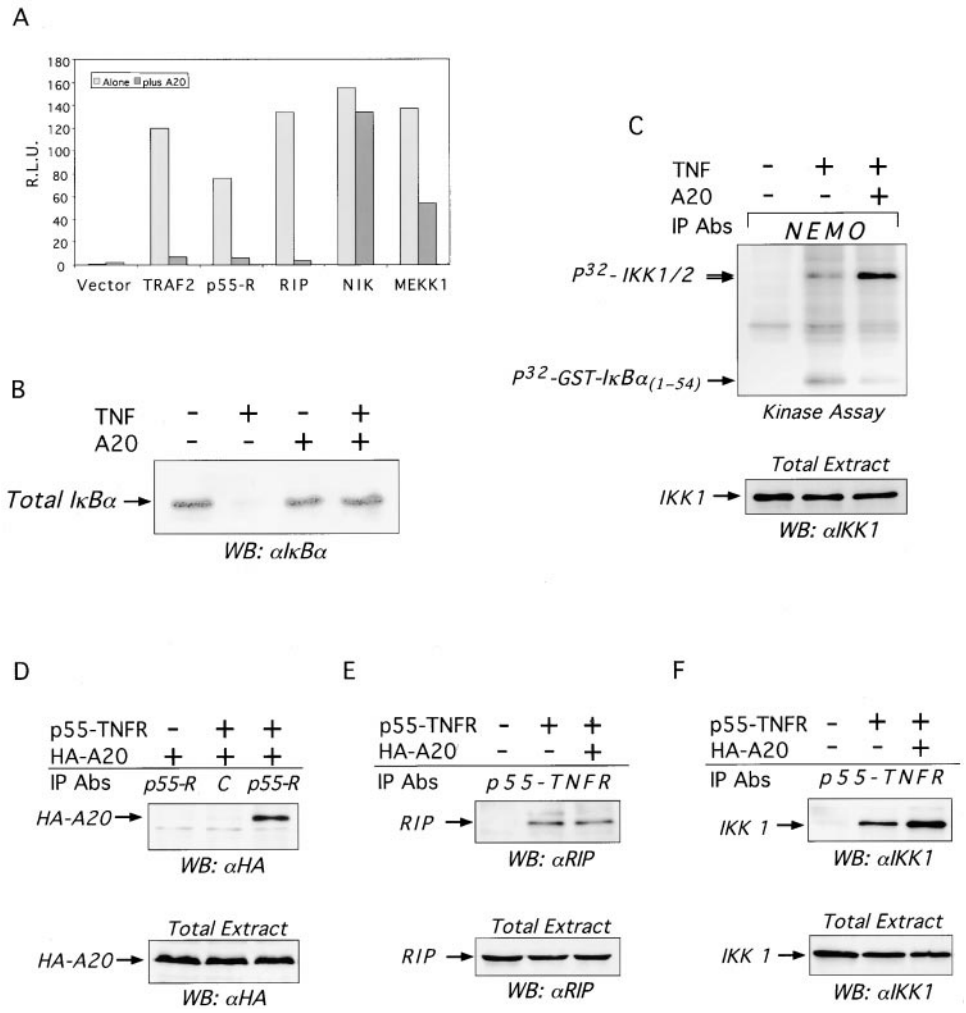


Figure 4. A20 Inhibits NF-κB Activation Induced by the p55 TNF Receptor, RIP, TRAF2, or MEKK1 and the Kinase Activity of the IκB Kinase Complex but Enhances the Recruitment of IKK1 to the Receptor

(A) Effect of A20 on NF-κB dependent reporter gene activity induced by transfection of HEK 293T cells with the p55 TNF receptor, RIP, TRAF2, NIK, or MEKK1.

