hUBC9 associates with MEKK1 and type I TNF-α receptor and stimulates NFκB activity

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Abstract hUBC9, an E2 ubiquitin conjugating enzyme, was identified by yeast two-hybrid screening and coprecipitation studies to interact with MEKK1 and the type I TNF-α receptor, respectively. Because both of these proteins regulate NFκB activity, the role of hUBC9 in modulating NFκB activity was investigated. Overexpression of hUBC9 in HeLa cells stimulated the activity of NFκB as determined by NFκB reporter and IL-6 secretion assays. hUBC9 also synergized with MEKK1 to activate NFκB reporter activity. Thus, hUBC9 modulates NFκB activity which, at least in part, can be attributed to its interaction with MEKK1 and the type I TNF-α receptor.

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Key words: Tumor necrosis factor-α; Ubiquitin conjugating enzyme; Yeast two-hybrid; Nuclear factor κB; MEKK1

1. Introduction

One mechanism by which the levels of certain proteins are regulated in the cell is through their ubiquination and subsequent degradation by proteosomes (as reviewed in [1–3]). The process by which proteins are ubiquinated involves the coordinated activity of three families of enzymes, designated E1, E2, and E3. Ten different E2 enzymes have been identified in yeast (designated ubiquitin conjugating enzyme, or UBC1–UBC10) [1]. UBC9 has been found to be an essential gene for cell cycle progression [4] and is involved in the degradation of S and M phase cyclins in yeast [4]. In addition, mammalian homologues of UBC9 have been found to interact with a variety of proteins, including ETS-1 [5], c-Jun [6], glucocorticoid receptor [6], RanBP2 [7], and Rad51 recombination protein [8]. The significance for most of these interactions, however, is unknown.

Recently it has been shown that UBC9 may be an E2 enzyme not for ubiquitin, but for the ubiquitin-like protein SMT3p [9–11]. The mammalian homologue of SMT3p, SUMO-1 (also known as sentrin [10]), has been found to be conjugated to Ran-GTPase activating protein RanGAP1 [12]. This conjugation has been shown to be necessary for the association of RanGAP1 to Ran-GTP binding protein RanBP2 at the nuclear pore complex [12].

We report on the identification of two additional proteins which interact with the human homologue of UBC9 (hUBC9), the type I tumor necrosis factor-α (TNF-α) receptor and MEKK1. The type I TNF-α receptor is a 55 kDa protein which is associated with a variety of functions when activated, including apoptosis, NFκB activation, and Jun N-terminal kinase activation [13,14]. The induction of both NFκB activity and apoptosis by type I TNF-α receptor is mediated through its intracellular 'death domain' region [14,15]. In the TNF-α mediated activation of NFκB, a pathway is stimulated in which the last step is the phosphorylation dependent degradation of IκB by proteosomes [16–19].

MEKK1 is a kinase which stimulates the stress activated protein kinase pathway [20,21]. It has been shown to phosphorylate SEK1, a stress kinase protein (SAPK) activator, which in turn phosphorylates and activates SAPK/JNK [22]. MEKK1 has also been shown to activate NFκB activity when overexpressed in cells [23–25]. The mechanism by which MEKK1 induces NFκB activity, however, is unclear.

Thus, because of the association of the type I TNF-α receptor and MEKK1 with NFκB activation, we also determined whether hUBC9 modulated TNF-α induced NFκB activity. In this report we show that hUBC9 overexpression induces NFκB activity and can synergize with MEKK1 for stimulating this activity.

2. Materials and methods

2.1. Cell culture

HeLa cells were obtained from ATCC (Rockville, MD). HeLa cells were grown in minimal essential medium with Earle's salts supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 U/ml streptomycin sulfate. Cells were grown at 37°C with 95% humidity and 5% CO_2. All cell culture reagents were purchased from Gibco-BRL (Gaithersburg, MD).

