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HOMEOSTATIC INTERACTIONS BETWEEN MEKK3 AND TAK1 INVOLVED IN NF- κ B SIGNALING

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Abstract

Several members of the mitogen-activated protein kinase kinase kinase (MAP3K) family including MEKK3 and TGF β -activating kinase (TAK1) play nonredundant roles in activation of the NF- κ B transcription factor. However, the mechanism by which MEKK3 mediates NF- κ B signaling is not fully understood. In this report we investigate the association of murine MEKK3 with other proteins and their roles in NF- κ B activation. Using tandem affinity purification TAK1 was identified as an endogenous protein that interacts with MEKK3. MEKK3-TAK1 interactions were confirmed by fluorescence resonance energy transfer and coimmunoprecipitation. MEKK3-TAK1 complexes contain non-phosphorylated forms of both molecules. Expression of non-phosphorylated TAK1 interferes with MEKK3 phosphorylation and NF- κ B reporter activity induced by transient MEKK3 expression or TNF α stimulation. Addition of TAB1 facilitates TAK1 autophosphorylation and reverses the inhibitory effects of TAK1 on MEKK3 phosphorylation and NF- κ B signal transduction in human 293 cells and TAK1 deficient mouse embryonic fibroblasts. The data provide insights into the homeostatic interactions that maintain basal NF- κ B levels by holding the enzymes MEKK3 and TAK1 in their inactive state.

Keywords

TAK1; TAB1; MEKK3; NF- κ B regulation; TNF α signaling; tandem affinity purification

1. Introduction

The mitogen-activated protein/ERK kinase kinase 3 (MEKK3) is a member of a family of MEKK/Ste11 serine/threonine protein kinases. One function of MEKK3 is to serve as an upstream regulatory kinase in the mitogen-activated protein kinase cascade, however, it also plays a critical role in other signaling pathways. MEKK3 regulates several important functions including cell survival and proliferation (1). This protein is remarkably well conserved from yeast to mammals. It is ubiquitously expressed in multiple tissues and has been found in complexes with scaffold proteins and other kinases (2).

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Responses to a diverse array of inflammatory mediators including the cytokines tumor necrosis factor α (TNF α) (3) or interleukin 1 β (IL-1 β) (4,5) and cellular stressors (6,7) provide an impetus for MEKK3-dependent signal transduction. This coupling to multiple input signals allows many opportunities for regulation and activation of downstream signaling cascades (2). MEKK3 couples these upstream signals with various outputs including activation of the ERK (8), p38 (4,7), JNK (9,10), ERK5 (11), NFAT (12), and NF- κ B (13) signal transduction pathways. The downstream components in many of these pathways are defined. However, the role of MEKK3 in NF- κ B signaling remains incompletely characterized.

Mouse embryos carrying a disruption of the gene encoding MEKK3 die at embryonic day 11 with defects in angiogenesis and cardiovascular development (14). Murine fibroblasts derived from MEKK3 deficient embryos demonstrate impaired NF- κ B activation following stimulation with TNF α or IL-1 β (3). Knockdown of MEKK3 in a macrophage cell line also resulted in impaired LPS-induced NF- κ B signaling and cytokine synthesis (15).

Phosphorylation of multiple MEKK3 sites was noted following stimulation of cells with TNF α and other exogenous stimuli (16,17). Serine residues within the activation loop regulate MEKK3 catalytic activity and serve as a sensor of LPS-induced NF- κ B signaling (17,18). Although the kinases responsible for *in vivo* MEKK3 phosphorylation remain poorly defined some evidence suggests phosphorylation of the activation loop may include a mechanism involving dimerization (19) and self-phosphorylation (17,18).

Overexpression of MEKK3 results in autophosphorylation, bypassing the need for an exogenous stimulus, and leads to NF- κ B activation (9,13). In the present experiments we examine the molecular mechanisms controlling MEKK3 phosphorylation and NF- κ B activation following ectopic expression. We noted a physical association between MEKK3 and TAK1 and now describe the functional parameters of this interaction.