(B) Effect of A20 on IκB degradation in response to TNF. 293T cells ($2 \times 10^5$) were transfected in 6 cm dishes with pcHA-A20 or with empty vector. After 24 hr, half of the samples were treated with 100 ng/ml TNF for 20 min as indicated. Cells were lysed on the dishes in SDS sample buffer, and one-tenth of each sample was subjected to conventional SDS-PAGE followed by electrotransfer and Western blotting with anti-IκB antiserum.

(C) Effect of A20 on kinase activity of the IKK complex. HeLa HtTA-1 cells ($1 \times 10^7$) were transfected with pcDNA3 (first and second lanes) or with pcHA-A20 (third lane). After 24 hr, the cells were stimulated with TNF (20 ng/ml) for 10 min (second and third lanes) or left untreated (first lane). The IκB kinase complexes were isolated by immunoprecipitation with 1 μg of anti-NEMO polyclonal antibodies, and the associated kinase activities were determined using GST-IκBα (1–54) as a substrate. The amounts of IKK1 in the immunoprecipitates were determined by immunoblotting with anti-IKK1 mAb (bottom panel).

(D) A20 associates with overexpressed p55 TNF receptor. HEK 293T cells ($1 \times 10^7$) were transfected with the indicated combinations of expression vectors for the p55 TNF receptor and with pcHA-A20. p35 expression vector was added to the p55 TNF receptor transfections. Cell lysates were prepared 24 hr later and subjected to immunoprecipitation and immunoblot analysis using the indicated antibodies.

(E and F) A20 expression augments the recruitment of IKK1 to the p55 TNF receptor. HEK 293T cells ($1 \times 10^7$) were transfected with the indicated plasmids. Cell lysates were immunoprecipitated with rabbit anti-p55 TNF receptor antibody. Coprecipitating endogenous RIP and IKK1 were detected by immunoblot analysis with anti-RIP mAb (E) and anti-IKK1 mAb (F).

Aliquots of total cell extracts (10 μl) were immunoblotted with anti-HA (D), anti-RIP (E), or anti-IKK1 (F) mAbs.

detectable only after application of TNF to the cell or upon overexpression of the p55 receptor (which results in receptor triggering, Boldin et al., 1995). RIP is recruited to the p55 TNF receptor upon its triggering (Hsu et al., 1996a) and so are NEMO and the two IKKs associated with it. Our data indicate that A20 also associates with the p55 TNF receptor. This association probably occurs through its binding not only to NEMO but also to TRAF2 (Song et al., 1996). It may thus assist the anchorage of NEMO and its associated proteins to the receptor complex. Cells expressing A20 indeed displayed enhanced recruitment of the IKK complex to the receptor (Figure 4F; see Figure 6 for a diagrammatic representation of the proposed protein interactions).

