A Role for NEMO/IKKγ Ubiquitination in the Activation of the IκB Kinase Complex by TNF-α

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SUMMARY

NEMO (NF-κB essential modifier)/IKKγ (IκB kinase-gamma) is required for the activation of the IκB kinase complex (IKK) by inflammatory stimuli such as tumor necrosis factor (TNF-α). Here we show that TNF-α stimulates the ubiquitination of NEMO in a manner that does not appear to target it for degradation, and that is impaired by mutations in the NEMO zinc finger. Mutations of the zinc finger are found in patients with hypohidrotic ectodermal dysplasia with immunodeficiency (HED-ID) and lead to the impairment of TNF-α-stimulated IKK phosphorylation and activation. In addition, the ubiquitination of NEMO is mediated by c-IAP1, an inhibitor of apoptosis protein that is a component of the TNF receptor signaling complex. Thus, the ubiquitination of NEMO mediated by c-IAP1 likely plays an important role in the activation of IKK by TNF-α. Also, defective NEMO ubiquitination may be responsible for the impaired cellular NF-κB signaling found in patients with HED-ID.
INTRODUCTION

The NF-κB/Rel family of transcription factors function in a wide range of biological activities including inflammation, immunity, and apoptosis and their activities are regulated by their interactions with IκB proteins (1-4). In unstimulated cells, NF-κB is kept inactive in the cytoplasm by virtue of the masking of its nuclear localization sequence by bound IκB protein. Exposure of cells to proinflammatory stimuli triggers the activation of a multisubunit IκB kinase (IKK) complex that phosphorylates IκB proteins on two serine residues (5-7). Phosphorylation of IκB proteins triggers their polyubiquitination and their subsequent recognition and degradation by the proteasome. Destruction of IκB proteins liberates NF-κB to enter the nucleus and activate gene expression (2,3,8).

The predominant IKK complex found in cell lines contains two catalytic subunits, IKKα (or IKK1) and I KKβ (or IKK2) (9-13), and a regulatory subunit, NEMO (or IKKγ) (14-17). I KKα and I KKβ are serine/threonine protein kinases, whereas NEMO contains several protein interaction motifs but no apparent catalytic domains. Subunit reconstitution experiments in yeast and mammalian cells suggest that IKK is composed of a NEMO homodimer bound together with either an I KKα/IKKβ heterodimer or an I KKβ homodimer (18). Although structurally similar, I KKα and I KKβ have distinct cellular functions. I KKβ phosphorylates IκB and is critical for IKK and NF-κB activation in response to proinflammatory stimuli (19-21). In contrast, I KKα phosphorylates the NF-κB2/p100 precursor and stimulates its processing in a fashion that is independent of the IKK complex (22). The biochemical mechanisms underlying the activation of IKK in response to proinflammatory stimuli are not well understood. Although it is known that phosphorylation of Ser 179 and Ser 181 in the activation loop of I KKβ is required and may be sufficient for IKK activation by proinflammatory stimuli (12,23), the biochemical mechanisms regulating these phosphorylation events are not clear.
NEMO was initially identified by complementation cloning in a rat cell line in which NF-κB could not be activated (16). It was subsequently also isolated by biochemical purification of the IKK complex and microsequencing (14,17). Structural predictions indicate that NEMO contains several coiled-coil domains, a leucine zipper, and a C-terminal zinc finger. Genetic studies suggest that NEMO is absolutely required for the activation of IKK and NF-κB in response to diverse stimuli (16,24-26). In humans, amorphic mutations in the NEMO gene that are caused by large genomic deletions are responsible for familial incontinentia pigmenti (IP) (27) whereas hypomorphic NEMO mutations are found in patients with hypohidrotic ectodermal dysplasia with immunodeficiency (HED-ID) (27-31). A subset of patients with HED-ID carry mutations in the predicted zinc finger domain of NEMO and the integrity of this domain has been shown to be important for full IKK activation in response to TNF-α in murine embryonic fibroblasts (32). Although it is known that NEMO is required for the formation of a high-molecular-weight IKK complex (16), the exact role of NEMO in IKK activation remains unclear. Because NEMO has been found to interact with a variety of signaling molecules, it has been proposed that NEMO may link upstream activators to the IKK complex (7).

In this report, we identify a role for the NEMO zinc finger in NEMO ubiquitination. We find that NEMO is ubiquitinated in response to TNF-α in a manner that does not appear to target it for degradation and that is dependent on an intact zinc finger. The inhibitor of apoptosis protein c-IAP1 mediates the ubiquitination of NEMO in response to TNF-α and functions as an E3 ligase to ubiquitinate NEMO both in vitro and in vivo. In addition, NEMO proteins containing mutations in the zinc finger show impaired ubiquitination both by c-IAP1 and in response to TNF-α. These mutants also demonstrate impaired abilities to rescue TNF-α-induced IKK activation in a NEMO-deficient cell line. Our results suggest that NEMO ubiquitination mediated by c-IAP1 is essential for the proper activation of the IKK signalsome complex by TNF-α.
EXPERIMENTAL PROCEDURES

Cell Culture - HEK 293T and Hela cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum. Jurkat cells and the NEMO-mutant JM4.5.2 cell line (gift of S.-C. Sun) were cultured in RPMI (Gibco) supplemented with 10% fetal bovine serum. All cells were grown in a 37°C incubator with 5% CO₂. Transfections of 293T and Hela cells were performed with LipofectAMINE (Life Technologies) according to the manufacturer's instructions. Transfections of the JM4.5.2 cell line was performed using DEAE-dextran.