2. Materials and Methods

2.1 Cell culture and reagents

HEK293T cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). TAK1 deficient MEF were a gift from Dr. S. Ghosh (Yale Medical School). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Monoclonal anti-MEKK3 antibody was purchased from BD Biosciences (San Diego, CA). Anti-FLAG antibody, FLAG peptide, HA peptide, and puromycin were bought from Sigma Chemical Co (St. Louis, MO) and anti-Myc antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human TNF α was purchased from R&D Systems (Minneapolis, MN). Anti-phospho-TAK1 (Thr184/187) antibody (90C7) was bought from Cell Signaling (Beverly, MA). Calf intestinal alkaline phosphatase (CIP) was from New England Biolabs (Ipswich, MA). The silver stain Kit and Nu-page gels were from Invitrogen (Carlsbad, CA).

2.2 Plasmids and mutagenesis

Mouse MEKK3 and TAB1 cDNA were purchased from ATCC. The TAB1 β expression construct was provided by Dr. J. Han (Scripps, La Jolla, CA). TAK1 cDNA was amplified from mouse brain cDNA. pcDNA3-YFP and pcDNA3-CFP were purchased from Addgene (Cambridge, MA). The Exchanger cloning system was purchased from Stratagene (La Jolla, CA). The plasmids encoding FLAG-MEKK3 were constructed by inserting the ClaI/ApaI fragment into the corresponding sites in the pCMV-3Tag-6 vector. All expression plasmids were verified by DNA sequencing. The primers used for MEKK3 mutagenesis were: S526A

(5'CATGTCAGGGACAGGAATTCGCGCTGTCACTGGCACACCCTAC 3') and K391M (5' CACAGGACGTGAACTTGCTTCTATGCAGGTCCAGTTTGACC 3').

2.3 Establishment of stable cell lines

MEKK3 with an N-terminal FLAG tag and BglII site plus a C-terminal hemagglutinin (HA) tag and EcoRV site plus a puromycin-resistance gene was constructed using the pExchange-core-2 system. The construct was transfected into HEK293T cells. After 48 hr 3 μ g/ml puromycin was added. 4–5 days later, single cells were picked and cultured for 8–10 days in medium containing 3 μ g/ml puromycin.

2.4 Tandem affinity purification

The tandem affinity purification kit was purchased from Sigma. MEKK3 stable HEK293T cells were cultured in 150 mm dishes, when cells grew to 80–90% confluence, they were serum starved in antibiotic-free DMEM. After 4 hr cells were stimulated with medium or 10 ng/ml TNF α for 15 min. Cells were harvested using RIPA lysis buffer (Sigma) containing a protease inhibitor (PI) cocktail (Roche, Indianapolis, IN). The lysate was rotated 1 hr at 4°C. Cellular debris was removed by centrifugation for 15–30 min at 15,000 rpm at 4°C. MEKK3 and its binding proteins were purified by overnight incubations with sequential FLAG and HA affinity columns. The resins were 3X washed with RIPA+PI buffer (5000 rpm at 4°C for 2 min) and eluted with FLAG (2.5 times the packed-resin volume with 150 ng/ μ l 3X-FLAG peptide) and HA peptide (1 μ g/ μ l), respectively. Protein samples were separated on a 4 to 12% NuPAGE Novex Bis-Tris 4–12% gel under denaturing conditions. The gel was silver stained using the Basic Staining Protocol (Invitrogen). The bands of interest were excised from the gel and the slices were twice washed with 1 ml 50% acetonitrile. Samples were analyzed at the Beth Israel Deaconess Medical Center mass spectrometry core facility. Protein identification was performed by liquid-chromatography tandem mass spectrometry (LC-MS/MS) and the data were searched against the International Protein Index and Genbank.

2.5 Cell transfection and luciferase activity assay

HEK293T cells grown on 24 or 96-well plates were transiently transfected as detailed elsewhere (20). After 48 hr the cells were serum starved for 4 hr and stimulated with cytokine for 6 hr. Luciferase activity was determined using the Dual Luciferase reporter system (Promega, Madison, WI) as previously described (20). Expression of transfected proteins were confirmed by immunoblotting with appropriate epitope-specific antibody. Data presented are the mean of triplicates from a representative experiment.