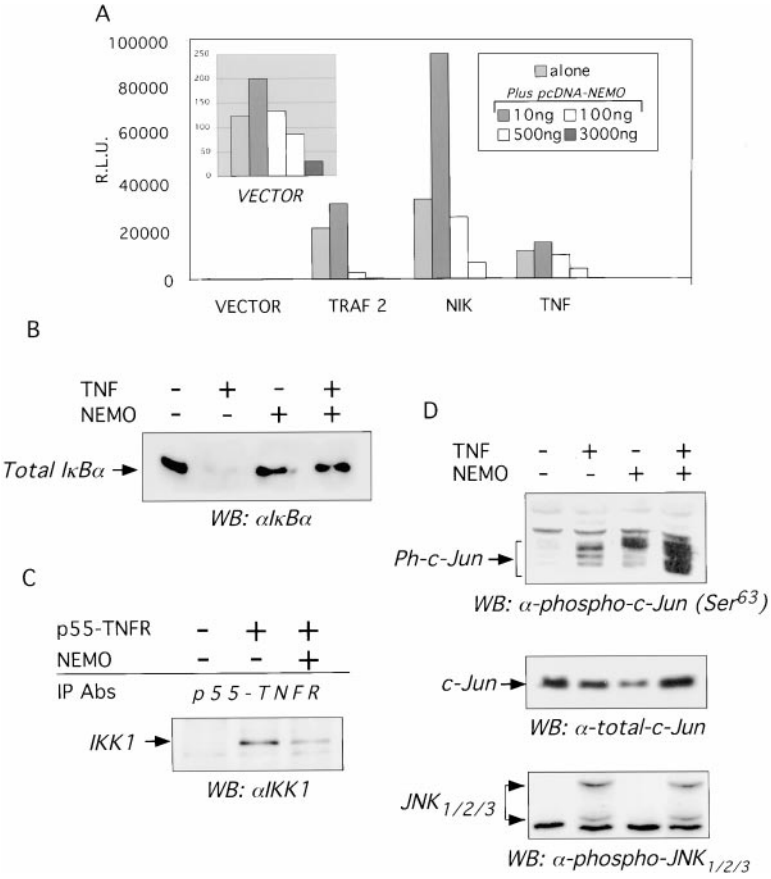


Figure 5. Modulation of NF-κB Activation and c-Jun Phosphorylation by Variation of the Cellular Amounts of NEMO

(A) Effects of NEMO on NF-κB-dependent reporter gene activity induced by transfection of HEK 293T cells with TRAF2 or NIK or their treatment with TNF and on its basal level (inset).

(B) Effect of high cellular amounts of NEMO on IκB degradation in response to TNF. 293T cells ($2 \times 10^5$) were transfected in 6 cm dishes with a NEMO expression plasmid or with an empty vector. After 24 hr, half of the samples were treated with 100 ng/ml TNF for 20 min. The cells were lysed on the dishes in SDS sample buffer, and one-tenth of each sample was subjected to conventional SDS-PAGE followed by electrotransfer and Western blotting with anti-IκB antiserum.

(C) Effect of high cellular amounts of NEMO (25 μg DNA/plate) on recruitment of the signalosome to the p55 TNF receptor.

(D) NEMO induces site-specific hyperphosphorylation of c-Jun without affecting the cellular amounts of c-Jun or the extent of JNK phosphorylation. The immunoblots prepared as described in (B) were probed with the indicated antisera. Results similar to those observed with the anti-phospho $Ser^{63}$ antibody were obtained when the blots were probed with anti-phospho $Ser^{73}$ antibody. Marked potentiation of the effect of NEMO on c-Jun phosphorylation was similarly observed in cells that, together with NEMO, overexpressed the p55 TNF receptor, the p75 TNF receptor, TRADD, RIP, TRAF2, or NIK.

Recruitment of the IKK complex to the receptor occurs soon after TNF application. It precedes receptor uptake (Figure 2E) and reaches almost its maximal extent within 2 min of TNF application. It therefore seems reasonable to assume that this recruitment constitutes an early event in the activation of the IKKs by the receptor. This notion is consistent with the knowledge that RIP, the molecule responsible for the recruitment, is required for NF-κB activation by the p55 receptor (Ting et al., 1996; Kelliher et al., 1998). It is also consistent with the evidence that the region in RIP that is involved in the recruitment (the intermediate domain) is the region known to be crucial for NF-κB activation (Hsu et al., 1996a; Ting et al., 1996). However, the phosphorylation of IκB by the kinases found in the receptor complex after TNF stimulation is much less effective than the phosphorylation observed with the kinase complex recovered from the whole cell lysate (Figure 3). Moreover, in cells that express A20, activation of the IKKs is blocked, in spite of their recruitment to the receptor. It seems likely that the recruitment does not suffice for full activation and that there is a need for some further modulation of the signalosome after its release from the receptor (Figure 6).

