DOCUMENT SUMMARY

This research paper by Deng et al. investigates the mechanism by which **TRAF6** activates the **IKB kinase (IKK) complex** in the **NF-κB** signaling pathway. The authors purified and identified a dimeric ubiquitin-conjugating enzyme complex, **Ubc13/Uev1A**, as the critical link between **TRAF6** and **IKK**. The study demonstrates that **TRAF6** functions as an E3 ubiquitin ligase, working with **Ubc13/Uev1A** to create unique **K63-linked polyubiquitin chains**, which are essential for **IKK** activation in a non-proteolytic regulatory role.

FILENAME

deng_2000_research_article_nf-kb_traf6_activation

METADATA

Category: RESEARCH

Type: research_article

Relevance: Reference

Update Frequency: Static

Tags: #nf-kb #traf6 #ubiquitination #ikk #signal-transduction #k63-linked-polyubiquitin #ubc13 #uev1a #e3-ligase

Related Docs: N/A

Supersedes: N/A

FORMATTED CONTENT

Deng_2000_Activation_of_the_IKB_Kinase_Complex_by_TRAF6_Requires_a_Dimeric_Ubiquitin-Conjugating_Enzyme_Complex_and_a_Unique_Polyubiquitin_Chain

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Summary

TRAF6 is a signal transducer in the **NF-κB** pathway that activates **IKB kinase (IKK)** in response to proinflammatory cytokines. We have purified a heterodimeric protein complex that links **TRAF6** to **IKK** activation. Peptide mass fingerprinting analysis reveals that this complex is composed of the **ubiquitin conjugating enzyme Ubc13** and the Ubc-like protein **Uev1A**. We find that **TRAF6**, a RING domain protein, functions together with **Ubc13/Uev1A** to catalyze the synthesis of unique **polyubiquitin chains** linked through **lysine-63 (K63)** of **ubiquitin**.

> Blockade of this **polyubiquitin chain** synthesis, but not inhibition of the proteasome, prevents the activation of **IKK** by **TRAF6**. These results unveil a new regulatory function for **ubiquitin**, in which **IKK** is activated through the assembly of **K63-linked polyubiquitin chains**.

Introduction

NF-KB provides a paradigm for a transcription factor that is regulated primarily via nuclear translocation (Sen and Baltimore, 1986; Baeuerle and Baltimore, 1988). In unstimulated cells, **NF-KB** is sequestered in the cytoplasm through association with an inhibitory protein of the **IKB** family. Upon stimulation of cells by various agonists, such as **tumor necrosis factor a (TNFα)** and **interleukin 1ß (IL-1ß)**, **IκB** proteins are rapidly phosphorylated by an **IKB kinase (IKK) complex** and then degraded by the **ubiquitin (Ub)-proteasome pathway** (reviewed by Ghosh et al., 1998). Following **IKB** degradation, **NF-KB** translocates into the nucleus where it regulates the expression of a wide spectrum of genes involved in immunity, inflammation, apoptosis, and other cellular processes.

The importance of **IKB** degradation in the **NF-KB** pathway is underlined by the observation that blockade of **IKB** degradation prevents activation of **NF-KB** by most, if not all, stimuli (reviewed by Chen and Maniatis, 1998). **IKB** is targeted to degradation by the proteasome through covalent modification by **Ub**. **Ubiquitination** of a protein is carried out by an enzymatic cascade composed of **E1 (Ub activating enzyme)**, **E2 (Ub-conjugating enzyme)**, and **E3 (Ub protein ligase)**. **Ub** is first activated by **E1** in the presence of ATP to form a high energy thioester bond. The activated **Ub** is then transferred to a member of the **E2** family, which, in conjunction with **E3**, conjugates **Ub** onto a lysine residue of the target protein to form an isopeptide bond (reviewed by Laney and Hochstrasser, 1999). Conjugated **Ub** itself can be further **ubiquitinated** through one of its seven lysine residues to form a **polyubiquitin (polyUb) chain**. A typical **Ub-Ub** linkage within a **polyUb** chain recognizable by the proteasome involves **lysine-48 (K48)** of one **Ub** and the carboxyl terminus of the next **Ub** (Chau et al., 1989). **PolyUb** chains linked through other lysines of **Ub** also exist in cells, but the function of these chains is poorly understood (reviewed by Pickart, 1997). The specificity in selecting target proteins for **ubiquitination** is determined to a certain extent by **E2s** and to a larger extent by **E3s**, the latter comprising a large and diverse family of proteins capable of binding to specific protein substrates. Recent studies have identified the **IKB-E2** as a member of the **Ubc4/5** family, and **IKB-E3** as a multiprotein complex composed

of Skp1, Cul1, Roc1, and an F-box/WD40 protein called **ßTrCP/Slimb**, which binds specifically to phosphorylated **IKB** (reviewed by Maniatis, 1999).