2.6 Immunoprecipitation and immunoblotting

Forty-eight hr after transfection cells were harvested using ice cold RIPA+PI buffer. Cellular debris was removed by centrifugation at 15,000 rpm for 10 min at 4°C. 500 μ g whole cell lysate was transferred to a fresh tube and cold RIPA+PI buffer was added to bring the total volume to 500 μ l. The cell lysates were precleared by addition of 20 μ l Protein G Plus-Agarose (Roche); the mixture was resuspended and incubated at 4°C for 30 min. The agarose beads were pelleted by centrifugation at 2500 rpm for 5 min at 4°C. The supernatant was transferred to a fresh tube and 0.2–2 μ g primary antibody was added then incubated overnight at 4°C. 20 μ l Protein G Plus-Agarose was added and the tubes were rocked for 3–4 hr at 4°C. Immunoprecipitates were pelleted at 2500 rpm for 5 min at 4°C. The pellet was washed 3–4 times with 0.5 ml RIPA buffer. After the final wash, the pellet was resuspended in 30 μ l of electrophoresis sample buffer. Samples were boiled for 5 min and then run on a 4–12% SDS-PAGE gradient gel (BioRad, Hercules, CA). Immunoblotting was performed as detailed previously (20).

2.7 Fluorescence resonance energy transfer (FRET)

MEKK3, RIP1, and TAK1 were subcloned into the pcDNA3/CFP or pcDNA3/YFP vectors. Immunoblotting confirmed equivalent expression of these constructs. Cells were grown on glass slide chambers (Sigma) for 48 hr. The slides were washed twice with PBS, then fixed with 4% paraformaldehyde (8 min at 37°C). After 3 washes the slides were mounted with coverslips. Cells were observed with an Olympus fluoview 1000 confocal microscope. Images were scanned and analyzed with FV19-ASW1.6 software. The fluorescence intensity of CFP (540–580 nm) and YFP (460–500 nm) were detected. YFP was bleached for 2500 msec per pulse with a laser at 515 nm. The difference of CFP fluorescence intensity before and after bleaching was analyzed.

2.8 In vitro kinase assay

HEK293T cells were plated in 6-well or 10-cm plates and transfected with FLAG-MEKK3, FLAG-MEKK3(S526A/K391M), Myc-TAK1, or Myc-TAB1 eukaryotic expression plasmids. After 48 hr the cells were harvested with RIPA+PI on ice (20 min). Cellular debris was removed by centrifugation at 13,000X g for 15 min. Equivalent amounts of proteins were immunoprecipitated with anti-FLAG or anti-Myc antibody. MEKK3 and mutant MEKK3 were eluted from protein G agarose with 3X-FLAG peptide. Myc-TAK1 or Myc-TAB1-protein G agarose complexes were washed 4–5 times with RIPA buffer and incubated with FLAG-MEKK3 or FLAG-MEKK3(S526A/K391M) in kinase buffer and 1 mM ATP (Cell Signaling) at 30°C for 2 hr. The beads were separated by centrifugation at 3,000X g for 5 min. Protein-bead complexes were washed 4–5 times and boiled in SDS loading buffer.

2.9 Statistical analysis

The two-tailed Student's *t* test was used to calculate sample distributions. The *F* statistic was used to analyze the homogeneity of variances.

3. Results

3.1. MEKK3 interacting proteins

Tandem affinity purification (TAP) was used to isolate cellular MEKK3 complexes and identify MEKK3 interacting proteins. The MEKK3 construct containing an N-terminal FLAG tag and a C-terminal HA tag was stably integrated into HEK293T cells by puromycin selection. Cells with low levels of MEKK3 integration were selected for preferential identification of biologically relevant molecular associations. Expression levels of MEKK3 protein in stably transfected cells was approximately double endogenous levels (Fig. 1A). Stably transfected cells were treated with either medium or TNF α (10 ng/ml) for 15 min. The tagged MEKK3 complexes were purified from cell lysates by sequential elution from anti-FLAG and anti-HA beads. Non-transfected 293 cells were included as a negative control. The MEKK3 associated endogenous proteins were separated from the complexes by SDS-PAGE electrophoresis. Multiple bands appeared in the fractions collected from medium or TNF-treated stably transfected 293 cells (Fig. 1B). However, no obvious band distinguished between the medium and TNF-treated cells. These findings are consistent with the observation that MEKK3 overexpression leads to cellular activation as monitored by NF- κ B activation (9,13). Fractions containing the selected prominent bands were excised from the gel and submitted for identification by LC-MS/MS. Several endogenous MEKK3-interacting proteins (HSP70, TRAF7, β -tubulin, and MEK5 β) were identified by amino acid sequence analysis from multiple independent peptide fragments (Fig. 1B). Attempts to identify proteins in fainter bands by LC-MS/MS were unsuccessful.