A process in which the IKK complex is repeatedly recruited and then released from the p55 receptor requires dynamic modulation of proteins that are involved in the recruitment. It was therefore particularly interesting to find that RIP, the protein through which this recruitment occurs, appears to be subject to some kind of rapid covalent modification, which is restricted to those RIP molecules that have associated with the receptor. This modification of RIP is reminiscent of a modification of IRAK, a serine threonine kinase that seems to play a role similar to that of RIP in the IL1-induced NF-κB activation cascade; namely, it links the receptor reversibly to molecules that act downstream in the signaling pathway (Cao et al., 1996). In the case of IRAK, these induced covalent changes could be shown to correspond to ubiquitination of the protein, which leads to its proteasomal degradation (Yamin and Miller, 1997; Li et al., 1999). The "ladder"-like appearance of the modified RIP molecule, which is characteristic of ubiquitinated proteins, suggests that it is subject to the same kind of modulation.

While the binding of RIP to NEMO may initiate the activation of the IKK complex by TNF, the binding of A20 to NEMO is likely to contribute to the inhibition of NF-κB by A20. The direct association of A20 with a core component of the IKK complex is consistent with the ability of this protein to suppress the activation of NF-κB by at least two different cytokines, TNF and IL1 (Jaattela et al., 1996), which employ distinct signaling pathways. The binding of A20 to TRAF2, an adapter protein found in the signaling complex induced by TNF but not in that induced by IL1, may specifically contribute to the inhibitory effect of A20 on TNF signaling. It is not crucial, however, since an A20 mutant that is incapable of TRAF2 binding can still block NF-κB activation by TNF (Song et al., 1996). It is likely that A20 affects the function of