Signal-induced phosphorylation of **IKBα** is a prerequisite for its **ubiquitination** and subsequent degradation (Chen et al., 1995). **IκBα** is phosphorylated at two specific serine residues (Ser32 and Ser36) following stimulation of cells (Brockman et al., 1995; Brown et al., 1995). A 700 kDa protein kinase complex was first identified as an activity that phosphorylates **IκBα** at the signaling serine residues (Chen et al., 1996). Surprisingly, the activity of this kinase complex can be induced by **ubiquitination**, a novel phenomenon suggestive of a regulatory role of **Ub**. Subsequently, this **IKB kinase complex** was found to be activated independently by phosphorylation through **MEKK1**, a kinase of the JNK/SAPK pathway (Lee et al., 1997). Recent studies have led to the identification of two catalytic subunits of the **IKB kinase complex** as **IKKα** and **IKKβ**, and a regulatory subunit as **NEMO/IKKy** (reviewed by Israel, 2000). Genetic studies demonstrate that **NEMO** and **IKKβ** are essential for **NF-KB** activation, whereas removal of **IKKα** does not significantly impair the **NF-kB** pathway. The mechanism by which **NEMO** regulates **IKK** activity is presently unknown.

The identification of the **IKK complex** opens the door to exploring the important question of how **IKK** integrates multiple signals from diverse pathways. While there are many different agents that can activate **NF-KB** through the **IKK complex**, the best characterized signaling cascades are those emanating from the cytokines **TNFα** and **IL-1ß**. For example, binding of **IL-1ß** to its receptor (**IL-1R**) leads to the formation of a complex between **IL-1R** and the receptor accessory protein (**IL-1RACP**), which in turn triggers the assembly of a submembraneous signaling complex containing an adaptor **MyD88**, a protein kinase **IRAK**, and a RING finger domain protein **TRAF6** (Cao et al., 1996). How **TRAF6** and other **TRAF** proteins transduce signals to the **IKK complex** is not understood. Although **MEKK1** and **NIK**, a kinase of the MEKK family (Malinin et al., 1997), have been postulated to link **TRAF** proteins to **IKK** activation, recent genetic studies found that deletion of **MEKK1** or **NIK** did not impair **IKK** or **NF-KB** activation (Shinkura et al., 1999; Xia et al., 2000). In contrast, genetic ablation of **TRAF6** completely abolishes **IKK** activation in response to **IL-1ß** and several other physiologic ligands, including CD40 and LPS (Lomaga et al., 1999; Naito et al., 1999). Thus, there is a significant gap in the pathway between **TRAF6** and the **IKK complex**.

We undertook a biochemical approach to filling in the gap between **TRAF6** and **IKK**. Our approach begins with the establishment of a cell-free system that activates **IKK** in a **NEMO**- and **TRAF6**-dependent manner. Biochemical fractionation of the cell extract reveals two distinct factors that are required to activate **IKK** by **TRAF6**. We now report the purification and characterization of one of these factors, which turns out to be a dimeric **ubiquitin-conjugating enzyme (E2) complex** composed of **Ubc13** and **Uev1A (Mms2)**. This **E2 complex** was previously shown to catalyze the assembly of unique **polyUb chains** linked through **lysine-63 (K63)** of **Ub** (Hofmann and Pickart, 1999). We find that recombinant **Ubc13/Uev1A** supports the activation of **IKK** by **TRAF6**, whereas depletion of **Uev1A** abolishes **TRAF6**-mediated activation of **IKK**. **TRAF6** facilitates the assembly of **K63-linked polyUb chains** by **Ubc13/Uev1A**, and this activity requires the RING finger domain of **TRAF6**. Furthermore, **Ub** mutants that fail to assemble into **K63-**

linked chains prevent activation of **IKK** by **TRAF6** and **Ubc13/Uev1A**, whereas inhibition of proteasome does not affect **IKK** activation. These results indicate that **TRAF6** functions as a **Ub ligase (E3)**, which, together with the **E2 Ubc13/Uev1A**, mediates the assembly of **K63-linked polyUb chains** required for **IKK** activation. Our results also unveil a novel nonproteolytic function for **Ub**, which in this case positively regulates a protein kinase activity.

