MEKK4 Is an Effector of the Embryonic TRAF4 for JNK Activation*

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Amy N. Abell and Gary L. Johnson

From the Department of Pharmacology and the Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7365

TRAF4 has previously been shown to activate JNK through an unknown mechanism. Here, we show that endogenous TRAF4 and MEKK4 associate in both human K562 cells and mouse E10.5 embryos. TRAF4 interacts with the kinase domain of MEKK4. However, this association does not require MEKK4 kinase activity. The interaction of MEKK4 and TRAF4 are further demonstrated by the colocalization of TRAF4 and MEKK4 in cells. Importantly, although TRAF4 has little or no ability to activate JNK independently, coexpression of TRAF4 and MEKK4 results in synergistic activation of JNK that is inhibited by a kinase-inactive mutant of MEKK4, MEKK4K1361R. MEKK4 binds the TRAF domain of TRAF4 and MEKK4/ TRAF4 activation of JNK is inhibited by expression of the TRAF domain. Furthermore, TRAF4 stimulates MEKK4 kinase activity by promoting MEKK4 dimerization and JNK activation can be stimulated by chemical induction of MEKK4 dimerization. The findings identify MEKK4 as the MAPK kinase kinase for TRAF4 regulation of the JNK pathway.

MAP2 kinase (MAPK) pathways are critical regulators of numerous cellular functions including cell proliferation, adhesion, migration, differentiation, and apoptosis. MEKK4 is a 180-kDa MAPK kinase (MAP3K) that phosphorylates and activates the MAPK kinases (MAP2K) MK3, MK4, MK6, and MK7. MKK4 and MKK7 phosphorylate and activate the MAPK JNK, whereas MKK3 and MKK6 phosphorylate and activate the MAPK p38. Both the upstream signaling molecules and the biochemical mechanisms that regulate MEKK4 activation of JNK are poorly defined.

TRAF4 (TNF receptor-associated factor) family proteins play important roles in development and immunity (1, 2). TRAF proteins function in part as scaffold to organize signaling complexes coupled to specific receptors at the plasma membrane. There are six proteins in mammalian genomes that encode TRAF proteins near their C terminus that mediate oligomerization of TRAF proteins and their association with specific membrane proteins. The null mutant of TRAF proteins organize signaling complexes involved in the regulation of MAPK and NF-kB activation (2). Mice with targeted gene disruption of TRAF2, -3, and -6 die shortly after birth, while disruption of the TRAF4 gene is embryonic lethal (3, 4). TRAF4-deficient mouse embryos display impaired neural tube closure defects and skeletal malformations similar to the embryonic lethal (3, 4). TRAF4-deficient mouse embryos display impaired closure of the neural tube in the embryo. MEKK4 oligomerization and JNK activation can be stimulated by chemical induction of MEKK4 dimerization. The findings identify MEKK4 as the MAPK kinase kinase for TRAF4 regulation of the JNK pathway.

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Materials and Methods

Cell Lines, Culture Conditions, and Transfections—K562 cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 1% penicillin and streptomycin. E10.5 embryos were isolated from timed matings of 129 Sv/Ev mice according to university and federal guidelines for the use of animals. 293 cells and COS-7 cells were cultured in Dulbecco's modified Eagle's high glucose medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin. Transfections of 293 cells were performed in 60-mm dishes using Lipofectamine Plus (Invitrogen) according to the manufacturer's specifications for 24–36 h. COS-7 cells were plated on coverslips and transfected as described for 293 cells.

Plasmids—Human TRAF4 cDNA was kindly provided by Nancy Raab-Traub (University of North Carolina, Chapel Hill). HA tagged full-length TRAF4 and the TRAF domain of TRAF4 (amino acids 308–470) in pCDNA3 were a kind gift from Dale Bredesen (Buck Center for Research in Aging). The TRAF4 N terminus (amino acids 1–307) and the TRAF4 Ring deletion mutant (amino acids 58–470) in pCDNA3 were kindly given by Wafik El-Deiry (Howard Hughes Medical Institute and University of Pennsylvania). HA-tagged JNK, HA-tagged full-length MEKK4, and HA-tagged MEKK4 kinase domain (amino acids 1301–1597) were as described previously (10). FLAG-tagged wild-type MEKK4 was constructed by subcloning MEKK4 into FLAG-pCDNA3. The kinase-inactive MEKK4, MEKK4K1361R, was constructed using PCR to replace the active site lysine with an arginine, producing a kinase-inactive MEKK4 that was verified by sequencing. FK506-binding protein (FKBP) MEKK4 fusion constructs were created by introduction of Sall sites on the wild-type MEKK4 kinase domain by PCR, and products were subcloned into pShi/SV-E-FKBP. A, a gift from David Spencer (Baylor) containing a point mutation Phe290→Val (FV) engineered in the FKBP results in 1000× higher binding affinity of the synthetic dimerizer AP20187 compared with the wild-type FKBP (11). Two Fv sites were inserted on the N terminus of the kinase domain of MEKK4 and a C-terminal HA tag.