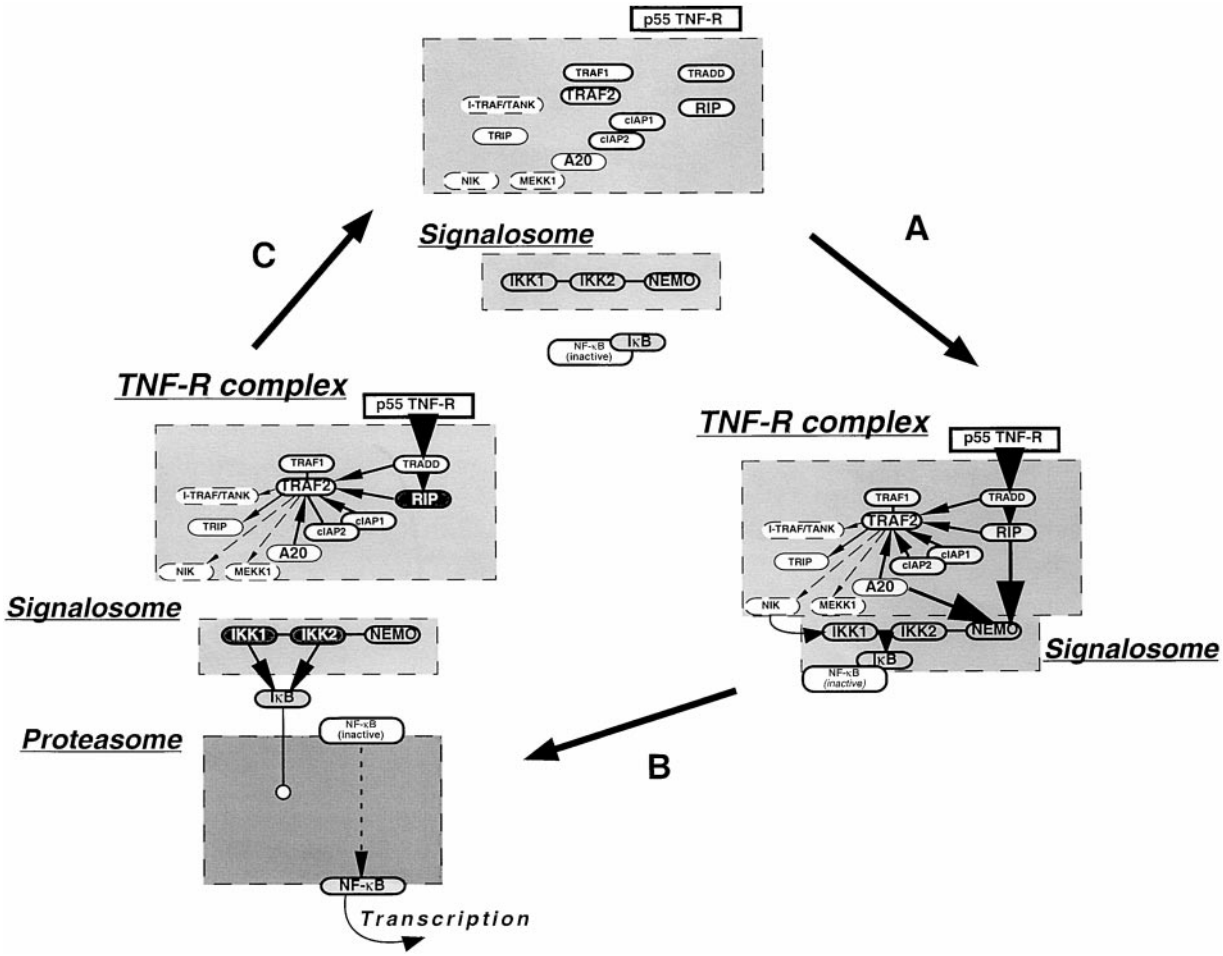


Figure 6. Diagrammatic Representation of the Protein Interactions Suggested to Take Part in the Recruitment of the Signalosome to the p55 TNF Receptor

the TNF receptor by more than one mechanism. Thus, while in the present study we observed inhibition of IκB phosphorylation in response to this protein, in another study it was shown that A20 can also inhibit the function of NF-κB at a step subsequent to IκB degradation (Heyninck et al., 1999). The mechanisms for these effects remain to be clarified. Two observations in the present study provide conflicting clues to the mechanism(s) by which A20 may inhibit IκB phosphorylation: (1) the inhibitory effect of A20 on NF-κB activation could be overcome by overexpressing NIK (also see Heyninck et al., 1999) and to some extent also by MEKK1, raising the possibility that A20 restricts the accessibility of the IKKs to these IKK-activating kinases; (2) while inhibiting the phosphorylation of IκB, A20 seems to augment IKK phosphorylation (Figure 4C), a process that may result in inhibition of these enzymes (Delhase et al., 1999).

Previous studies have indicated that NEMO plays an important role in the regulation of IKK function but give no clue as to the mechanisms by which this molecule acts (Yamaoka et al., 1998; Rothwarf et al., 1998; Li et al., 1999; Mercurio et al., 1999). The present study suggests that NEMO acts to link the IKKs to regulatory molecules and does so in a way that allows them to affect IKK function. There are, in principle, two ways in which such linkage may result in functional modulation. The mere translocation of the signalosome complex to the vicinity of other proteins may allow them to modulate IKK function. Its recruitment to the receptor may bring the IKKs into close proximity with kinases, such as NIK or MEKK1, which seem to associate with the receptor and have the capacity to phosphorylate and thus activate the IKKs (Lee et al., 1997; Ling et al., 1998). Likewise, the physical association of A20 and the complex may allow proteins associated with A20 (De Valck et al., 1999; Heyninck et al., 1999) to exert inhibitory effects on IKK function.

Alternatively, NEMO may act as a transduction element. Its binding to regulatory proteins may induce conformational changes in NEMO, which in turn transmit inhibitory or stimulatory effects to its associated IKKs.