Plasmids - The human NEMO cDNA was PCR amplified from an IMAGE Consortium EST clone (#41072) and inserted into pcDNA3-HA. The ∆ZF (codons 1 to 392) and C417R (Cys 417 mutated to Arg) NEMO mutants were created using standard PCR procedures. Human Ub cDNA were PCR amplified from a human lymphocyte/leukocyte cDNA library (Clonetech) and subcloned into pcDNA3-FLAG and pcDNA3-AU1. The Ub 7R, K48R, and G76A mutants were created by standard PCR procedures. c-IAP1 and XIAP cDNAs were PCR amplified and subcloned into pcDNA3-FLAG and pcDNA3-Myc vectors. The Δ121 (codons 122-618), Δ321 (codons 322-618), 1-396 (codons 1 to 396), ΔRING (codons 1 to 570), C605A (Cys 605 mutated to Ala), and RING (codons 562 to 618) c-IAP1 mutants were constructed using standard PCR procedures. Mutations were verified by automated DNA sequencing (University of Michigan DNA Sequencing Core).

Immunoprecipitations - For the visualization of endogenous modified NEMO protein, Hela cells were lysed in IP lysis buffer (50 mM Tris pH 7.6, 250 mM NaCl, 1% Triton X-100, 0.5% NP-40, 3 mM EGTA, 3 mM EDTA, 10% glycerol, 0.1 mM Na₃VO₄, 10 µM NEM) supplemented with a cocktail of protease inhibitors (Roche). Lysates were incubated with NEMO antibody and Protein G sepharose and immunoprecipitates were washed four times with IP lysis buffer and once with IP lysis buffer supplemented with 3M urea. All immunoprecipitates were boiled in SDS-PAGE sample buffer and resolved on a
7.5% polyacrylamide gel. Proteins were transferred to PVDF membrane and probed with Ub or NEMO antibodies. To minimize the detection of the IgG heavy chain, protein A conjugated to horseradish peroxidase (Zymed) was used as a secondary antibody for anti-NEMO immunoblots.

For coimmunoprecipitation assays, cells were lysed in EBC150 (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 0.1 mM Na3VO4, 1 mM DTT) supplemented with a cocktail of protease inhibitors (Roche) and lysates were incubated with the appropriate antibody for 2 hrs at 4°C and with Protein G sepharose for 1 additional hr. All immunoprecipitates were boiled in SDS-PAGE sample buffer and resolved on a 7.5% polyacrylamide gel.

**RNAi knockdown experiments** - For the knockdown of c-IAP1 expression, we employed a strategy similar to that described previously (33). An oligo corresponding to a hairpin siRNA targeting a unique 19 bp c-IAP1 mRNA sequence (GGAAAUGCUGCGGCCAACA) was subcloned into pTKO, a vector that directs the expression of siRNAs driven by the H1 RNA promoter and contains a puromycin selection marker (E.T. and K.G., manuscript in prep). One day following transfection of 293T cells with pTKO plasmids, transfected cells were selected for by incubation in media containing puromycin (3 ug/ml) (Sigma) for 72 hrs. Whole cell lysates were subsequently prepared for immunoblot or immunoprecipitation analysis.

**Ub conjugation assays** - Ubiquitination reactions were performed in 20 µl reactions containing 25 mM Tris (pH 7.5), 50 mM NaCl, 10 mM MgCl2, 2 mM ATP, and 0.5 mM DTT. Where necessary, rabbit E1 (Boston Biochemical) (40 ng), bovine Ub (Sigma) or His6-tagged Ub (1 µg), His6-UbcH5C or related E2 (0.2 µg), GST-c-IAP1 (0.2 µg), and MBP-NEMO (0.1 µg), and mutant variants were included. Reactions were incubated at 30°C for 1 hr, terminated by addition of SDS-PAGE sample buffer, and separated on 7.5% polyacrylamide gels. Proteins were transferred to PVDF membrane and probed with MBP antibody. AU1-tagged wild-type and mutant Ub cDNAs were cloned into pET3E-His.
Wild-type and mutant NEMO coding sequences were cloned into pMAL-c2 (NEB) for expression as MBP fusion proteins. c-IAP1 wild-type and mutant cDNAs were cloned into pGEX-KG for expression as GST fusion proteins. GST fusion, MBP fusion, and His$_6$-tagged proteins were expressed in the *E. Coli* strain BL21(DE3)RIL (Stratagene) which harbors a helper plasmid which encodes the AGA-specific ArgU tRNA. His-tagged UbcH3, UbcH6, UbcH10, and UbcH13, and UbcH7 were purchased from Boston Biochemical.

**Retroviral Infections** - JM4.5.2 cells stably expressing NEMO proteins were created by retroviral infections. HA-tagged wild-type and mutant (C417R) NEMO cDNAs were PCR amplified and subcloned into the retroviral expression vector pPGS-CMV-CITE-neo (gift of G. Nabel). Amphotropic Phoenix packaging cells (gift of G. Nolan) in 6-well plates were transfected with 2 µg of retroviral expression plasmid using Lipofectamine 2000 reagent (Gibco). 48 hrs following transfection, the viral supernatants were supplemented with polybrene (9 mg/ml) and filtered through a 0.45 µm filter. Viral supernatants were incubated with 1 x 10$^6$ JM4.5.2 cells and spun at 1800 rpm for 45 min at room temperature. Infected cells were resuspended in regular growth media containing 1 µg/ml G418 (Gibco) and stably infected cells were selected for one week.