Results

A Cell-Free System that Activates IKK in Response to TRAF6

Overexpression of **TRAF6** in cells activates **NF-KB** through the **IKK complex** (Cao et al., 1996; Regnier et al., 1997). We sought to recapitulate the **TRAF6**-dependent activation of **IKK** in vitro in order to identify the missing link between **TRAF6** and **IKK** through biochemical fractionation. As shown in Figure 1A, addition of recombinant **TRAF6** to cell extracts prepared from 70Z pre-B cells led to the phosphorylation of endogenous **IκBα** that could be detected with a phospho-**IκBα**-specific antibody (lane 2). This reaction is strictly dependent on **NEMO**, since cell extracts prepared from 1.3E2, a **NEMO**-deficient line derived from 70Z cells (Courtois et al., 1997), failed to phosphorylate **IκBα** in the presence of **TRAF6** (lane 5). Moreover, addition of recombinant **NEMO** to the 1.3E2 extract restored **IKBα** phosphorylation in response to **TRAF6** (lane 6). Genetic experiments have previously demonstrated that **NEMO** is essential for **IKK** activation by most stimuli, including components of the **TRAF6** pathway (Yamaoka et al., 1998; Rudolph et al., 2000). Thus, the in vitro **TRAF6**-inducible system described here mimics the stringent requirement of **NEMO** for **IKK** activation.

NF-KB agonists trigger the phosphorylation of **IκBα** at both serines 32 and 36. To determine whether **IKBα** is phosphorylated at these serine residues in the in vitro **TRAF6**-inducible system, we tested in vitro translated, 35S-labeled **IκBα** serine mutants in our assay. While wild-type **IκBα** was fully phosphorylated, as indicated by the electrophoretic mobility shift (lane 2), mutation at both serines 32 and 36 abolished this phosphorylation (lane 8). Single point mutation at either S32 or S36 led to an intermediary gel shift (lanes 4 and 6), which is indicative of single site phosphorylation at the remaining wild-type serine residue. Therefore, the in vitro **TRAF6**-inducible system retains the specificity of **IKBα** phosphorylation.

TRAF6-Dependent Activation of IKK Requires Intermediary Factors

To determine whether **TRAF6** activates **IKK** directly, we purified the **IKK complex** from unstimulated HeLa cells or from calf thymus through several chromatographic steps, including an ATP-affinity column step. The purified **IKK complex** from HeLa cells contains **IKKα**, **IKKβ**, and **NEMO** (data not shown) and can be activated directly by **MEKK1** (Figure 2A, lane 5) (Lee et al., 1998). The ability to purify this latent **IKK complex** from HeLa cells allows us to search for upstream factors required for its activation. One of the candidate upstream activators is **TRAF6**. However, unlike **MEKK1**, **TRAF6** did not activate the purified **IKK complex** directly (Figure 2A, lanes 4 and 5). In contrast, the crude extract from 293 cells can be activated by **TRAF6** (lane 2),

suggesting the existence of intermediary factors required for **IKK** activation by **TRAF6** in the extract. As an initial step to identify these factors, we fractionated HeLa cytosolic extract (S100) on Q-Sepharose through step elution with increasing concentration of NaCl (Figure 2B). Significantly, the activation of the **IKK complex** by **TRAF6** requires at least two fractions, the unbound fraction (Fr.I) and the 0.1-0.2 M NaCl eluate (Fr.lla, lane 3). Omission of either fraction abolishes **IKK** activation by **TRAF6** (lanes 4 and 5). The factor present in Fr.l is hereby referred to as **TRAF6-Regulated IKK Activator 1 (TRIKA1)**, whereas the factor present in Fr.lla is referred to as **TRIKA2**.

Purification and Identification of TRAF6-Regulated IKK Activator 1 (TRIKA1)

We have purified **TRIKA1** from HeLa cytosolic extracts to apparent homogeneity through six steps of conventional chromatography (Figure 3A). Silver staining of fractions from the last MonoQ step is shown in Figure 3B, and the **IKK** stimulatory activity assay of the same fractions shown in Figure 3C. This factor has an apparent molecular size of 35 kDa based on gel filtration chromatography (data not shown), and is separated into two bands on SDS-PAGE with molecular sizes of 16 and 19 kDa, respectively (Figure 3B). The intensity of these bands correlated with **IKK**-stimulatory activity, indicating that they are candidate subunits of **TRIKA1** (a and ß subunits).

The 16 and 19 kDa bands were excised for mass spectrometric analysis following digestion with trypsin. Peptide mass fingerprinting (MALDI-TOF) of the 16 kDa protein identified 9 peptides whose masses matched those predicted from human **Ubc13**, a **Ub-conjugating enzyme** (Figure 3D). The 19 kDa protein has 11 peptides whose masses matched those of an isoform of human Uev1 such as **Uev1A** (Figure 3E), a **Ub-conjugating E2 enzyme variant (UEV)** that is structurally related to E2 enzymes but lacks the catalytic cysteine residue found in a typical E2 (Rothofsky and Lin, 1997; Sancho et al., 1998).