A rather surprising finding was the marked site-specific ($Ser^{63}$/$Ser^{73}$) phosphorylation of c-Jun in cells overexpressing NEMO. The effect of NEMO was synergistic with that of TNF and occurred without any increase in the phosphorylation of JNK at its activation sites ($Thr^{183}$/$Tyr^{185}$). It thus appears to involve a mechanism distinct from the one thought to account for the effect of TNF

on c-Jun phosphorylation. It may perhaps occur through activation of JNK by a mechanism other than its phosphorylation, or by activation of another as yet unknown Jun kinase, or by inhibition of a Jun phosphatase. Although it is difficult to place this unexpected overexpression effect in the context of the normal functioning of the signalosome, its existence indicates that NF-κB activation is not the sole signaling role of this complex. A role of the signalosome in controlling c-Jun phosphorylation was also indicated in a previous study in which NIK, a protein kinase that seems to play an important role in the activation of the signalosome, was shown to have the capacity to activate AP1, a transcription complex containing c-Jun, by a mechanism that seems to be independent of enhanced Jun kinase activity (Natoli et al., 1997).

While the function of RIP, to which NEMO was found to bind, is most probably restricted to the signaling activity of the TNF receptors, the function of NEMO itself is certainly not restricted to signaling induction by TNF. Rather, it acts as a general regulator of signalosome function (Yamaoka et al., 1998). It thus seems possible that, besides binding RIP, NEMO has the ability to interact with components of various other signaling pathways, in each case imposing translocation of the core signalosome to another signaling complex and transmitting the regulatory effects of these different complexes on its functions.

## Experimental Procedures

### Cells and Materials

HeLa S3, the HeLa HtTA-1 clone (Gossen and Bujard, 1992), and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The anti-FLAG epitope monoclonal antibody (mAb) M2 and the anti-His epitope mAb were purchased from Sigma (St. Louis, MO). The anti-HA epitope mAb 12CA5 was from Boehringer Mannheim. The anti-RIP and anti-IKKα mAbs were from PharMingen (San Diego, CA). Rabbit anti-NEMO and anti-HA polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-IKK2 antibody was a gift from Dr. A. Manning, Signal Pharmaceuticals (San Diego, CA). The rabbit antiserum and mAbs (clones number 34 and 39) against the p55 TNF receptor extracellular domain were produced in our laboratory (Engelmann et al., 1990a, 1990b). The rabbit polyclonal anti-total IκBα, anti-total c-Jun, anti-phospho c-Jun ($Ser^{63}$), anti-phospho c-Jun ($Ser^{73}$), and anti-phospho JNK1/2/3 ($Thr^{183}/Tyr^{185}$) antibodies were purchased from New England Biolabs (Beverly, MA). Recombinant human TNF was a gift from Dr. G. Adolf, Boehringer Institute, Vienna, Austria.

### Expression Vectors

The cDNAs for human p55 TNF receptor, RIP, TRAF2, NEMO, A20, and NIK were cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) with or without an additional 5′ sequence encoding the FLAG, HA, or His epitope. The expression vector for the antisense of NEMO was kindly provided by Drs. David Rothwarf and Michael Karin (San Diego, CA), for the catalytic domain of MEKK1 (residues 360–672) by Drs. Michael Kracht and Helmut Holtmann (Hannover, Germany), and for SEK1(K129R) by Dr. Rony Seger (Rehovot, Israel). The N-terminal deletion mutant of RIP (559–671) expression plasmid was generated by PCR.

### Yeast Two-Hybrid Screening

The cDNA corresponding to the complete ORF of human RIP was cloned into the GAL4 DNA-binding domain vector pGBT9 (Clontech, Palo Alto, CA). The resulting plasmid pGBT9-RIP was used as bait in a two-hybrid screen of a human B cell cDNA library (Clontech) in *Saccharomyces cerevisiae* HF7c. The isolation of positive clones and subsequent two-hybrid interaction analyses were performed according to the manufacturer's instructions (Matchmaker Two-Hybrid System Protocol, Clontech). The binding properties of NEMO and A20 as well as of the other examined proteins were assessed in the yeast SFY526 reporter strain (Clontech) using the pGBT9 GAL4-DBD and pGAD GH GAL4-AD vectors. Deletion constructs for two-hybrid mapping were made by PCR.