2.2. Yeast two-hybrid screening and binding studies

Screening was performed using standard yeast two-hybrid methodology [26–28]. A human Jurkat T cell cDNA library (2.5×10^6 individual recombinants) in the vector pPC86 [29] was screened using as bait the cytoplasmic domain of the human type I TNF-α receptor [30,31], obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) and cloned into pPC97 [29]. The library and bait were cotransformed into the yeast two-hybrid strain Y190 (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3,112, gal4D, gal80D, cyh^r2, LYS2::$GAL1_{uas}$-$HIS3_{tata}$-HIS3, URA3::$GAL1_{uas}$-$GAL1_{tata}$-lacZ) using a standard lithium acetate-polyethylene glycol method [32]. A total of 2×10^6 clones were screened. Positive library clones (growth on His^- medium, and LacZ reporter activity) were rescued, verified, and subjected to DNA sequencing.

For domain mapping, type I TNF-α receptor bait plasmids containing the death domain (nucleotides 1321–1579) or the cytoplasmic region from the transmembrane to the death domain (nucleotides 958–1320) were prepared by PCR, subcloned into pPC97 and analyzed as described above.

A plasmid containing the murine MEKK1 [20], pECE-MEKK1

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(gift of Dr. Jacques Pouysségur, Université de Nice, Nice, France) was used to transfer the MEKK1 coding sequence to the two-hybrid DNA binding domain vector pGBT9 (Clontech, Palo Alto, CA). This plasmid was used to screen a HeLa cell cDNA library in pGAD-GH (Clontech) in the yeast strain yCM17 (MATa, ura3, trp1, leu2, his3, lys2, ade2, gal4, gal80, met16::URA3-GAL1-10LacZ). Positive clones were treated as described above.

hUBC9 binding domain mapping of MEKK1 was accomplished by cloning fragments of the MEKK1 cDNA corresponding to the regulatory (amino acids 1–369) and kinase domains (amino acids 363–673), as well as a fragment encoding amino acids 284–673 into pGBT9. These constructs were cotransformed with the hUBC9 activation domain plasmid obtained from the yeast two-hybrid screen, as described above, and scored for interaction.

2.3. Glutathione S-transferase (GST)-hUBC9 precipitation analysis

hUBC9 cDNA was cloned into pGEX2T (Pharmacia Biotech, Piscataway, NJ). The resulting 45 kDa GST-hUBC9 fusion protein produced was purified by glutathione-Sepharose chromatography [33]. Coupled transcription/translation (TNT-Quick; Promega, Madison, WI) was performed on DNA templates with a T7 promoter upstream of the appropriate gene fragment containing an in-frame Kozak translation initiation sequence [34], using [^{35}S]methionine (Amersham, Arlington Heights, IL) as a label. Precipitation reactions were prepared using either 5 μg of hUBC9-GST or GST for each reaction, prebound to glutathione-Sepharose. The prebound hUBC9-GST and GST, respectively, were blocked in 200 μl of 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 2% bovine serum albumin for 1 h at 4°C. After this incubation, 25 μl of the respective type I TNF-α receptor or MEKK1 ^{35}S-labeled transcription/translation products was added. The precipitation reactions were incubated for 2 h further at 4°C with gentle mixing. The resin containing bound proteins was then pelleted followed by extensive washing (3×) with 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20, with pelleting after each wash. The resin was then resuspended in SDS-polyacrylamide gel loading dye and boiled for 5 min, followed by electrophoresis on 4–20% precast Tris-glycine polyacrylamide gels (Novex, San Diego, CA). After electrophoresis, the gels were fixed in acetic acid/methanol for 30 min, followed by a 30 min incubation in enhancing reagent (Amplify, Amersham). The gels were then vacuum dried onto filter paper and autoradiographed for 24 h.