Recombinant Ubc13/Uev1A Complex Supports the Activation of IKK by TRAF6

To verify that **Ubc13** and **Uev1A** are indeed components of **TRIKA1**, we expressed these proteins in *E. coli* and purified the recombinant proteins to apparent homogeneity with the aid of a hexahistidine (His) affinity tag. Recombinant **Ubc13** and **Uev1A** form a heterodimer that migrates at ~45 kDa on a gel filtration column (data not shown). In the presence of **TRAF6**, the recombinant **Ubc13** and **Uev1A** activated **IKK** in a manner that correlated with the formation of the heterodimer (Figure 4A). Furthermore, mutation of the active site Cys-87 of **Ubc13** (C87A) abolished its ability to support **IKK** activation by **TRAF6** (Figure 4B). Like the native **TRIKA1**, recombinant **Ubc13/Uev1A** stimulated **IKBα** phosphorylation by **IKK** at both serines 32 and 36 (Figure 4C). We also tested whether other E2s could support **TRAF6**-mediated **IKK** activation (Figure 4D). Among several E2s tested, only **Ubc13/Uev1A** was capable of activating **IKK** together with **TRAF6**. These results confirm that **Ubc13/Uev1A** is the **TRIKA1** that links **TRAF6** to **IKK** activation.

Ubc13/Uev1A Is Required for IKK Activation by TRAF6

The tight binding between **Ubc13** and **Uev1A** makes it feasible to deplete endogenous **Uev1A** from crude cell extracts with immobilized GST-**Ubc13**, thus providing an

opportunity to address the consequence of removing **Uev1A**. This is particularly useful in light of the fact that the antibodies generated against **Ubc13** or **Uev1A** fail to immunoprecipitate the respective proteins, although they can detect the proteins by immunoblotting (data not shown). As shown in Figure 5A, cell extracts were devoid of **Uev1A** after passage through GST-**Ubc13**-Sepharose (lane 2), whereas GST-Sepharose did not retain any **Uev1A** from the extracts (lanes 3 and 5). Consequently, cell extracts depleted of **Uev1A** are severely defective in **IKK** activation in the presence of **TRAF6** (Figure 5B, lane 7), whereas control extracts retain full **IKK** activation potential (lanes 3 and 10). Addition of recombinant **Ubc13/Uev1A** to the **Uev1A**-depleted extracts restored **IKK** activation in response to **TRAF6** (lane 8). These results indicate that **Ubc13/Uev1A** is required for **IKK** activation by **TRAF6** in vitro.

To determine whether **Ubc13/Uev1A** is required for **NF-KB** activation in living cells, we transfected an expression construct encoding the **Ubc13(C87A)** mutant into 293 cells together with a reporter gene that expresses luciferase under the control of three tandem repeats of **NF-KB** binding sites (Figure 5C). The transfected cells were stimulated with **IL-1ß**, **TNFα**, or by cotransfection with expression constructs encoding **TRAF6**, **TRAF2**, **NIK**, or the **TAX** protein of the human T cell leukemia virus (HTLV1). As a control, the expression of a luciferase reporter gene driven by Gal4 was also examined, using hybrid transcription activators composed of the DNA binding domain of Gal4 and the transactivation domain of c-Rel (Gal4-Rel) or VP16 (Gal4-VP16). Remarkably, overexpression of **Ubc13(C87A)** led to potent inhibition of **NF-KB** activation by **IL-1ß**, **TNFα**, **TRAF6**, or **TRAF2**. In contrast, **Ubc13 (C87A)** had little effect on **NF-KB** activation by **NIK** or **TAX**, or on Gal4-dependent transcription by Rel or VP16. Thus, **Ubc13/Uev1A** appears to be involved primarily in the pathways that require **TRAF** proteins (**TRAF2** for **TNFα**, and **TRAF6** for **IL-1ß**). Taken together, these results strongly suggest that **Ubc13/Uev1A** is required for **NF-KB** activation by **TRAF** proteins in the IL-1 and TNF pathways.

TRAF6 Facilitates the Assembly of K63-Linked polyUb Chains in Conjunction with Ubc13/Uev1A

Previous studies have shown that **Ubc13** and Mms2, a yeast homolog of **Uev1A**, form an E2 complex to catalyze the synthesis of unique **polyUb chains** linked through **lysine-63 (K63)** of **ubiquitin** (Hofmann and Pickart, 1999). This finding, together with our present results that this E2 complex is involved in **IKK** activation by **TRAF6**, raises the possibility that **TRAF6** may also be involved in **ubiquitination**. In fact, **TRAF6** contains a **RING finger domain** and five repeats of zinc fingers at its N terminus (Cao et al., 1996). Several other **RING finger proteins** have previously been shown to function as **ubiquitin ligases** in **polyubiquitination** (Lorick et al., 1999). To test whether **TRAF6** has a role in **ubiquitination**, we carried out an in vitro **ubiquitination** assay in the presence of E1, **Ubc13/Uev1A** (as an E2), ATP, and **Ub** (Figure 6A). When all components were present, **polyUb chain** synthesis was readily detectable with a **Ub**-specific antibody (lane 5). In the absence of any of the components, no significant **polyubiquitination** was observed (lanes 1, 2, 3, and 4). (The Ub_2 seen in lanes 1-5 is principally a contaminant in the commercial **Ub**). Inclusion of a **Ub** mutant (KO) in which all seven lysines were mutated to arginine prevented **polyUb chain** formation (lanes 6-