To identify additional molecules potentially obscured by the major dual MEKK3 bands at 80 kDa we tested parallel samples by Western blotting with anti-TAK1 or anti-RIP1 antibody. An endogenous 80 kDa TAK1 molecule was detected in the TAP-purified MEKK3 complexes (Fig. 1C). Negative controls included Western blotting with anti-RIP1 antibodies (data not shown).

3.2 Fluorescence resonance energy transfer (FRET) detects the interaction between MEKK3 and TAK1

To independently demonstrate the association of MEKK3 with TAK1 we transiently transfected the CFP-MEKK3 and YFP-TAK1 constructs into 293 cells. Colocalization was measured directly by the FRET photobleach method. The CFP intensity increased after bleaching YFP indicating the close association between MEKK3 and TAK1 in living cells (Fig. 2). We could not detect associations between fluorochrome-tagged MEKK3 and RIP1 using the same methodology (data not shown).

3.3 TAK1 regulates MEKK3-induced NF- κ B activity and controls MEKK3 phosphorylation

To explore the functional consequence of MEKK3-TAK1 interactions on NF- κ B signaling we transiently expressed TAK1 along with MEKK3 or a mutant MEKK3 in which the critical S526 phosphorylation site and K391 catalytic site were mutated MEKK3(S526A/K391M) into 293 cells. Cells were either treated with medium or 10 ng/ml TNF α . Expression of wild type MEKK3 in control cells resulted in increased expression of the NF- κ B luciferase reporter whereas transfection of kinase dead mutant MEKK3 had no effect (Fig. 3A). Overexpression of TAK1 alone had little effect on reporter activity, however, concomitant expression of TAK1 inhibited MEKK3-induced NF- κ B activation (Fig. 3A). TNF α treatment induced NF- κ B promoter activity and overexpression of MEKK3 further increased NF- κ B activity. Expression of TAK1 also inhibited MEKK3-induced NF- κ B reporter activity in TNF α treated cells (Fig. 3A).

To investigate the mechanisms involved in the regulation of MEKK3-induced NF- κ B activity we transiently expressed FLAG-MEKK3 with or without Myc-TAK1, 48h later cell lysates were prepared and treated with buffer or phosphatase (CIP). We visualized MEKK3 by Western blotting with anti-FLAG antibody. Overexpressed wild type MEKK3 ran as two bands the slower migrating band disappeared following phosphatase treatment indicating that MEKK3 overexpression induced a phosphorylated MEKK3 species which could be identified by decreased mobility on SDS-PAGE (Fig. 3B).

Overexpression of a mutant MEKK3 construct (in which the critical S526 phosphorylation site was destroyed) resulted in appearance of a single band that migrated along with the rapidly migrating MEKK3 species and was not sensitive to the effects of CIP supporting the conclusion that the upper MEKK3 band represents phosphorylated MEKK3. Importantly, coexpression of MEKK3 with TAK1 resulted in appearance of only the more rapidly migrating band indicating that TAK1 inhibited MEKK3 phosphorylation (Fig. 3B).

Catalytically active TAK1 exists in a complex with a TAK1 binding protein (TAB1) (21,22). To examine the effects of TAB1 on MEKK3 phosphorylation we transiently expressed TAB1 and TAK1 in 293 cells. MEKK3 phosphorylation was completely restored after TAB1 was cotransfected with TAK1 (Fig. 4A).

We used an *in vitro* kinase assay to examine the role of MEKK3 and TAB1 in TAK1 phosphorylation. TAK1 autophosphorylation is dependent on the presence of TAB1 *in vitro* (Fig. 4B). Under the experimental conditions employed MEKK3 did not phosphorylate TAK1

(Fig. 4B and data not shown). Addition of wild type or mutant MEKK3 had no effect on TAB1-dependent TAK1 phosphorylation (Fig. 4B).