2.4. Plasmid constructs

The NFκB reporter construct, (Igκ)3-conaluc, which contains three copies of the immunoglobulin κ chain enhancer κB site upstream of the minimal conalbumin promoter [35], was a generous gift from Dr. Alain Israël (Institut Pasteur, Paris, France).

hUBC9 [8], hUBC5 [36], and IκB [37] constructs for mammalian cell expression (designated with pC prefix; e.g. pC-hUBC9) utilized the pCDNA 3.1 vector (Invitrogen, Carlsbad, CA) with a hemaglutanin tag at the NH_2-terminus. The catalytic domain construct of murine MEKK1 used for mammalian cell expression, HA-ΔMAPKKK in pECE [38] (designated in this paper as pC-MEKK1CAT), was a gift of Dr. Jacques Pouysségur (Université de Nice) and contained a hemaglutanin tag at the NH_2-terminus of the catalytic domain of the kinase [20].

2.5. Mutagenesis

The mutagenesis of hUBC9 was performed using the Transformer site directed mutagenesis kit (Clontech) and the mutagenesis oligo 5′-CTTCGGGGACAGTGTCCCTGTCCATCTTAGAGG-3′. The underlined sequence corresponds to the Cys (TGC) to Ser (TCC) codon change. The presence of the mutation was confirmed by DNA sequencing. DNA sequencing was performed using an ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) following the protocol described by the manufacturer. Automative DNA sequence analysis was performed using a 3T3 DNA sequencer (Applied Biosystems).

2.6. Transfection studies

HeLa cell transfections were performed in 6 well dishes using a calcium phosphate transfection kit (Gibco-BRL) following the protocol described by the manufacturer. Transfection medium was replaced, 24 h following the transfection, with complete medium, followed 8 h later by placing the cells in serum free medium. After a 16 h incubation in serum free medium, medium was changed to complete medium which, when indicated, contained 100 ng/ml TNF-α (Gibco-BRL). 24 h after treatment, cells were harvested for measurement of luciferase. Luciferase assays were performed using a luciferase assay system (Promega) according to the manufacturer's instructions. Luminescence was measured using an ICN Titertek Luminoskan (Labsystems, Needham Heights, MA). In experiments in which expression vector constructs were transfected, expression of the respective proteins was confirmed by Western analysis [39], using a hemaglutanin specific antibody (BAbCO, Richmond, CA).

2.7. Interleukin 6 (IL-6) ELISA assays

For IL-6 measurements, a 200 μl aliquot of medium (total volume 2 ml) was analyzed for each experimental sample. IL-6 levels were quantitated using a Duoset IL-6 ELISA kit (Genzyme, Cambridge, MA) using the protocol supplied by the manufacturer. ELISA measurements were obtained using an EL 312e microtiter plate reader (Bio-Tek Instruments, Winooski, VT).

3. Results

3.1. hUBC9 interacts with type I TNF-α receptor and MEKK1

Two separate yeast two-hybrid screens were performed to identify binding partners for the human type I TNF-α receptor and MEKK1, respectively. For the type I TNF-α receptor, the cytoplasmic domain of the receptor was used as bait to screen a human Jurkat cDNA library. In the MEKK1 screening, the murine clone was used to screen a HeLa cell cDNA library. Both screens isolated hUBC9 as a binding partner for their respective bait. The interaction was confirmed by the reintroduction of clones for hUBC9 into yeast with their respective bait (Fig. 1).

As an additional confirmation of the interaction of hUBC9 with these proteins, it was determined if the transcribed/translated product of the respective protein could interact with GST-hUBC9. Both ^{35}S-labeled type I TNF-α receptor and MEKK1 were found to bind to GST-hUBC9 (Fig. 2A), but not GST alone (Fig. 2B).

3.2. hUBC9 binds to the catalytic domain of MEKK1 and the death domain of type 1 TNF-α receptor

Deletion mutants of MEKK1 and the type I TNF-α receptor, respectively, were characterized for their ability to interact with hUBC-9, using the yeast two-hybrid system (Fig. 1) and GST-hUBC9 pull down experiments (Fig. 2). For MEKK1 it was determined that the catalytic domain interacted with hUBC9. For the type I TNF-α receptor, it was found that hUBC9 interacts with the 'death domain' of the molecule.