Immunoprecipitations, Western Blot Analysis, Measurement of JNK Activity, and Kinase Assays—Cells were lysed in cold Buffer A containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-1, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 0.05 mM dithiothreitol, 1 μM MgCl2, and 17 μg/ml aprotinin. 500 μg of lysate was immunoprecipitated for 1.5 h with the indicated antibodies, followed by incubation with protein G-Sepharose (Zymed Laboratories Inc.) for 1.5 h. Immunoprecipitates were washed four times with cold Buffer A. Lysates and immunoprecipitates were probed with anti-TRAF4 (Santa Cruz Biotechnology), anti-MEKK4, anti-FLAG (Sigma), anti-HA (12CA5) and phosphospecific antibodies against phospho-JNK (Cell Signaling). For kinase assays, lysates were prepared and immunoprecipitated as described above. Immunoprecipitates were washed three times with cold lysis buffer and once with cold kinase buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 10 mM β-glycerophosphate, and 0.5 mM ATP. Reactions were incubated with inactive His-MKK6 or GST-c-Jun at 30 °C for 20 min and probed with anti-FLAG, anti-TRAF4, anti-HA, anti-phospho-c-jun, and anti-phospho-MKK3 (Cell Signaling) antibodies. Chemical dimerization experiments using AP20187 provided by Ariad Pharmaceuticals (www.araid.com) were performed as described previously (11).

 Immunofluorescence—For TRAF4 and MEKK4 staining, COS-7 cells plated on coverslips were fixed for 10 min in 3% paraformaldehyde containing 3% sucrose and phosphate-buffered saline pH 7.4 and were permeabilized for 6 min in 0.1% Triton X-100 in phosphate-buffered saline. Coverslips were washed, blocked in 10% donkey serum, and incubated for 1 h with murine anti-FLAG and goat anti-TRAF antibodies diluted 1:500. Coverslips were washed and incubated with DAPI (0.04 ng/ml), Cy3 donkey anti-goat diluted 1:500, and fluorescein isothiocyanate donkey anti-mouse. Imaging was performed using an Axiovert 200 microscope from Zeiss. Imaging software from Intelligent Imaging Innovations (Denver, Colorado) was used to perform nearest neighbors deconvolution on 0.1-μm sections.
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RESULTS AND DISCUSSION

TRAF4, an Atypical Embryonic TRAF, Binds MEKK4—Immunoprecipitation assays using K562 cells that endogenously express high levels of both TRAF4 and MEKK4 demonstrated that endogenous MEKK4 was specifically coprecipitated with TRAF4 (Fig. 1A). Similarly, immunoprecipitation of endogenous MEKK4 from K562 cells revealed an association of TRAF4 (data not shown). Both TRAF4 and MEKK4 are expressed at low levels in most adult mouse tissues, whereas expression is strong throughout embryogenesis with particularly high levels in the developing neuroepithelium (5, 7, 8). Significantly, TRAF4 and MEKK4 coprecipitated in lysates prepared from E10.5 embryos showing the association of MEKK4 and TRAF4 during development (Fig. 1B). These data demonstrate an in vivo association of TRAF4 and MEKK4 and suggest a role for this interaction in MEKK4 and TRAF4 function.

Analysis of TRAF4 and MEKK4 Interaction Domains—Analysis of the domains of MEKK4 that bind full-length TRAF4 revealed that the N terminus of MEKK4 did not bind TRAF4 (data not shown). The kinase domain alone of MEKK4 was sufficient for the association of MEKK4 with TRAF4 (Fig. 2A). However, binding of TRAF4 to MEKK4 was not dependent on MEKK4 kinase activity as TRAF4 coimmunoprecipitated equally with FLAG-tagged wild-type MEKK4 or a kinase-inactive MEKK4 where the active site lysine is substituted with an arginine (MEKK4K1361R) (Fig. 2B). Coprecipitation of endogenous TRAF4 with MEKK4 from lysates prepared from E10.5 embryos. Embryo lysates prepared from embryos isolated in timed matings were immunoprecipitated with either an anti-MEKK4 antibody or anti-TRAF4 antibody and immunoblotted with antibodies to TRAF4 and MEKK4. MEKK4 specifically precipitated with TRAF4 (Fig. 2C). Coprecipitation of endogenous TRAF4 with MEKK4 from lysates prepared from E10.5 embryos. Embryo lysates prepared from embryos isolated in timed matings were immunoprecipitated with either an anti-MEKK4 antibody or anti-TRAF4 antibody and immunoblotted with antibodies to TRAF4 and MEKK4. MEKK4 is highly sensitive to proteolysis. The lower bands represent cleavage fragments of MEKK4.