8). Restoration of a lysine at position 48 (K48) on an otherwise lysine-less background was not sufficient to restore **polyubiquitination** (lane 9). In contrast, restoration of a lysine at position 63 (K63) restored **polyubiquitination** (lane 10).

> These results show that **TRAF6** facilitates the synthesis of **polyUb chains** through **K63** rather than **K48**. In this regard, **TRAF6** is a **Ub ligase (E3)** that partners with **Ubc13/Uev1A (E2)**.

We also found that **TRAF2** functioned together with **Ubc13/Uev1A** to catalyze the synthesis of **K63-linked polyUb chains** (data not shown), consistent with the obligatory role of **Ubc13/Uev1A** in **NF-KB** activation by **TNFα** and **TRAF2** (Figure 5C).

To determine whether the **RING finger domain** of **TRAF6** is important for its **Ub-conjugating activity**, we mutated the highly conserved cysteine and histidine residues in the **RING domain** to generate two point mutants, C70A and C85A/H87A. These constructs were expressed in 293 cells as Flag-tagged proteins and immunopurified for **ubiquitination** assays (Figure 6B). While wild-type **TRAF6** catalyzed the assembly of **K63-linked polyUb chains** from a K63 **Ub** mutant in the presence of E1 and **Ubc13/Uev1A** (lane 4), the **RING finger** mutations abolished this **Ub-conjugating activity** (lanes 6 and 8). These same mutations also abolished the ability of **TRAF6** to activate the expression of an **NF-KB** reporter gene (luciferase) when overexpressed (Figure 6C), suggesting that the **NF-KB**-inducing activity of **TRAF6** is linked to its **Ub-conjugating activity** through the use of the **RING domain**.

K63-Linked polyUb Chains Are Involved in IKK Activation by TRAF6

Our findings that **TRAF6** and **Ubc13/Uev1A** are involved in **IKK** activation, and that they catalyze the synthesis of **K63-linked polyUb chains**, led us to investigate the role of these unique **Ub chains** in **IKK** activation. We first examined a panel of **Ub** mutants for their ability to activate **IKK** in vitro (Figures 7A and 7B). These mutants include a lysine-less mutant (Figure 7B, lane 2, KO), single-lysine mutants, which contain one lysine with the remaining six lysine residues mutated to arginine (K6, K11, K27, K29, K33, K48, and K63; lanes 3-9), and single K/R mutants, which contain a single point mutation from lysine to arginine with the remaining lysines intact (R11, R29, R48, and R63; lanes 10-13). In addition, we also tested methylated **Ub** (MeUb, lane 1), in which all seven lysine residues were blocked by methylation, thus preventing elongation of **Ub chains**. Strikingly, MeUb, KO, and all single-lysine mutants except for K63 fail to support **IKK** activation by **TRAF6** (lanes 1-9), indicating that K63 is the only important lysine on **Ub** required for this function. In further support of this conclusion, a single point mutation at position 63 from Lys to Arg (R63) abolished the stimulatory activity of **Ub** (lane 13), whereas mutations at other lysines had no effect (lanes 10-12). These results strongly suggest that **K63-linked polyUb chains** play an important role in **TRAF6**-mediated **IKK** activation.

To determine whether the assembly of **K63-linked polyUb chains** is required for **IKK** activation by **TRAF6** in crude cell extracts, we tested several **Ub** lysine mutants for their ability to interfere with **IKK** activation in the extracts (Figure 7C). Addition of wild-type or

K63 **Ub** had no effect on endogenous **IκBα** phosphorylation stimulated by **TRAF6** (lanes 3 and 6). In contrast, **Ub** mutants lacking any lysine (KO) or retaining only one lysine at position 48 (K48) exert a dominant-negative effect on endogenous **IKK** activation by **TRAF6** (lanes 4 and 5). These mutants did not interfere with **IKK** activation triggered by **MEKK1** or **IKKβ**. Taken together, these results further strengthen the conclusion that **TRAF6**-mediated activation of **IKK** requires the assembly of **K63-linked polyUb chains**.