3.3. hUBC9 induces NFκB activity

Because the activation of MEKK1 and type I TNF-α receptor has been associated with the activation of NFκB, experiments were performed to determine if hUBC9 can modulate NFκB activity. When hUBC9 was overexpressed in HeLa cells cotransfected with an NFκB-luciferase reporter construct, an increase in basal and TNF-α induced luciferase activity was observed over control transfected samples (Fig. 3a). Coexpression of IκB with hUBC9 completely inhibited both hUBC9 stimulated and TNF-α induced NFκB reporter activity, indicating that the induction of reporter activity was due to NFκB activation (Fig. 3a). Other reporter constructs tested (e.g. heat shock and serum response) were not stimulated by hUBC9 (data not shown), indicating that the effects seen with hUBC9 were specific for NFκB.

In order to further confirm that the overexpression of

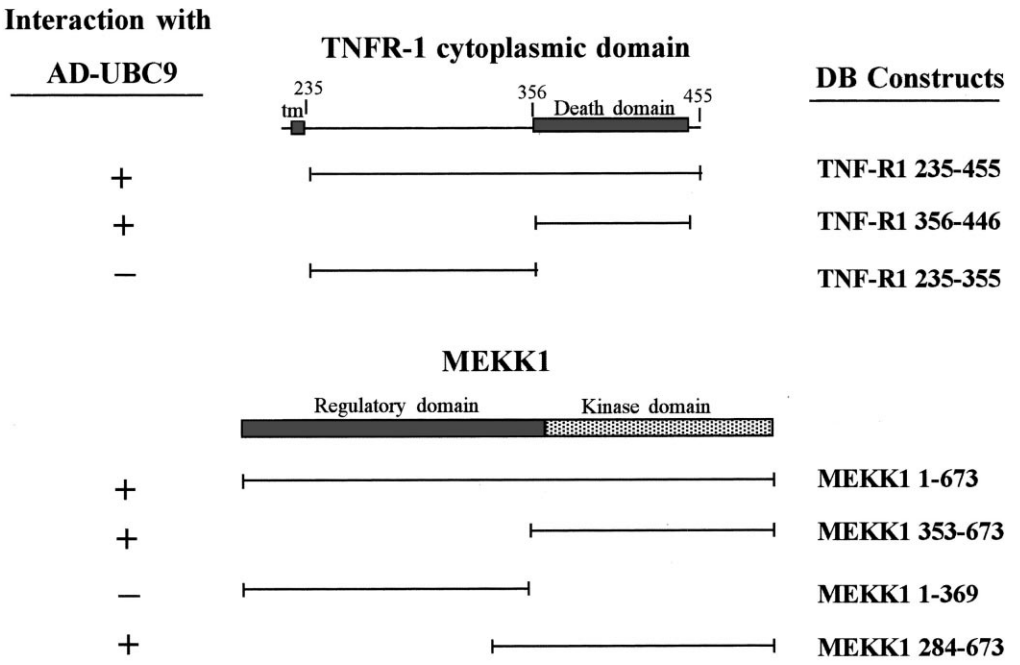


Fig. 1. Yeast two-hybrid binding analysis of the interaction of hUBC9 with the TNF-α receptor type I and MEKK1. Interaction experiments were performed by using the portions of the TNF-R1 and MEKK1 indicated, as constructs in a two-hybrid GAL4 DNA binding domain vector (DB Constructs) cotransformed with hUBC9 in a GAL4 activation domain vector, as described in Section 2. Interactions are scored as a + and no interaction as a −.

hUBC9 induces NFκB activity, NFκB stimulation of IL-6 secretion was tested. When HeLa cells overexpressing hUBC9 were treated with TNF-α, a significant increase in IL-6 secretion was observed, compared to vector alone transfected cells (Fig. 3b).