**Kinase assays** - To measure IKK activity, cells were lysed in kinase lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1mM EDTA, 30 mM NaF, 2 mM sodium pyrophosphate, 0.1 mM Na$_3$VO$_4$, 1 mM DTT) and anti-NEMO immunoprecipitates were incubated in 20 µl reactions containing 50 mM Tris pH 7.5, 10 mM MgCl$_2$, 15 µM ATP, 1 mM DTT, 0.5 µg GST-IκBα(1-54), and 5 µCi [$\gamma$-$^32$P]ATP (ICN) at 30°C for 20 min. Reactions were terminated by the addition of SDS-PAGE sample buffer and resolved on a 10% polyacrylamide gel. Proteins were transferred to PVDF membrane and exposed to X-ray film. GST-IκBα(1-54) was purified from DH5α bacteria transformed with pGEX-IκBα(1-54) (gift of M. Karin).
RESULTS AND DISCUSSION

Ubiquitination of NEMO in cells - An earlier report described the purification of a multiprotein IκBα kinase complex that could be activated in vitro by a ubiquitination event (34). One possibility is that ubiquitination of IKK may regulate its activity. In order to investigate whether IKK may be ubiquitinated during the course of its activation by TNF-α, we immunoprecipitated individual IKK subunits from Hela cells following stimulation for various time periods. Interestingly, TNF-α treatment induced the accumulation of a ladder of slower migrating forms of NEMO (Fig. 1A). These modifications on NEMO paralleled the phosphorylation and degradation of IκBα. On the other hand, we failed to detect similar modification of IKKα or IKKβ under the same conditions (data not shown).

Immunoblotting using an ubiquitin (Ub) antibody suggested that TNF-α stimulated the modification of NEMO by different numbers of Ub molecules (Fig. 1B). NEMO-Ub conjugates were also detected in transient transfection experiments using 293T cells in which both epitope-tagged Ub and NEMO were expressed (Fig. 1C). An "inactive" Ub mutant that contained a substitution of Ala for the terminal Gly residue (G76A) (35) was unable to support NEMO ubiquitination.

Because the experiments described above were performed in the absence of proteasome inhibitor, the NEMO-Ub conjugates seen in vivo were apparently not targets for immediate destruction by the proteasome. To examine this idea further, we compared NEMO-Ub conjugates with E2F-4-Ub conjugates, which are known to be targeted for degradation by the Ub-proteasome pathway (36). As expected, E2F-4-Ub conjugates were detectable only when cells were incubated with a proteosome inhibitor (Fig. 1D). In contrast, NEMO-Ub conjugates were readily visible in the absence of proteasome inhibitor and were only slightly altered in the presence of proteasome inhibitor. Thus, the ubiquitination of NEMO may have primarily a regulatory role, rather than a role in protein stability.
Ubiquitination of NEMO is dependent on its zinc finger - All NEMO orthologs identified to date contain predicted zinc finger motifs at their extreme C-termini (5-7) (Fig. 2A). The zinc finger has been found to be the frequent target of mutations found in the NEMO gene loci of patients with the congenital disorder hypohidrotic ectodermal dysplasia with immunodeficiency (HED-ID) (27-31). Cells from these patients display defective NF-κB signaling (29,30). Recently, the zinc finger was reported to be partially required for IKK activation in response to TNF-α in mouse embryonic fibroblasts (32). We found, however, that deletion of the zinc finger in NEMO did not affect its binding to IKKα, IKKβ, RIP, or A20 in coimmunoprecipitation assays (data not shown). To test whether the zinc finger might be important for NEMO ubiquitination, we examined two NEMO mutants. One mutant contained a deletion of the last 27 residues comprising the predicted zinc finger (ΔZF) and another had a substitution of Arg for Cys 417 (C417R) as seen in an HED-ID patient (29-31) (Fig. 2A). Cys 417 is one of the predicted zinc coordinating residues of the zinc finger. When wild-type and mutant NEMO proteins were coexpressed with Ub in cells, the ubiquitination of wild-type NEMO was enhanced by TNF-α, as seen with endogenous NEMO (Fig. 2B). In contrast, the ubiquitination of neither of the two zinc finger mutants was enhanced by TNF-α treatment. An intact zinc finger in NEMO therefore appears to be necessary for the stimulation of NEMO ubiquitination by TNF-α.

Engagement of tumor necrosis factor receptor 1 (TNFR1) by TNF-α leads to the recruitment of several molecules to the receptor complex including TRAF2, c-IAP1 and the IKK complex (37-39). TRAF2 and c-IAP1 can both function as E3s (Ub ligases) via their RING fingers motifs (40-42). E3s function together with E2s (Ub-conjugating enzymes) to catalyze protein ubiquitination (43). We examined whether expression of either of these two Ub ligases could affect NEMO ubiquitination. Transfection of c-IAP1, but not TRAF2, markedly enhanced the levels of NEMO-Ub conjugates without decreasing the steady-state levels of NEMO (Fig. 3A). Another related inhibitor of apoptosis protein, XIAP, had a relatively smaller effect on NEMO ubiquitination. Since the E3 activity of c-IAP1 is
dependent on its C-terminal RING finger domain (42), we examined whether the RING finger was required for c-IAP1 to enhance NEMO ubiquitination. Deletion of the RING finger prevented c-IAP1 from enhancing NEMO-Ub conjugates (Fig. 3B). A fragment of c-IAP1 containing the RING finger alone was significantly less active in elevating NEMO ubiquitination, suggesting that other sequences of c-IAP1 were important also. Thus, the stimulation of NEMO ubiquitination by c-IAP1 is RING finger-dependent and may depend on the E3 activity of c-IAP1.

**Ubiquitination of NEMO in vitro by c-IAP1 and UbcH5C** - Next, we investigated whether c-IAP1 might be able to function as an E3 to ubiquitinate NEMO using purified components *in vitro*. In our ubiquitination assay we used rabbit E1 (Ub activating enzyme), bovine Ub, and bacterially expressed UbcH5C, c-IAP1, and NEMO. A UbcH5 family member had been previously shown to both function as an E2 in conjunction with c-IAP1 to ubiquitinate proteins (42) and mediate IKK activation in vitro (34). Inclusion of E1, UbcH5C, and c-IAP1 in ubiquitination reactions was sufficient to catalyze the ubiquitination of NEMO (Fig. 3C). Ubiquitination was absolutely dependent on E1, UbcH5C, and c-IAP1 each because no ubiquitination was observed when any one of these components was omitted.