The critical role of K63 rather than K48 of **Ub** in **IKK** activation raises the question of whether proteasomal degradation plays any role at all in **IKK** activation, since **K48-linked polyUb chains** are preferentially recognized by the proteasome (Chau et al., 1989). In our in vitro reconstitution system, no proteasome was added to the assay, suggesting that **IKK** activation does not require proteasome. To rule out the possibility that any residual proteasomal activity might be required for **IKK** activation, we added proteasome inhibitors MG132 (Figure 7D, lanes 4-6) or lactacystin (lanes 7-9) to the assay. No inhibition of **IKK** activation was observed in the presence of MG132 or lactacystin at 20 μM, a concentration known to completely block proteasomal activity (Chen et al., 1995). As a control, the kinase inhibitor staurosporin (STS) significantly inhibited **IKK** activity in the extract (lane 11). This experiment, together with previous results that proteasome inhibitors do not interfere with **IKB** phosphorylation in vivo (Palombella et al., 1994), shows that **IKK** is activated by **TRAF6** through a mechanism involving **K63-linked polyUb chains**, but not proteasomal degradation.

Discussion

In this report, we describe the purification and identification of a protein complex that links **IKK** to its upstream activator **TRAF6**. This complex is composed of **Ubc13** and **Uev1A (Mms2)**, a dimeric **Ub-conjugating enzyme** previously shown to catalyze the synthesis of **K63-linked polyUb chains** (Hofmann and Pickart, 1999). Several lines of evidence suggest that **Ubc13/Uev1A** is indeed a critical factor that mediates **IKK** activation by **TRAF6**. First, recombinant **Ubc13/Uev1A** dimer supports **IKK** activation in a reconstituted system; second, mutation of the catalytic cysteine of **Ubc13** abolishes its ability to activate **IKK**; third, among several known E2s tested, **Ubc13/Uev1A** is uniquely capable of activating **IKK** in vitro, consistent with the purification data showing that only a single peak of **TRIKA1** activity was detected throughout the fractionation steps; fourth, removal of **Uev1A** renders cell extracts defective in activating **IKK** in response to **TRAF6**; and finally, interference of **Ubc13/Uev1A** in cells blocks **NF-KB** activation by **IL-1ß** and **TNFa**, two agonists known to transduce signals through **TRAF** proteins.

Consistent with a previous report (Chen et al., 1996), we have found that another **Ub-conjugating enzyme** of the **Ubc4/5** subfamily can also activate a partially purified **IKB kinase** fraction. Immunodepletion of **IKK** from the partially purified fraction abolishes the **Ub**-dependent phosphorylation of **IκBα** (C. W. and Z. J. C., unpublished data), indicating that **IKK** is the kinase that is activated by **ubiquitination**. However, highly purified **IKK complex** can no longer be activated directly by **Ubc4/5**, although it can be activated directly by **MEKK1** (data not shown). Significantly, when the purified **IKK**

complex was supplemented with a fraction that is depleted of **IKK**, **Ubc4/5**-dependent activation of **IKK** was restored, suggesting the existence of a factor required for **IKK** activation by **Ubc4/5** (C. W. and Z. J. C., unpublished data). This factor, which remains to be characterized, does not support the activation of **IKK** by **Ubc13/Uev1A** and **TRAF6**. Conversely, **Ubc4/5** cannot substitute for **Ubc13/Uev1A** in the activation of **IKK** by **TRAF6** (Figure 4D). Unlike **TRAF6**, whose essential role in the **NF-KB** pathway is well established, it is presently unknown how **Ubc4/5** is connected to the known **NF-KB** pathways.

The involvement of **Ubc13/Uev1A** as a **Ub-conjugating enzyme** in **TRAF6**-mediated **IKK** activation led us to investigate the potential role of **TRAF6** in **ubiquitination**. Indeed, **TRAF6** greatly facilitates the synthesis of **K63-linked polyUb chains** by **Ubc13/Uev1A**. This E3 activity of **TRAF6** requires an intact **RING domain**, a structural motif that is also critical for **TRAF6** to induce **NF-kB** in cells. Several **RING finger proteins** have recently been shown to possess **Ub-conjugating activity** in conjunction with other E2s (Lorick et al., 1999). However, the role of these **RING finger proteins** appears to be linked to targeting proteins for degradation. **TRAF6**, on the other hand, functions together with **Ubc13/Uev1A** to mediate the synthesis of **K63-linked polyUb chains**. Rad5, a RING-containing helicase, may function similarly in the yeast DNA repair pathway (Ulrich and Jentsch, 2000). **K63 chains** have been previously implicated in stress response and DNA repair in yeast (Spence et al., 1995; Hofmann and Pickart, 1999). Interestingly, conditions such as UV and DNA damaging agents that trigger stress response and DNA repair also lead to **IKK** activation.