3.4. *Characterization of the induction of NFκB activity by hUBC9*

Studies were performed to determine if the induction of NFκB activity by hUBC9 was a general feature of E2 enzymes. When hUBC5, an E2 enzyme associated with P53 degradation [36], was overexpressed in HeLa cells no stimulation of TNF-α induced NFκB activity was observed. (Fig. 4a). This finding indicates that the stimulation of NFκB activity by hUBC9 was not a general property of E2 enzymes.

To determine the importance of E2 enzymatic activity in hUBC9's ability to stimulate NFκB activity, HeLa cells were transfected with a mutated form of hUBC9 in which a conserved cysteine residue important for thiolester formation [1] was mutated to serine (Cys^{93}-Ser). As shown in Fig. 4b, the Cys^{93}-Ser mutant of hUBC9 was also able to stimulate TNF-α induced NFκB reporter activity. The general conclusion from these experiments is that the induction of NFκB activity by hUBC9 is not due to its E2 enzymatic properties.

3.5. *hUBC9 synergizes with MEKK1 to activate NFκB activity*

Since it has been reported that MEKK1 can stimulate NFκB activity [23,25], it was tested whether hUBC9, since it can interact with MEKK1, can enhance the ability of MEKK1 to induce NFκB activity. Transfection of the catalytic domain of MEKK1 into HeLa cells gave a 100-fold activation of TNF-α induced NFκB reporter activity (Fig. 5). Cotransfection of the catalytic domain of MEKK1 with hUBC9 resulted in a 150-fold activation of TNF-α induced NFκB reporter activity, indicating a synergistic effect of the two proteins on NFκB activity.

4. Discussion

In this report, we describe the interaction of the human homologue of UBC9 with two proteins associated with the induction of NFκB activation, MEKK1 and the type 1 TNFα receptor. In addition, we show that overexpression of hUBC9 in HeLa cells results in the activation of NFκB activity.

UBC9 is a member of a family of ubiquitin conjugating enzymes involved in the transfer of ubiquitin to cellular substrates [1,2]. The transfer of ubiquitin to proteins has been found to be an essential element in the process by which

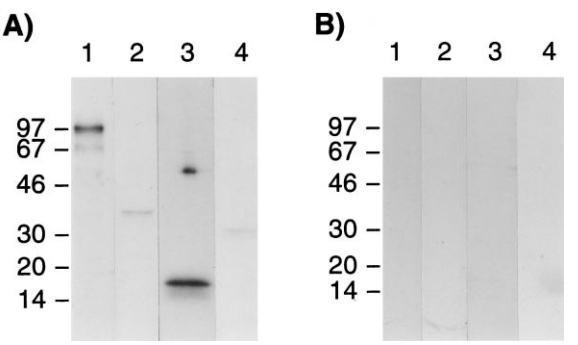


Fig. 2. Interaction of ^{35}S-labeled MEKK1 and TNF-α receptor with GST-hUBC9. ^{35}S-labeled translation products for (1) MEKK1, (2) MEKK1 CAT, (3) death domain of type I TNF-α receptor, and (4) cytoplasmic domain of type I TNF-α receptor with either (A) GST-hUBC9 or (B) GST. ^{35}S-labeled translation products which bound to GST fusion proteins were analyzed by SDS-PAGE electrophoresis, as described in Section 2.