To evaluate the functional consequence of TAB1 on TAK1 inhibition of MEKK3-induced NF- κ B transcriptional activity 293 cells were transfected with various combinations of MEKK3, TAK1 and TAB1. Transfection with MEKK3 alone stimulated NF- κ B reporter activity while cotransfection of MEKK3 and TAK1 displayed little activity (Fig. 4C). As reported previously (21) transfection of TAK1 plus TAB1 also stimulated NF- κ B transcriptional activity. Addition of TAB1 reversed the inhibitory effect of TAK1 on MEKK3-induced NF- κ B reporter activity. The combination of MEKK3 plus TAK1 and TAB1 enhanced NF- κ B activity compared to MEKK3 alone (Fig. 4C). To examine specificity we compared TAB1 with TAB1 β , the natural splice variant of TAB1 which lacks the C-terminal TAK1 binding domain and does not support TAK1 phosphorylation or activation of TAK1 kinase activity (23). Cotransfection of TAB1 β with MEKK3 and TAK1 did not augment NF- κ B reporter activity (Fig. 4C). Comparable results were also noted with TNF α treated cells (Fig. 4C). The combined results suggest that nonphosphorylated TAK1 regulates MEKK3 phosphorylation and NF- κ B transcriptional activity.

3.4 MEKK3 activity in TAK1 $^{-/-}$ cells

To investigate the position of MEKK3 relative to TAK1 we examined NF- κ B signaling in TAK1 $^{-/-}$ MEF. Transfection of MEKK3 significantly activated NF- κ B reporter activity in TAK1 deficient cells (Fig. 5), indicating that MEKK3-mediated signal transduction was independent of TAK1 and implied that MEKK3 acts downstream or independently of TAK1. Restoration of TAK1 completely inhibited MEKK3-induced NF- κ B reporter unless cells were also transfected with TAB1 (Fig. 5).

As previously demonstrated (24,25), TAK1 $^{-/-}$ cells do not display NF- κ B signaling following TNF α treatment and responsiveness can be restored by TAK1 transfection. The inhibitory interaction of MEKK3 was clearly noted in TNF α stimulated TAK1 $^{-/-}$ MEF since MEKK3 or TAK1 transfection induced NF- κ B transcriptional activity but cotransfection of MEKK3 with TAK1 was completely inactive. As expected the combination of MEKK3 with TAK1 and TAB1 stimulated NF- κ B reporter activity (Fig. 5).

3.5 TAB1 regulates TAK1 phosphorylation and TAK1-MEKK3 interaction

Finally, we examined the effects of MEKK3 on TAK1 phosphorylation. Various combinations of epitope-tagged constructs of TAK1, MEKK3, mutant MEKK3, and TAB1 were transfected into 293 cells. Anti-TAK1 antibody was used to immunoprecipitate the TAK1 complexes which were analyzed by Western blotting. Coexpression of TAB1 facilitated TAK1 phosphorylation; addition of wild type or mutant MEKK3 did not affect TAK1 phosphorylation (Fig. 6A). *In vivo* TAK1 phosphorylation is manifest as a single band although two phosphorylated species are observed after *in vitro* TAK1 autophosphorylation (Fig. 4B) suggesting differential phosphorylation mechanisms.

The TAK1 immunoprecipitates were also probed with anti-FLAG antibody to detect MEKK3-TAK1 complexes. A reciprocal relationship was noted between MEKK3-TAK1 complexes and TAB1-dependent TAK1 phosphorylation. Only those samples of MEKK3 that contained non-phosphorylated TAK1 were found in complexes. These conclusions were supported by reciprocal experiments in which phosphorylated-TAK1 was not detected in anti-FLAG immunoprecipitated MEKK3 complexes (Fig. 6B). Furthermore, additional transfection with TAB1 to phosphorylate TAK1 resulted in loss of the MEKK3-TAK1 complexes (Fig. 6C). Thus, non-phosphorylated TAK1 interacts with MEKK3 but after TAB1 cotransfection TAK1

becomes phosphorylated and the MEKK3-TAK1 association disappears. Analysis of lysates demonstrates that activated TAK1 facilitates MEKK3 phosphorylation (Fig. 6A).