We propose that **K63-linked polyUb chains** play an important regulatory role in **IKK** activation, based on the following lines of evidence. First, a single point mutation at K63, but not at other lysine residues, abolishes the ability of **Ub** to support **IKK** activation by **TRAF6**; second, restoration of a single lysine at position 63, but not at any other position, is sufficient to activate **IKK** in a reconstituted system; third, **Ub** mutants that fail to assemble into **K63-linked polyUb chains** exert dominant negative effects on **IKK** activation in cell extracts; fourth, inhibition of the proteasome has no effect on **IKK** activation. These results, together with the aforementioned findings that **IKK** activation requires the catalytic cysteine of **Ubc13** and the **RING domain** of **TRAF6**, provide strong support for a critical role of **K63-linked polyUb chains** in **IKK** activation.

Although **TRAF6** facilitates the assembly of free K63 chains in conjunction with **Ubc13/Uev1A**, free K63 chains do not activate **IKK** (see below). Thus, the **K63-linked chains** that function in **IKK** activation are evidently linked to a target protein that remains to be identified. Further purification and characterization of **TRIKA2** may lead to the identification of such a target. We have not detected **ubiquitination** of components of the **IKK complex** including **NEMO**, **IKKα**, and **IKKβ**. However, some proteins known to participate in the **NF-KB** pathway have been reported to undergo **ubiquitination** following engagement of ligands. For example, stimulation of cells with **TNFa** led to "some kind of covalent modification" of the protein kinase **RIP** that resembles **ubiquitination** (Zhang et al., 2000). The conjugated form of **RIP** appears within one minute of **TNFα** treatment, and then decays with kinetics that coincides with transient activation of **IKK**. Interestingly, there is no apparent degradation of **RIP** throughout the

time course when **IκBα** is rapidly degraded. It remains to be determined whether this form of **RIP** modification is related to **IKK** activation. Recently, a protein associated with atypical PKCs called **p62** has been shown to be involved in **IKK** activation in both the TNF and IL-1 signaling pathways (Sanz et al., 2000). The mechanism by which **p62** activates **IKK** in these pathways is not clear, but is thought to involve the binding of **p62** to **RIP** and **TRAF6**, respectively. Intriguingly, independent studies have found that **p62** is a **polyUb chain binding protein** (Vadlamudi et al., 1996). Further studies are needed to determine whether the **Ub** binding property of **p62** is relevant to its stimulatory function in the **NF-KB** pathway.

How does **ubiquitination** lead to **IKK** activation? Two possible scenarios come to mind. In the first scenario, covalent modification of a target protein by **K63-polyUb chains** could activate (in the case of an activator) or inactivate (in the case of an inhibitor) such a protein, just as phosphorylation can regulate the function of a target protein. In the second scenario, **K63-linked polyUb chains** could function as a tag to recruit other proteins in the **IKK** pathway, just as phosphotyrosine is used extensively to recruit SH2-domain-containing proteins in various signaling cascades. The latter possibility is consistent with our preliminary results showing that **IKK** activation in vitro can be inhibited by free **Ub** tetramers linked through K63 [(K63)4], but not by (K48)4 (data not shown). Presumably free (K63)4 chains, which cannot be conjugated to target substrates due to the blockade of the C terminus, function in a dominant-negative manner by sequestering proteins that normally bind to **polyubiquitinated** targets. While the exact mechanism by which **K63-linked polyubiquitination** activates **IKK** requires further investigation, our results clearly show that proteasomal degradation is not required to activate **IKK**. This does not mean that the target proteins conjugated by K63 chains are not ultimately degraded, but it does mean that degradation, even if it occurs, is not necessary for **IKK** activation. These results reaffirm the notion that **ubiquitination**, but not degradation, plays a regulatory role in **IKK** activation (Chen et al., 1996).

Protein kinases are known to have complex modes of regulation. Besides the well characterized phosphorylation cascades that can positively or negatively regulate protein kinases, some kinases can also be regulated by DNA, RNA, lipids, small molecules (i.e., Ca^{2+} and cAMP), or by protein-protein interaction (i.e., activation of cyclin-dependent kinases by cyclins).

> Our results uncover another mode of kinase regulation by **ubiquitination**. This mode of enzymatic regulation by **Ub** is unprecedented.

Mono-ubiquitination, on the other hand, could serve regulatory function by targeting certain receptors for internalization and subsequent lysosomal, rather than proteasomal, degradation (reviewed by Hicke, 1999).