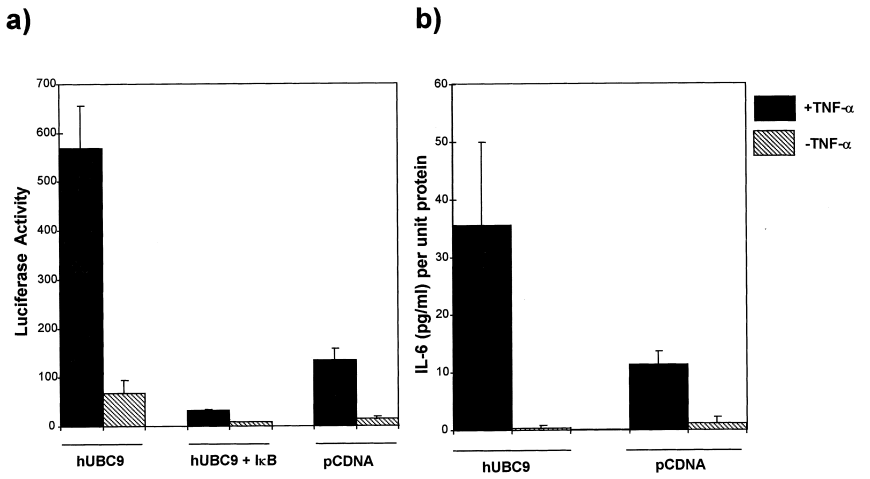


Fig. 3. Activation of NFκB activity by hUBC9. a: HeLa cells were cotransfected with NFκB reporter construct and either pC-hUBC9 and pCDNA 3.1 (2:1:1 ratio), pC-hUBC9 and pC-IκB (2:1:1 ratio), or pCDNA 3.1 (1:1 ratio). After 48 h, cells were untreated or treated with 100 ng/ml TNF-α. Cells were then analyzed, 24 h later, for luciferase activity as described in Section 2. b: HeLa cells were cotransfected with NFκB reporter construct and either pC-hUBC9 or pCDNA 3.1 (1:1 ratio). Cells, 48 h after transfection, were either untreated or treated with TNF-α (100 ng/ml). After an additional 24 h medium was analyzed for IL-6 secretion, as described in Section 2. Data are representative of three separate experiments ± S.E.M.

such proteins are degraded by the proteosome. More recently, hUBC9 has been shown to be an E2 enzyme for SUMO-1 [9–11], a ubiquitin-like molecule that is crosslinked to proteins associated with nuclear transport [12].

It would be interesting to speculate, especially with the findings on SUMO-1, that the E2 enzymatic activities of hUBC9 are involved in its ability to stimulate NFκB activity. However, data presented in this report do not support this theory. The NFκB stimulatory activity of hUBC9 cannot be inhibited by mutating the Cys^{93} site of hUBC9 to Ser. The Cys^{93} site is conserved amongst E2 enzymes and is necessary for thiolester formation [1]. The finding that the E2 enzyme defective mutant of hUBC9 can still stimulate transcriptional activity is similar to what was seen by Hahn et al. [5] on the activation of ETS-1 transcriptional activity by hUBC9.

The implication of these results is that hUBC9 has additional properties which are not dependent on its E2 enzymatic activity. Given the findings that this protein interacts with a wide range of other proteins, one may speculate that it may play a role as a scaffolding protein. In the case of MEKK1, hUBC9 may aid the ability of MEKK1 to phosphorylate given substrates by bringing MEKK1 and the given substrate together. Such a model would be analogous to that proposed for the MAP kinase cascade which is associated with the pheromone-response pathway in yeast [40]. In this model, Ste5 acts as a scaffold to bring the MAP kinase components Ste11, Ste7, and Fus3p together to promote the formation of a kinase complex.

Recently, Tashiro et al. [41] showed that the mouse homologue of UBC9 interacts with IκB. They found that the over-