4. Discussion

There is considerable information regarding the cascade of events that occur downstream of MEKK3 (6,26–28) but little is known about the mechanisms which regulate MEKK3 activation. Ectopically expressed MEKK3 activates the NF- κ B pathway without addition of an exogenous agonist (3,8). This suggests that some process intrinsic to MEKK3 is sufficient for activation of signal transduction. Thus, there should be a mechanism that maintains this powerful kinase in an inactive state.

We demonstrated MEKK3 associations with endogenous TAK1 by purification in TAP assays (Fig. 1C) and confirmed that these molecules form a physical complex in living cells by FRET analysis (Fig. 2). Coimmunoprecipitation also demonstrated that TAK1 interacted with both wild type MEKK3 and a kinase dead MEKK3 mutant molecule (Fig. 6). Although a previous report (29) using TAP analysis to define MEKK3 interacting molecules failed to detect TAK1 the remaining MEKK3 interacting molecules including HSP70, TRAF7, β tubulin, and MEK5 β were detected (Fig. 2B). One difficulty in detecting TAK1 is that it migrates at the same position as MEKK3 and is obscured by the dense MEKK3 bands, thus we used Western blot to identify TAK1 in the MEKK3 complexes. TAK1 was shown to directly interact with the N-terminal regulatory domain of MEKK3 in a yeast two-hybrid interaction assay (30). Furthermore, MEKK3-TAK1 associations were previously noted by coexpression (30).

Several previous reports demonstrated interactions between overexpressed RIP1 and MEKK3 by coimmunoprecipitation (14,31–33). Since RIP1 also migrates at approximately the same rate as MEKK3 on SDS-PAGE we examined the TAP derived MEKK3 complexes by Western blotting with anti-RIP1 antibody. Consistent with the report of Bouwmeester, et al (29) we were unable to detect endogenous RIP1 in TAP-derived MEKK3 complexes from normal or TNF α stimulated cells (data not shown). The failure to detect MEKK3-RIP1 associations supports the specificity of the interactions between MEKK3 and TAK1. Attempts to identify MEKK3-RIP1 associations by FRET were also unsuccessful.

MEKK3 and TAK1 are serine/threonine kinases of the MAP3K family and both are involved in TNF α -mediated NF- κ B signaling (3,34,35). The activation of MEKK3 or TAK1 catalytic activity requires phosphorylation of critical residues within their activation loops (17,18,21, 22). The mechanisms controlling phosphorylation of the activation loop are poorly understood and the upstream kinases remain undefined. Most studies of MEKK3-dependent NF- κ B signaling involve overexpression, a condition which can promote oligomer formation. Dimers of MAP3K proteins can induce autophosphorylation and catalytic activity (19). These observations led to the notion that autophosphorylation is also involved in activation of MEKK3 (17,18). The interaction between MEKK3 and TAK1 has the potential to restrain the autophosphorylation of MEKK3. Thus, in cells we only detected interactions between non-phosphorylated MEKK3 and TAK1 molecules (Fig. 6). Furthermore, addition of TAK1 blocked MEKK3-induced NF- κ B signaling (Fig. 3A) and inhibited MEKK3 phosphorylation (Fig. 3B).

Activation of TAK1 catalytic activity requires TAB1, a TAK1-binding protein. The association of these two proteins promotes autophosphorylation of multiple residues within the activation loop (21,22). TAK1-TAB1 complexes induce NF- κ B activation (Fig. 4C) plus TAK1 autophosphorylation *in vitro* (Fig. 4B) and *in vivo* (Fig. 6A). Importantly, addition of TAB1 reverses TAK1 inhibition of MEKK3 phosphorylation (Fig. 4A) and NF- κ B transcriptional activity (Fig. 4C) and correlates with dissolution of MEKK3-TAK1 complexes (Fig. 6A and

6C). These conclusions are consistent with a report that concludes TAB1 is required for TAK1 and MEKK3 phosphorylation (30).