Given that an intact zinc finger in NEMO is required for its ubiquitination in response to TNF-α (Fig. 2B), we examined whether the zinc finger might be important for ubiquitination in vitro by c-IAP1. We compared the ubiquitination of wild-type NEMO and zinc finger mutants by UbcH5C/c-IAP1. Like wild-type NEMO, both the ΔZF and C417R mutants were also ubiquitinated by UbcH5C/c-IAP1, but less efficiently (Fig. 3D). Thus, an intact zinc finger appears to be important for NEMO to be efficiently recognized by UbcH5C/c-IAP1 as a substrate for ubiquitination.

Ubiquitination of NEMO was also observed in reactions where His₆-tagged Ub purified from bacteria was used as a source of Ub. However, these reactions yielded higher migrating conjugates than those seen when using bovine Ub, a difference that may be
attributable to the presence of inhibitors of ubiquitination in the bovine Ub purification (Fig. 3E). In order to determine whether NEMO was conjugated to polyUb chains in vitro by UbcH5C/c-IAP1, we tested a lysine-less Ub mutant (7R) in which all 7 Lys residues are replaced by Arg residues (Fig. 3F). In contrast to wild-type Ub, the 7R mutant cannot be used to extend a polyUb chain. Reactions using wild-type Ub contained higher molecular weight conjugates not seen when using the 7R mutant, suggesting that NEMO-polyUb conjugates were assembled (Fig. 3G). The ability of UbcH5C to catalyze polyubiquitination of NEMO was specific among several E2 enzymes that were tested (Fig. 3E). Also, unlike c-IAP1, the other RING finger E3s ROC1 and HDM2 failed to polyubiquitinate NEMO under similar conditions (data not shown).

As expected, the ability of c-IAP1 to function as an E3 was absolutely dependent on its RING finger because deletion of the RING finger (∆RING) or substitution of Cys 605 with Ala (C605A) rendered c-IAP1 inactive in ubiquitinating NEMO (Fig. 3H). The RING finger alone could also promote polyubiquitination, albeit less effectively than wild-type c-IAP1. We have found previously that the ROC1 RING finger alone is sufficient to activate UbcH5C in catalyzing polyubiquitin chains (44). Our results here suggest that the c-IAP1 RING finger may function in a similar manner. Taken together with our observation that the RING finger alone is also less effective than full length NEMO in stimulating ubiquitination in vivo (see Fig. 3B), it appears that sequences outside of the c-IAP1 RING finger are required for optimal substrate ubiquitination, perhaps by serving as a NEMO binding site (see below).

Ubiquitination of NEMO by non-Lys 48-linked polyUb chains - Most protein ubiquitination studied to date involves the conjugation of Lys 48-linked polyUb chains to proteins, leading to their subsequent recognition and degradation by the proteasome. Given our findings that ubiquitinated NEMO is not targeted for degradation, we examined the polyUb chains assembled by c-IAP1 in more detail. ROC1 is a RING finger protein that is a component of the multisubunit E3 SCF complex that targets proteins such as IκB and β-
catenin for degradation. We found previously that ROC1 alone is sufficient to function as an E3 with UbcH5C to catalyze polyUb chains (44). When c-IAP1 and ROC1 were tested for their abilities to function as E3s in \textit{in vitro} assays, we found that Lys 48 in Ub was required for polyUb assembly by ROC1 but not by c-IAP1 (Fig. 3I). Thus, the identity of the particular RING finger E3 can dictate the structure of the polyUb chains that can be assembled in conjunction with UbcH5C.

Further experiments using single lysine Ub mutants (Fig. 3F) demonstrated that c-IAP1 could specifically assemble Lys 6-linked polyUb chains (Fig. 3J). When NEMO was included as a substrate, c-IAP1/UbcH5C was found to be able to catalyze the modification of substrate by Lys 6-linked polyUb chains (Fig. 3K). Of note, the assembly of Lys 6-linked chains was noticeably less efficient than the assembly of wild-type polyUb chains (Fig. 3J). This disparity can likely be attributed to the effect of the six lysine to arginine substitutions on protein structure. However, we cannot rule out the possibility from these experiments that other lysines in Ub other than Lys 6 can also be used in polyUb chains.

\textit{Role of c-IAP1 in IKK activation by TNF-\(\alpha\)} - Given the ability of c-IAP1 to stimulate NEMO ubiquitination, we wanted to examine if c-IAP1 could stimulate NF-\(\kappa\)B activity as well. Overexpression of c-IAP1 in cells was able to activate NF-\(\kappa\)B and this activity was dependent on the RING finger of c-IAP1 (Fig. 4A). Expression of the c-IAP1 RING finger mutants \(\Delta\)RING and C605A had varying effects on NF-\(\kappa\)B activation by TNF-\(\alpha\), with the latter mutant having a more potent dominant interfering effect (Fig. 4A). Also, the ability of c-IAP1 to activate NF-\(\kappa\)B was dependent on the presence of NEMO, as suggested in experiments with NEMO-deficient cells (Fig. 4B). When coexpressed in 293T cells, c-IAP1 and NEMO could coimmunoprecipitate together (Fig. 4C). Endogenous c-IAP1 and NEMO were also found to associate together in Hela cells in a stimulus-independent manner (Fig. 4D). Past reports have suggested that c-IAP1 is recruited to the receptor complex by binding to TRAF2 (39,45). We compared the abilities
of TRAF2 and NEMO to bind to several deletion mutants of c-IAP1 in transient transfection assays. These results suggested that TRAF2 and NEMO bind to distinct domains outside of the RING finger of c-IAP1 (Fig. 4E). Experiments performed with these c-IAP1 deletion mutants suggested that both the RING finger and the ability to bind NEMO was required to activate NF-κB (Fig. 4A). Since TRAF2 directly binds to c-IAP1 and is required for recruitment of both c-IAP1 and NEMO to the signaling complex (38,45), we examined whether c-IAP1 could bridge an interaction between TRAF2 and NEMO. When NEMO and TRAF2 were coexpressed in the absence of transfected c-IAP1, a relatively small amount of NEMO was found bound to TRAF2 (Fig. 4F). However, coexpression of c-IAP1 significantly enhanced the NEMO-TRAF2 interaction. A c-IAP1 mutant that was defective in binding to TRAF2 but not NEMO (Δ121) (see Fig. 4E) failed to increase the interaction. Thus, c-IAP1 may mediate the recruitment of NEMO to TRAF2 and the TNFR1 signaling complex as well as regulate NEMO ubiquitination.