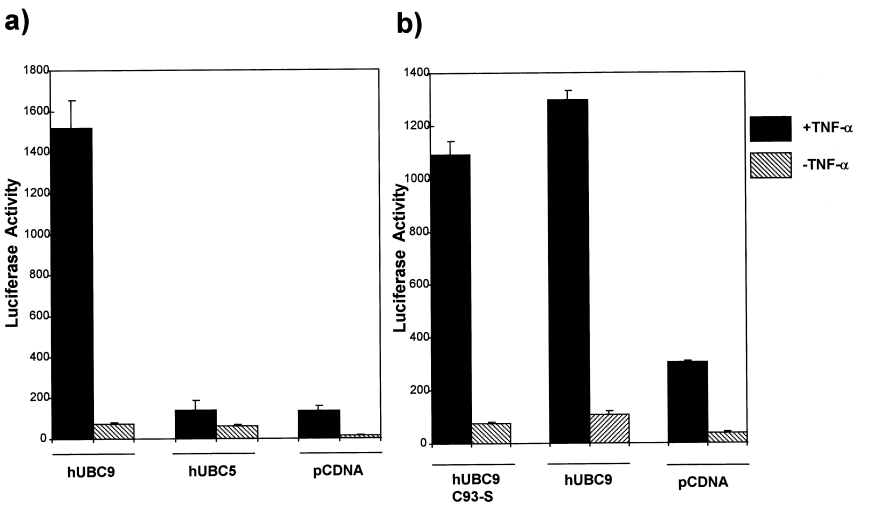


Fig. 4. Effect of hUBC5 or Cys^{93}-Ser mutation of hUBC9 on NFκB activation. a: HeLa cells were cotransfected with NFκB reporter and either pC-hUBC9, pC-hUBC5, or pCDNA 3.1 (1:1 ratio) and then either untreated or treated with 100 ng/ml TNF-α 48 h later. Cells were then analyzed 24 h later for luciferase activity. b: HeLa cells were cotransfected with NFκB reporter and either pC-C93-S hUBC9, pC-hUBC9, or pCDNA 3.1 (1:1 ratio) and analyzed, as described above, for the stimulation of NFκB activation. Results are representative of duplicate determinations ± S.E.M.

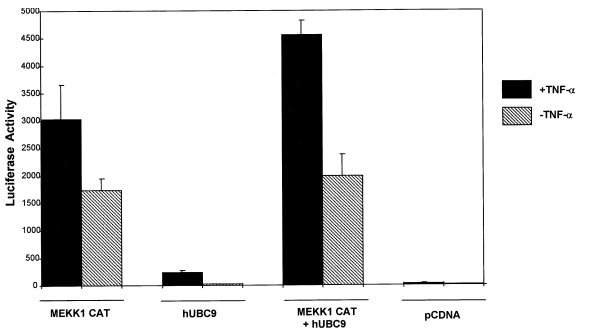


Fig. 5. hUBC9 synergizes with MEKK1 to stimulate NFκB activity. HeLa cells were cotransfected with NFκB reporter and either pC-MEKK1CAT and pCDNA 3.1 (2:1:1 ratio), pC-hUBC9 and pCDNA 3.1 (2:1:1: ratio), pC-MEKK1CAT and pC-hUBC9 (2:1:1 ratio), or pCDNA (1:1 ratio). Cells, 48 h after transfection, were either untreated or treated with 100 ng/ml TNF-α, and then processed 24 h later for luciferase activity. Data are representative of duplicate experiments ± S.E.M.

expression of an E2 defective mutant of mouse UBC9 delayed the TNF-α induced degradation of IκB and induction of NFκB DNA binding activity produced within the first 11 min of treatment. However, in our studies, where measurements were not taken until at least 2.5 h following TNF-α treatment, an E2 defective mutant of hUBC9 induced NFκB activity. In summary, these results infer a complex role of hUBC9 in the regulation of NFκB activity whereby its E2 activity may be important in the initial stimulation of NFκB, but other properties of this protein, such as scaffolding, may be important in later stages.

In conclusion, we have identified hUBC9 as a protein which is involved in the regulation of NFκB activity. The ability of hUBC9 to stimulate NFκB activity does not appear to be due to its enzymatic properties, but potentially through its ability to associate with proteins which are involved in the activation of NFκB, such as MEKK1 and type I TNF-α receptor. The understanding of the mechanism by which this activation occurs will provide additional insight into the regulation of NFκB activity and hUBC9 function.

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