MEKK3 is generally positioned somewhere between RIP1 and the I κ B kinase (IKK) complex in the TNF-induced NF- κ B signaling pathway (3,31,36). It has been difficult to position MEKK3 relative to TAK1. Cells lacking either MEKK3 or TAK1 demonstrate defective TNF α mediated signaling (3,24,25). Overexpression of MEKK3 stimulates NF- κ B activity in TAK1 deficient MEF (Fig. 5). These findings suggest that MEKK3 either operates downstream of TAK1 (30) or in parallel pathways (5). In support of the latter model, Qin, et al (27) demonstrated that imidazoquinoline compounds trigger TLR8-mediated NF- κ B responses in a MEKK3-dependent but TAK1-independent fashion while Yao, et al. (5) reported that IL-1 induced both MEKK3-dependent and TAK1-dependent NF- κ B signaling. The present observation that phosphorylation of MEKK3 or TAK1 correlates with dissociation of MEKK3-TAK1 complexes and subsequent NF- κ B activation is consistent with a bifurcation model in which ligand activation disrupts the MEKK3-TAK1 complexes facilitating MEKK3 oligomerization and/or TAK1-TAB1 complex formation leading to autophosphorylation of either MEKK3, TAK1, or both. In our model (Fig. 7) the MEKK3-TAK1 complexes are inactive in unstimulated cells preventing basal NF- κ B signaling because of the antagonistic effects of TAK1 on MEKK3 (Fig. 4A and 6A). Proinflammatory activation leads to disruption of MEKK3-TAK1 complexes via TAB1 allowing both TAK1 and MEKK3 to transduce biochemical signals. The activated MEKK3 and TAK1 kinases eventually stimulate the IKK complex leading to IKK phosphorylation and NF- κ B activation (5,13).

5. Conclusions

We demonstrate that non-phosphorylated MEKK3 forms a complex with non-phosphorylated TAK1. The MEKK3-TAK1 complex may maintain basal levels of NF- κ B activity. TAB1 regulates the interaction of MEKK3 and TAK1. Effective activation of TAK1 autophosphorylation leads to MEKK3 phosphorylation, disruption of the MEKK3-TAK1 complex, and subsequent NF- κ B activation.

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Figure 1. MEKK3-TAK1 association in stably transfected cells

(A) Expression levels of MEKK3 in parental (lane 1) and stably transfected HEK293T cells (lane 2). In the upper panel, MEKK3 was immunoprecipitated and immunoblotted with anti-MEKK3 antibody. In the bottom panel, MEKK3 was precipitated with anti-FLAG antibody and immunoblotted with anti-HA. (B) TAP assay detects MEKK3 associated proteins. Silver-stained SDS-PAGE gel of tandem affinity purified MEKK3 complexes from parental HEK293T cells, FLAG-MEKK3-HA stably transfected cells, and TNF α -treated FLAG-MEKK3-HA cells. M1-M5 represent gel slices submitted for analysis by mass spectrometry and the major protein identified in each slice. (C) TAP detects association of MEKK3 with endogenous TAK1. Cell lysates from parental 293 cells, FLAG-MEKK3-HA stably transfected

cells, and TNF-treated FLAG-MEKK3-HA cells were Western blotted and stained with anti-TAK1 antibody.

Figure 2. FRET analysis of MEKK3-TAK1 interactions

CFP-MEKK3 and YFP-TAK1 tagged proteins were transiently transfected into HEK293T cells. 48 hr later cells were washed and fixed. (A) Distribution of CFP-MEKK3 and YFP-TAK1 proteins before bleaching. Dashed red line indicates sections used for FRET analysis. The fluorescence intensity of CFP and YFP before (B) and after (C) photobleaching.

Figure 3. TAK1 inhibits MEKK3-induced NF- κ B activity and MEKK3 phosphorylation
 (A) Effects of MEKK3, inactive MEKK3, and TAK1 expression on NF- κ B transcriptional activity. FLAG-MEKK3 or FLAG-MEKK3(S526A/K391M) were transfected into HEK293T cells with or without TAK1-Myc plus a NF- κ B luciferase reporter gene. The indicated groups were stimulated for 6 hr with TNF α . Relative luciferase activity was determined and normalized to *Renilla* luciferase activity. Experiments were performed in triplicate. Graphs include data from three independent experiments. An asterisk (*) indicates statistical significance ($P < 0.01$) between the indicated groups. (B) TAK1 regulates MEKK3 phosphorylation. FLAG-MEKK3 was transfected into HEK293T cells with or without Myc-TAK1, 48 hr later, cell lysates were prepared and some groups were incubated with calf intestinal alkaline phosphatase (CIP) for 30 min at 37°C. Protein samples were immunoblotted and stained for MEKK3 with anti-FLAG antibody.