Now that we show the **IKK complex** could be independently activated by phosphorylation or by **ubiquitination** in vitro, which of these mechanisms might be responsible for **IKK** activation in vivo? Strong support for **IKK** being activated by

phosphorylation comes from the observation that both **IKK** and **IKKβ** contain canonical MAPK phosphorylation sites, and that mutation of these sites significantly interferes with **IKK** activation in response to various stimuli (Mercurio et al., 1997). This and other experiments led to the idea that there is an upstream kinase (IKKK) that phosphorylates **IKK** and is responsible for its activation. In fact, the in vitro **TRAF6**-inducible system we set up was initially designed to identify a putative IKKK downstream of **TRAF6**. However, upon fractionation, it became clear that the factors mediating **IKK** activation in response to **TRAF6** were **ubiquitination** enzymes. We had also attempted to identify an IKKK through fractionation of **TNFα**-stimulated cell extracts. However, we found that the only fraction capable of activating **IKK** (isolated from unstimulated cells) is the activated **IKK** itself (from **TNFa**-stimulated cells, L. D. and Z. J. C., unpublished data). Thus, activated **IKK** appears to be capable of activating another **IKK** to amplify the signals, raising the possibility that the activation loop of **IKK** is needed to maintain full activity of **IKK**. Consistent with this possibility, we found that a recombinant **IKKβ** mutant lacking MAPK phosphorylation sites in the activation loop (S177A/S181A) retains significant **IKB** kinase activity (Z. J. C., unpublished data). All of these data can be reconciled in a model whereby full activation of **IKK** involves two steps, an initiation step through **TRAF6**-mediated **ubiquitination**, and an amplification step through autoactivation of **IKK** as a result of phosphorylation at the activation loop. It is possible that in some signaling pathways, an upstream kinase may directly phosphorylate and activate **IKK**, thus bypassing the requirement for **ubiquitination**. However, at least in the **TRAF6** pathway, our data show that **ubiquitination** by **TRAF6** plays a key role in triggering the initial activation step. How **TRAF6** and other **TRAF** proteins are activated is presently unknown, but it may involve receptor-mediated dimerization or oligomerization of these proteins through the conserved C-terminal **TRAF** domains (Baud et al., 1999).

The enzymatic pathways for the synthesis of **K63-linked polyUb chains** are conserved from yeast to man. Homologs of **Ubc13** and **Uev1A** are present in yeast, and homologs of **Ubc13**, **Uev1A**, and **TRAF6** can also be found in *Drosophila*, which possesses highly conserved dorsoventral patterning and immunity pathways that resemble those of **NF-κB/IκB** in vertebrates. Perhaps the formation of **K63-linked polyUb chains** is an evolutionarily conserved defense mechanism for an organism to protect against stressful insults ranging from heat and UV damage to pathogens (innate immunity).

Figures

- **Figure 1. A Cell-Free System that Activates IKK in Response to TRAF6 and NEMO:** This figure shows that recombinant **TRAF6** induces phosphorylation of endogenous **IκBα** in wild-type cell extracts but not in **NEMO**-deficient extracts, and that this phosphorylation is specific to serines 32 and 36.
- **Figure 2. Activation of IKK by TRAF6 Requires Intermediary Factors:** This figure demonstrates that **TRAF6** does not directly activate purified **IKK complex**, unlike MEKK1. It also shows that fractionation of cell extract separates the activity into two required components, **TRIKA1** and **TRIKA2**.
- **Figure 3. Purification and Identification of TRIKA1:** This figure outlines the purification scheme for **TRIKA1** and shows silver staining of the purified protein,

revealing two subunits. Mass spectrometry identified these subunits as the **ubiquitin-conjugating enzyme Ubc13** and the Ubc-like protein **Uev1A**.

- **Figure 4. Reconstitution of TRAF6-Dependent IKK Activation with Recombinant TRIKA1 (Ubc13/Uev1A):** This figure confirms that a recombinant heterodimer of **Ubc13** and **Uev1A** can substitute for native **TRIKA1** in activating **IKK** in the presence of **TRAF6**, and that the catalytic activity of **Ubc13** is essential.
- **Figure 5. TRIKA1 Is Required for IKK Activation by TRAF6:** This figure shows that depleting **Uev1A** from cell extracts abolishes **TRAF6**-mediated **IKK** activation. It also demonstrates that a dominant-negative mutant of **Ubc13** inhibits **NF-κB** activation by **TRAF**-dependent pathways in living cells.
- **Figure 6. TRAF6 Facilitates the Assembly of K63-Linked polyUb Chains in Conjunction with Ubc13/Uev1A:** This figure shows that **TRAF6** functions as an E3 ligase with **Ubc13/Uev1A** to synthesize **polyubiquitin chains** specifically through **lysine-63 (K63)**. This activity requires an intact **RING finger domain** in **TRAF6**.
- **Figure 7. K63-Linked polyUb Chains Mediate the Activation of IKK in Response to TRAF6:** This figure uses **ubiquitin** mutants to show that **K63** is necessary and sufficient for **IKK** activation by **TRAF6**. It also demonstrates that proteasome inhibitors do not block this activation, confirming a non-degradative role for **ubiquitination**.

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