To see if endogenous c-IAP1 was required for IKK activation by TNF-α, we used RNA interference (RNAi) to deplete endogenous c-IAP1. We examined the TNF-α response of 293T cells that were transfected with a hairpin short interfering RNA (siRNA) expression plasmid that reduces the expression of c-IAP1 but did not effect the expression of the related proteins c-IAP2 or XIAP (Fig. 4G). Cells transfected with the c-IAP1 siRNA plasmid displayed impaired IKK activation and IκBα phosphorylation in response to TNF-α (Fig. 4H, J). Expression of the RING finger C605A mutant of c-IAP1 was able to function as a dominant interfering mutant in suppressing IKK activation as well (Fig. 4I). Furthermore, cells transfected with the c-IAP1 siRNA plasmid displayed impaired the ubiquitination of endogenous NEMO in response to TNF-α, suggesting that NEMO ubiquitination is mediated by c-IAP1 in vivo (Fig. 4J). Of note, we did not find any differences in rates of apoptosis between cells transfected with c-IAP1 siRNA plasmid and control cells (data not shown). These data together suggest an important role for c-IAP1 in mediating both IKK activation and NEMO ubiquitination in response to TNF-α.
Requirement of the NEMO zinc finger in TNF-α-induced IKK activation - Given that cells from patients with HED-ID have been reported to display defective NF-κB signaling (29,30), we sought to determine if the NEMO zinc finger was important for TNF-α-induced NF-κB activation. We compared the functional activities of wild-type NEMO and zinc finger mutants in JM4.5.2 cells, a T cell line that lacks expression of NEMO (46). These cells have been previously shown to be defective in activating NF-κB in response to PMA/ionomycin or the retroviral oncoprotein Tax (46,47). JM4.5.2 cells also failed to activate NF-κB in the response to TNF-α, and this defect was restored by the expression of wild-type NEMO (Fig. 5A). In contrast, both zinc finger NEMO mutants, ΔZF and C417R, demonstrated impaired abilities to restore the activation of an NF-κB luciferase reporter by TNF-α, suggesting that an intact zinc finger is important for NF-κB activation (Fig. 5A).

To examine the importance of the NEMO zinc finger in IKK function, we stably expressed wild-type NEMO or the C417R mutant in JM4.5.2 cells by retroviral infection. As expected, the C417R mutant displayed defective TNF-α-stimulated ubiquitination when compared to wild-type NEMO (Fig. 5B). Significantly, we found that in contrast to wild-type NEMO, the C417R mutant was unable to restore TNF-α-induced phosphorylation of IκBα (Fig 5C). The activation of IKK was also not restored (Fig. 5D) even though IKK complexes displayed similar levels of IKKα, IKKβ, and NEMO proteins (data not shown). Thus, the zinc finger of NEMO is necessary for proper IKK activation, but not for its incorporation into the IKK complex. Furthermore, wild-type but not mutant NEMO protein could restore TNF-α-induced phosphorylation of IKKβ on Ser 181 in its activation loop (Fig. 5D). The defect in restoring NF-κB activation by the NEMO mutants can therefore be attributed to defective IKK phosphorylation and activation in these cells.

In summary, our results suggest that the conserved zinc finger of NEMO is required for both NEMO ubiquitination and IKK activation by TNF-α. The inhibitor of apoptosis protein c-IAP1, which is recruited to the TNF receptor complex, is an important mediator of
IKK activation and NEMO ubiquitination in response to TNF-α. Thus, NEMO ubiquitination by c-IAP1 appears to be a critical event in the activation of IKK in response to TNF-α. In addition, our data from in vitro Ub assays suggest that the identity of the RING finger E3 can dictate the lysine of Ub that is used in the polyUb chains that are assembled. A recent report found that mutation of two cysteines in the zinc finger of NEMO distinct from Cys 417 impaired TNF-α but not IL-1-induced IKK activation in MEFs (32). These results are in accordance with our findings and together with our data suggests that NEMO ubiquitination by c-IAP1 plays a specific role in TNF-α signaling.

Interestingly, previous work described the identification of a purified IKK complex that could be activated by a ubiquitination event involving a UbcH5 family member and an unidentified factor (34). Based on our findings, c-IAP1 may be this unidentified factor and NEMO may be the target for ubiquitination. TRAF2 is another E3 that plays an important role in IKK activation in response to TNF-α (38). Unlike, c-IAP1, TRAF2 does not appear to mediate NEMO ubiquitination. TRAF2 may instead play a role upstream of IKK activation in TNF-α signaling by ubiquitinating itself with Lys 63-linked polyUb chains, in a similar mechanism as has been postulated for TRAF6 in the IL-1 signaling pathway (48-50).