Figure 4. TAB1 reverses TAK1-mediated inhibition

(A) TAK1 modulates MEKK3 phosphorylation. FLAG-MEKK3 was transfected into HEK293T cells with TAK1 or TAK1 plus TAB1, 48 hr later, cell lysates were prepared. Protein samples were immunoblotted and MEKK3 was detected with anti-FLAG antibody. Further details are included in Fig. 6A. (B) *In vitro* phosphorylation of TAK1. FLAG-MEKK3, FLAG-MEKK3(S526A/K391M), Myc-TAK1, and Myc-TAK1 proteins were expressed in HEK293T cells. The wild type and mutant MEKK3 proteins were purified and eluted with 3 $\times$ FLAG peptide. The TAK1 and TAB1 proteins were isolated on anti-Myc agarose beads. After the kinase assay the beads were washed and phospho-TAK1, TAK1, and TAB1 were detected by Western blot. Input levels of MEKK3 are presented. (C) TAB1 reverses TAK1 inhibition of

MEKK3-induced NF- κ B activity. HEK293T cells were transfected with the indicated constructs. Groups were treated with medium or TNF α for 6 hr. Relative luciferase activity was determined and normalized to *Renilla* luciferase activity. Graphs depict data from three independent experiments. An asterisk (*) indicates statistical significance ($P < 0.01$) between groups.

Figure 5. Effects of TAK1 and TAB1 on MEKK3-induced NF- κ B reporter activity in TAK1 $^{-/-}$ MEF

The indicated combinations of FLAG-MEKK3, Myc-TAK1, and Myc-TAB1 were transfected into TAK1 $^{-/-}$ MEF cells along with the NF- κ B luciferase reporter gene. 48 hr later, cells were serum starved for 4 hr then treated with medium or 10 ng/ml TNF α for 6 hr. Relative luciferase activity was determined and normalized to *Renilla* luciferase activity. Experiments were performed in triplicate. An asterisk (*) indicates statistical significance (P<0.01), a triangle (Δ) indicates (P<0.05).

Figure 6. Association between non-phosphorylated MEKK3 and non-phosphorylated TAK1
 (A) TAB1 regulates TAK1 phosphorylation and MEKK3-TAK1 interaction. The indicated epitope tagged constructs were transfected into HEK293T cells. After 48 hr cells were treated with medium or TNF α for 10 min. Cell lysates were prepared and the TAK1-Myc complex was immunoprecipitated with monoclonal anti-TAK1 antibody. Phosphorylated (Thr184/187) TAK1 was detected with anti-phosphoTAK1 antibody, MEKK3 associated with TAK1 was detected with anti-FLAG antibody, TAK1 and TAB1 were detected with anti-Myc antibody. (B) Reciprocal immunoprecipitation of MEKK3-TAK1 complexes. The indicated constructs were transfected into HEK293T cells. After 48 hr some groups were treated with TNF α for 15 min. FLAG-MEKK3 and FLAG-MEKK3(S526A/K391M) complexes were

immunoprecipitated with anti-FLAG antibody. (C) MEKK3 interacts with non-phosphorylated TAK1. The indicated constructs were transfected into HEK293T cells. After 48 hr cell lysates were prepared and immunoprecipitated with the indicated antibodies.

Figure 7. Model of MEKK3 interactions

MEKK3 interacts with endogenous TAK1 in unstimulated cells to prevent basal NF- κ B signaling. Following receptor activation or ectopic expression of MEKK3 or TAK1-TAB1 the MEKK3-TAK1 complex dissociates favoring bifurcation of independent MEKK3 and TAK1 signaling pathways. Complexes containing either MEKK3 oligomers or TAK1-TAB1 are autophosphorylated. The latter phosphorylated complexes directly or indirectly phosphorylate components of the IKK complex leading to NF- κ B transcriptional activity.