### Reporter Gene Assays

HEK 293T cells ($2 \times 10^5$ cells/well) were seeded into 35 mm plates. On the following day, cells were transfected by the calcium phosphate precipitation method (Sambrook et al., 1989). Each dish was transfected with 1 μg HIV LTR-luciferase, 1 μg pSV-β-galactosidase, 1 μg expression vector for the tested protein and, when indicated, 2 μg HA-A20 expression vector (pcHA-A20). The amount of transfected DNA was kept constant (5 mg/well) by supplementation with pcDNA3 "empty" vector. Twenty-four hours after transfection, the cells were treated, whenever indicated, for 4 hr with TNF (100 ng/ml), then rinsed twice with phosphate-buffered saline (PBS) and harvested. Reporter gene activity was determined with the luciferase assay system (Promega, Madison, WI). β-galactosidase activity was measured to normalize transfection efficiencies.

### Immunoprecipitations and Immunoblotting

The choice of cell lines in these experiments was defined by the following considerations. To assess the interaction of proteins expressed by transfected constructs, we used HEK 293T cells, which are highly transfectable and express the transfected constructs at very high levels. The HEK 293T cells, however, express very little p55 TNF receptor. The interactions of endogenous proteins and their recruitment to the p55 TNF receptor were therefore assessed using HeLa cells. In experiments aimed at assessing the effects of transfected proteins on the endogenous proteins, we used the HtTA-1 clone of HeLa cells, which is effectively transfectable (>50% of the cells), or the HEK 293T cells. In other experiments we preferred the HeLa S3 variant, since for some reason the background of proteins nonspecifically recovered in the immunoprecipitation procedure was particularly low with lysates of these specific cells. In the absence of highly sensitive antibodies against A20, we could detect it only by transfection of constructs for A20 fused with FLAG or HA tag.

For coimmunoprecipitation of endogenous proteins, HeLa S3 cells ($0.5–2 \times 10^8$) were rinsed in warm PBS and incubated for 5 or 10 min in the presence or absence of TNF (100 ng/ml). Cells were lysed for 45 min at 4°C in 1 ml lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate, 1× protease inhibitor cocktail (Complete, Boehringer Mannheim)). Cellular debris was removed by centrifugation, performed twice at 10,000 × g for 5 min. The cell lysates were precleared with 50 μl protein A-Sepharose beads and then incubated for 2 hr at 4°C with 2 μg anti-p55 TNF receptor polyclonal antibodies, 8 μg anti-p55 TNF receptor mAb 34 plus mAb 39 (1:1), or 2 μg anti-NEMO polyclonal antibodies, then mixed with 40 μl of a slurry of protein G-Sepharose (Pharmacia, 1:1 with PBS) and incubated for another 2 hr. The Sepharose beads were washed twice with 1 ml lysis buffer, twice with 1 ml high-salt (1 M NaCl) lysis buffer, and twice more with the regular lysis buffer. Aliquots corresponding to one-fourth of each sample were fractionated on 10% SDS-PAGE and transferred to a nitrocellulose membrane and probed with the indicated Abs. The antibodies were visualized with horseradish peroxidase–coupled sheep anti-mouse or anti-rabbit immunoglobulin, using the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Little Chalfont, UK), according to the manufacturer's instructions.

For coimmunoprecipitation of transfected proteins, HEK 293T cells ($2 \times 10^6$) were plated on 10 cm dishes and on the following day were transfected by the calcium phosphate precipitation method with the indicated expression plasmids, keeping the total amount of DNA constant (10 μg/dish) by supplementation with pcDNA3 vector. After 24 hr the cells were rinsed once with PBS and lysed in 1 ml lysis buffer (50 mM HEPES-NaOH [pH 7.6], 250 mM

NaCl, 0.1% NP-40, 5 mM EDTA). Lysates were incubated with 1 μg anti-FLAG, anti-His, or control mouse IgG at 4°C for 2 hr. The lysates were mixed with 25 μl of a 1:1 slurry of protein G-Sepharose and incubated for 2 hr. The Sepharose beads were washed with lysis buffer and then subjected to 10% SDS-PAGE and Western analysis as described above.