It remains to be determined what the exact role of the NEMO zinc finger in NEMO ubiquitination and IKK activation is. We have found that removal of the zinc finger does not affect the binding of NEMO to c-IAP1 (E.T. and K.G., unpublished data). It is possible that the zinc finger may facilitate the assembly of other ubiquitination factors such as UbcH5C. The ubiquitination of NEMO may in turn lead to the recruitment of an IKKβ kinase such as MEKK3 or ζPKC (51,52) to the IKK complex, thereby leading to IKKβ phosphorylation and consequently IKK activation.

ABBREVIATIONS- The abbreviations used are: TNF-α, tumor necrosis factor alpha; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; IKK, IκB kinase; MEF, mouse embryonic
fibroblasts; MEKK1, mitogen-activated protein kinase kinase 1; TAK1, transforming growth factor-β-activated kinase 1; ζPKC, zeta isoform of protein kinase C; DTT, dithiothreitol.

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FIGURE LEGENDS

FIG. 1 NEMO is ubiquitinated in vivo. (A) Hela cells were stimulated with human TNF-α (10 ng/ml) for the indicated time periods and anti-NEMO immunoprecipitates were probed with NEMO antibody (Santa Cruz Biotechnology, FL-419) as indicated. Both short and long exposures after immunoblotting with NEMO antibody are shown. Lysates were immunoblotted with phospho-IκBα (Cell Signaling) and IκBα (Santa Cruz Biotechnology, C-21) antibodies. (B) Hela cells were stimulated with TNF-α (10 ng/ml) for 5 min where indicated and lysed. Anti-NEMO immunoprecipitates were probed a monoclonal Ub antibody (Santa Cruz Biotechnology, P4D1) (top) or NEMO antibody (bottom). (C) FLAG-tagged wild-type Ub or Ub (G76A) were coexpressed in 293T cells with HA-NEMO where indicated. Extracts were prepared as described previously (53) under denaturing conditions and anti-HA (Santa Cruz Biotechnology, Y-11) immunoprecipitates were probed with FLAG M2 antibody (Sigma). (D) HA-NEMO or HA-E2F-4 was coexpressed in 293T cells with FLAG-Ub as indicated. Cells were treated with MG132 (10
µM) or carrier (DMSO) 6 hrs prior to lysis. Immunoprecipitations were performed as in (C) and immunoblotting was performed with HA (Covance) or FLAG M2 antibody.

**FIG. 2 An intact zinc finger in NEMO is required for ubiquitination in vivo.** (A) Depicted is an alignment of the sequences of the predicted zinc fingers found in the C-termini of NEMO orthologs from *H. sapiens* (residues 393 to 419), *M. musculus* (residues 386 to 412), *X. laevis*, and *D. melanogaster* (residues 359 to 387). The residues that are conserved in all species are in bold. The position of the predicted zinc-coordinating residues are indicated by the asterisks. The arrow indicates the position of Cys 417 in human NEMO. The peptide sequence from *X. laevis* NEMO was derived from an EST sequence from a partial cDNA clone and ends with the predicted terminal residue. Accession numbers for the sequences are 6685695 (*H. sapiens*), 6093481 (*M. musculus*), 15044130 (*X. laevis*), and 9937530 (*D. melanogaster*). (B) HA-tagged wild-type and mutant NEMO proteins were coexpressed in 293T cells with FLAG-Ub as indicated. Transfected cells were treated with TNF-α (10 ng/ml) prior to lysis. Immunoprecipitations were performed and analyzed as in Fig. 1D.

**FIG. 3 c-IAP1 functions as an E3 to ubiquitinate NEMO.** (A) FLAG-c-IAP1, FLAG-XIAP, and FLAG-TRAF2 were coexpressed in 293T cells with AU1-Ub and HA-NEMO where indicated. Immunoprecipitations and cell lysates were analyzed as in Fig. 1b with HA, AU1, or FLAG M2 antibody. (B) HA-NEMO and various FLAG-tagged c-IAP1 proteins were coexpressed with AU1-Ub in 293T cells as indicated. Immunoprecipitations were analyzed as in (A). (C) Ubiquitination assays were performed with E1, His6-UbcH5C, GST-c-IAP1, MBP-NEMO, and bovine Ub where indicated. Reactions were probed with MBP antibody. (D) Ubiquitination assays containing E1, His6-UbcH5C and bovine Ub were performed in the presence or absence of GST-c-IAP1 as indicated. MBP or recombinant wild-type NEMO or mutant zinc finger proteins were tested. (E)
Ubiquitination assays were performed as in (C) except that His$_6$-tagged Ub was used. His$_6$-UbcH5C, His$_6$-UbcH3, His$_6$-UbcH6, UbcH7, His$_6$-UbcH10, or His$_6$-UbcH13 was also included where indicated. (F) Schematic representation of Ub mutants used in this study. (G) Ubiquitination assays were performed using E1, His$_6$-UbcH5C, MBP-NEMO, and GST-c-IAP1 where indicated. His$_6$-tagged wild-type Ub (WT) or a lysine-less (7R) was tested for conjugation to MBP-NEMO. (H) Ubiquitination assays were performed with E1, His$_6$-UbcH5C, His$_6$-Ub, MBP-NEMO, and either wild-type, ΔRING, C605A, or RING GST fusion proteins as indicated. (I) Ubiquitination assays were performed with E1, His$_6$-UbcH5C (lanes 2-4, 6-8), GST-c-IAP1 (lanes 1-4), and GST-ROC1 (lanes 5-8) as shown. His$_6$-tagged wild-type Ub (WT) or the lysine-less (7R) or K48R mutant were included where indicated. Reactions were probed with Ub antibody. (J) Ubiquitination assays were performed with E1, His$_6$-UbcH5C, GST-c-IAP1 (lanes 2-10), and His$_6$-tagged wild-type Ub (WT), lysine-less (7R), or single lysine Ub mutants as indicated. (K) Ubiquitination assays were performed with E1, His$_6$-UbcH5C, GST-c-IAP1, and MBP-NEMO where indicated. His$_6$-tagged wild-type Ub (WT), lysine-less (7R), or single lysine Ub mutants were included as indicated. MBP-NEMO was immunoprecipitated from reactions using anti-NEMO antibody and probed with Ub antibody.

**FIG. 4** TNF-α-stimulated IKK activation and NEMO ubiquitination are mediated by c-IAP1 in vivo. (A) 293T cells were transfected with pNF-κB-luciferase, pCMV-β-gal, and pcDNA3 (vector) or FLAG-tagged wild-type and mutant c-IAP1 proteins as indicated. 24 hrs following transfection, extracts were analyzed for luciferase activity. Results were normalized to β-gal activity readings. Where indicated, TNF-α (10 ng/ml) was added 6 hrs prior to extract preparation. Samples were performed in duplicate and results shown are representative of three independent experiments. (B) NEMO-deficient JM4.5.2 cells were transiently transfected with HA-NEMO and FLAG-c-IAP1 or FLAG-c-IAP1(C605A) where indicated along with pNF-κB-luciferase and pCMV-β-gal. 48 hrs after transfection,
cell extracts were prepared and analyzed for luciferase activity as in (C). (C) HA-NEMO and Myc-c-IAP1 were coexpressed in 293T cells as indicated. Anti-Myc immunoprecipitates were separated by SDS-PAGE and immunoblotted with HA antibody. (D) Hela cells treated or untreated with 10 ng/ml of TNF-α for 10 min were lysed and immunoprecipitates prepared using the antibodies indicated. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with NEMO antibody. (E) The horizontal bars signify the sequence of c-IAP1 with BIR domains and RING finger (RG) indicated. The interactions between Myc-tagged wild-type and mutant c-IAP1 with FLAG-TRAF2 and HA-NEMO were discerned by coimmunoprecipitation assays in 293T cells. A plus sign indicates that an interaction was seen while a minus sign indicates that an interaction was not detected. Protein expression was confirmed by immunoblotting of cellular lysates. (F) HA-NEMO was coexpressed with FLAG-TRAF2 and Myc-c-IAP1 or Myc-c-IAP1Δ121 in 293T cells where indicated. Anti-HA immunoprecipitates were examined as in (E) using HA or FLAG antibody. (G) 293T cells transfected with empty vector (pTKO) or an c-IAP1 siRNA expression plasmid were selected for using puromycin. Whole cell extracts were probed with c-IAP1, c-IAP2 (R&D Systems), or XIAP (BD Transduction Laboratories) antibodies. (H) 293T cells transfected with empty vector (pTKO), an c-IAP1 siRNA expression plasmid (c-IAP1 RNAi) or control siRNA plasmid (TRAF6 RNAi) were selected for using puromycin and treated with TNF-α (10 ng/ml) where indicated. Anti-NEMO immunoprecipitates were assayed for IKK activity (top) or probed for IKK subunits by immunoblotting (bottom) using IKKα (Santa Cruz Biotechnology, H-744), IKKβ (BD Transduction Laboratories) or NEMO antibodies. (I) 293T cells transfected empty vector (lanes 1, 2) or Myc-c-IAP1(C605A) (lanes 3, 4) and FLAG-IKKβ (lanes 1-4) were lysed and anti-FLAG immunoprecipitates were assayed for IKK activity (top) or FLAG-IKKβ protein. (J) 293T cells transfected with pTKO or c-IAP1 siRNA expression plasmid were selected for using puromycin and anti-NEMO immunoprecipitates were
probed using Ub or NEMO antibodies. Also, lysates were examined for phosphorylated IκBα or total IκBα protein.

**FIG. 5 The NEMO zinc finger is required for IKK activation by TNF-α.** (A) The NEMO-deficient JM4.5.2 cell line was transiently transfected with pNF-κB-luc, pCMV-β-gal, and pcDNA3 (vector) or HA-tagged wild-type NEMO (WT), ΔZF mutant, or C417R mutant as indicated. 40 hrs following transfection, cells were stimulated with TNF-α (10 ng/ml) for 8 hrs (lanes 5-8) or untreated (lanes 1-4) and lysed. Extracts were assayed for luciferase activity as in Fig. 4A. The results shown are the average of three independent experiments. (B) Stably infected JM4.5.2 cells expressing wild-type NEMO or the C417R mutant was untreated or treated with TNF-α (10 ng/ml) for 5 min. Lysates from 5 x 10⁶ cells were prepared and anti-NEMO immunoprecipitates were analyzed as in Fig. 1B using Ub or NEMO antibodies. (C) Stably infected JM4.5.2 cells were untreated or treated with TNF-α (10 ng/ml) for 5 min. Lysates prepared from 5 x 10⁶ cells stably expressing the indicated NEMO proteins were prepared and were run out by SDS-PAGE. Immunoblotting was performed with phospho-IκBα antibody. (D) IKK activity was measured from stably infected JM4.5.2 cells untreated or treated with TNF-α (10 ng/ml) for indicated times (top and bottom). Alternatively, immunoprecipitates were probed with a phospho-IKKβ antibody (Cell Signaling) (top). Phosphorylation of GST-IκBα(1-54) was analyzed on a phosphoimager and the intensity of bands was calculated using the Image Quant Microsoft system.