To examine the effect of the RIP DD and of the antisense of NEMO on the recruitment of IKK1 to the p55 TNF receptor, HeLa HtTA-1 cells were plated on 15 cm dishes ($5 \times 10^6$/plate) and transfected by the calcium phosphate precipitation method with a total of 30 μg DNA containing the appropriate expression plasmids. After 24 hr, cells were stimulated with TNF (100 ng/ml) for 10 min or left untreated. They were then pooled in batches of two plates ($\sim 10^7$ cells) and lysed in 1 ml lysis buffer. Immunoprecipitations were performed as described above, using 2 μg anti-p55 TNF receptor polyclonal antibodies.

To examine the association of A20 with the p55 TNF receptor complex and its effect on the recruitment of IKK1 to the receptor and to examine the effect of NEMO on IKK1 recruitment, HEK 293T cells were seeded on 10 cm plates ($2 \times 10^6$/plate). On the following day they were transfected by the calcium phosphate precipitation method with the indicated expression plasmids, applying a total of 25 μg DNA/plate. After 24 hr, cells were pooled in batches of five plates ($\sim 10^7$ cells) and lysed in 1 ml lysis buffer. Immunoprecipitations were performed as described above for the coimmunoprecipitation of endogenous proteins, using 2 μg anti-p55 TNF receptor polyclonal antibodies. In all cases presented, immunoprecipitation was performed at least thrice, with qualitatively identical results.

### In Vitro Kinase Assays

HeLa S3 cells ($5 \times 10^7$/sample) were stimulated with TNF (20 ng/ml) for 10 min or left untreated. Cell lysates were subjected to immunoprecipitation with 2 μg anti-p55 TNF receptor or 1 μg anti-NEMO polyclonal antibodies, as described above. The immunoprecipitates were further washed twice with 1 ml kinase buffer (20 mM HEPES-NaOH [pH 7.6], 2 mM DTT, 20 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM EDTA, 1 mM NaF, 20 mM β-glycerophosphate, and 0.1 mM $Na_3VO_4$). In vitro kinase reaction was allowed to proceed at 30°C for 45 min in 20 μl kinase buffer supplemented with 10 μCi [$\gamma$-$^{32}$P]ATP and 1 μg bacterially expressed GST-IκBα (1–54). The reaction was stopped with 20 μl SDS sample buffer, boiled for 5 min, fractionated on 10% SDS-PAGE, and visualized by autoradiography.

To examine the effect of A20 on IKK kinase activity, HeLa HtTA-1 cells were plated on 15 cm dishes ($5 \times 10^6$/dish) and transfected with the appropriate expression plasmids (a total of 30 mg DNA/dish) by the calcium phosphate precipitation method. After 24 hr, cells were stimulated with TNF (20 ng/ml) for 10 min. Dishes were paired and their cells ($\sim 10^7$) lysed in 1 ml lysis buffer. Immunoprecipitations were performed using 1 μg anti-NEMO polyclonal antibody, and the kinase assay was performed as described above.

### Acknowledgments

We thank Drs. Mark Boldin, Marcus Schuchmann, Eugene Varfolomeev, Rony Seger, Michael Kracht, and Helmut Holtmann and Ms. Tania Goncharov for their helpful suggestions. This work was supported in part by grants from Inter-Lab, Ness Ziona, Israel; Ares Trading SA, Switzerland; the Israeli Ministry of Arts and Sciences; and the Alfred and Ann Goldstein Foundation.

Received December 30, 1999; revised February 14, 2000.

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