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Fig. 1

A

TNF-\(\alpha\) (min): 0 2 5 10 20 30

NEMO-U\(b_n\)

NEMO

p-I\(\kappa\)B\(\alpha\)

total I\(\kappa\)B\(\alpha\)

B

TNF-\(\alpha\) (min): 0 5

NEMO-U\(b_n\)

NEMO

C

FLAG-U\(b\): + - - + -
FLAG-U\(b\)(G76A): - + - - +
HA-NEMO: - - + + +

184

121

86

69

NEMO-U\(b_n\)

IP: HA

IB: FLAG

D

NEMO

E2F-4

FLAG-U\(b\):

MG132:

NEMO-U\(b_n\)

E2F-4-U\(b_n\)

E2F-4
Fig. 2

A

*H. sapiens*  PDFCCPKCQYQAPDMDTLQIHVMECIE
*M. musculus*  PDFCCPKCQYQAPDMDTLQIHVMECIE
*X. laevis*  PDFSCPKCYKAPDMTLQIHVMDCIQ
*D. melanogaster*  TTLRCPICSFSNALSVLQSHVNDCLDKN

B

|               | WT  | ΔZF | C417R |
|---------------|-----|-----|-------|
| TNF-α(min)    | 0   | 0   | 5     |
|               | 10  | 10  | 0     |

**IF: HA**

**IB: FLAG**

ubiquitated NEMO proteins

NEMO proteins
Fig. 3

A

AU1-Ub:
HA-NEMO:

IP: HA
IB: AU1

IB: HA

Lysate
IB: FLAG

1  2  3  4  5

IP: HA
IB: HA

IP: HA
IB: HA

B

AU1-Ub:
HA-NEMO:

IP: HA
IB: AU1

IB: HA

Lysate
IB: FLAG

1  2  3  4  5

IP: HA
IB: HA

IP: HA
IB: HA

C

E1:
UbcH5C:
c-IAP1:

200
97

D

c-IAP1:

MBP
WT
ΔZF
C417R

NEMO
proteins

200
97

E

E1:
UbcH3
UbcH6
UbcH7
UbcH10
UbcH13

200
97

NEMO
proteins

MBP

NEMO
proteins

NEMO
proteins

NEMO-Ub

NEMO-Ub

NEMO

NEMO

NEMO-Ub

NEMO

MBP
Fig. 3 (cont)

**F**

\[
\begin{array}{cccccccc}
6 & 11 & 27 & 29 & 33 & 48 & 63 \\
\text{Ub} & - & - & - & - & - & - & - \\
7\text{R} & - & - & - & - & - & - & - \\
K6 & - & - & - & - & - & - & - \\
K11 & - & - & - & - & - & - & - \\
K27 & - & - & - & - & - & - & - \\
K29 & - & - & - & - & - & - & - \\
K33 & - & - & - & - & - & - & - \\
K48 & - & - & - & - & - & - & - \\
K48\text{R} & - & - & - & - & - & - & - \\
\end{array}
\]

**G**

![Western blot gel with bands labeled NEMO-polyUb and NEMO-Ub.](image)

**H**

![Western blot gel with bands labeled NEMO-Ub.](image)

**I**

| c-IAP1  | ROC1  |
|---------|-------|
| UbcH5C  | UbcH5C|
| WT      | WT    |
| 7\text{R} | 7\text{R} |
| K48\text{H} | K48\text{H} |

**J**

![Western blot gel with bands labeled polyUb.](image)

**K**

![Western blot gel with bands labeled polyUb and IP: anti-NEMO.](image)
Fig. 4 (cont)

F

|           | c-IAP1 | c-IAP1,Δ121 |
|-----------|--------|-------------|
| c-IAP1    |        |             |
| vector    |        |             |
| c-IAP1    |        |             |
| IP: FLAG  |        |             |
| IB: HA    |        |             |
| Lysate    |        |             |
| IP: FLAG  |        |             |
| IB: FLAG  |        |             |

G

|        | pTKO | c-IAP1 RNAi | TRAF6 RNAi |
|--------|------|-------------|------------|
| c-IAP1 |      |             |            |
| NEMO   |      |             |            |
| c-IAP2 |      |             |            |
| XIAP   |      |             |            |

H

TNF-α (5 min):

|        | pTKO | c-IAP1 RNAi |
|--------|------|-------------|
| GST-IκBα |      |             |
| NEMO   |      |             |
| IKKα   |      |             |
| IKKβ   |      |             |

I

TNF-α:

|        | C605A |
|--------|-------|
| TNF-α:| -     | +     |
| GST-IκBα |      |       |
| IKKβ   | 1     | 2     | 3     | 4     |

J

TNF-α (min):

|        | pTKO | c-IAP1 RNAi |
|--------|------|-------------|
| NEMO   |      |             |
| p-IκBα |      |             |
| IκBα   |      |             |
Fig. 5

A

Fold Induction

| TNF-α (min): | vector | WT | ΔZF | C417R | vector | WT | C417R |
|-------------|-------|----|-----|-------|-------|----|-------|
| 0           |       |    |     |       |       |    |       |
| 5           |       |    |     |       |       |    |       |
| 10          |       |    |     |       |       |    |       |
| 20          |       |    |     |       |       |    |       |
| 30          |       |    |     |       |       |    |       |

B

WT  C417R

TNF-α (min): 0 2 5 0 2 5

C

NEMO protein-Ub

D

GST-IκBα

p-IκB

TNF-α (min): 0 2 5 10 20 30

Fold activation:

WT

C417R

1 3.3 5.2 6.6 3.4 3.9

WT

C417R

1 0.9 0.6 1.0 1.0 0.9
A role for NEMO/IKKγ Ubiquitination in the activation of the IκB kinase complex by TNF-α
Eric D. Tang, Cun-Yu Wang, Yue Xiong and Kun-Liang Guan

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