Colocalization of MEKK4 and TRAF4 in the Cytoplasm of COS-7 Cells—Previous reports have yielded conflicting results regarding the localization of TRAF4 within the cell. Some studies have shown a nuclear localization of TRAF4, whereas others have shown that TRAF4 localizes specifically to the cytoplasm (12, 13). MEKK4 has been shown to localize to the cytoplasm and to perinuclear, Golgi-associated vesicles (14). To determine whether TRAF4 and MEKK4 localize to similar regions within the cell, COS-7 cells were cotransfected with TRAF4 and FLAG-MEKK4wt or kinase-inactive FLAG-MEKK4K1361R, lysates were immunoprecipitated with an anti-FLAG antibody and then probed with antibodies to HA and TRAF4. B, coprecipitation of TRAF4 with MEKK4 does not require MEKK4 kinase activity. 293 cells were transiently cotransfected with FLAG-MEKK4 and full-length TRAF4, TRAF4 N terminus, TRAF4 TRAF domain, or a RING deletion mutant of TRAF4. Lysates were immunoprecipitated as indicated with either an anti-N-terminal or anti-C-terminal TRAF4 antibody and then probed with antibodies to FLAG and TRAF4. C, coprecipitation of MEKK4 with the TRAF domain of TRAF4. 293 cells were transiently cotransfected with FLAG-MEKK4 and full-length TRAF4, TRAF4 N terminus, TRAF4 TRAF domain, or a RING deletion mutant of TRAF4. Lysates were immunoprecipitated as indicated with either an anti-N-terminal or anti-C-terminal TRAF4 antibody and then probed with antibodies to FLAG and TRAF4. MEKK4 is highly sensitive to proteolysis. The lower bands represent cleavage fragments of MEKK4. Full-length TRAF4 and the RING deletion mutant are sensitive to proteolysis. Smaller bands represent cleavage fragments of TRAF4.

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colocalization of TRAF4 and MEKK4 is consistent with their ability to coprecipitate from cell lysates.

**TRAF4 Promotes MEKK4 Activation of the JNK Pathway—**Similar to previous findings, expression of TRAF4 weakly activates JNK, and MEKK4 expressed alone modestly activates JNK producing only a 3-fold increase in JNK relative to basal (Fig. 4, A and B) (9, 10). However, coexpression of TRAF4 and MEKK4 resulted in synergistic activation of JNK producing a 7-fold induction in phosphorylation of JNK (Fig. 4, A and B). JNK activation by coexpressed TRAF4 and MEKK4 was markedly inhibited by the coexpression of MEKK4K1361R (Fig. 4C). Additionally, the TRAF domain of TRAF4, the site that binds MEKK4, was able to inhibit activation of JNK by MEKK4 (Fig. 4D). Interestingly, TRAF4 does not promote MEKK4 activation of p38 (data not shown). Furthermore, the TRAF domain of TRAF4 inhibits JNK, but not p38, activation by MEKK4 indicating that TRAF4/MEKK4 complexes are specific for JNK signaling (data not shown).

TRAF4 Increases MEKK4 Kinase Activity by Promoting Dimerization of MEKK4—In *in vitro* kinase assays demonstrate the ability of TRAF4 to increase MEKK4 kinase activity. Coexpression of TRAF4 with MEKK4 resulted in a 2.2-fold stimulation of MEKK4 phosphorylation of purified His-MKK6 as compared with expression of MEKK4 alone (Fig. 5A). One mechanism to explain the ability of TRAF4 to increase MEKK4 kinase activity and MEKK4-dependent JNK activation is through the oligomerization of MEKK4 by TRAF4. This mechanism would also explain the ability of the kinase-inactive MEKK4K1361R to block activation of JNK by TRAF4 and MEKK4. To test this hypothesis, we examined the association of wild-type HA-tagged MEKK4 with FLAG-tagged MEKK4K1361R and found that wild-type MEKK4 coprecipitated weakly with the kinase-inactive MEKK4K1361R and that coprecipitation of wild-type and kinase-inactive MEKK4K1361R is significantly enhanced by the presence of TRAF4 (Fig. 5B). To further ascertain the ability of dimerization of MEKK4 to promote signaling to JNK, we utilized a cell permeable synthetic dimerizer AP20187. The kinase domain of MEKK4 was fused to two FKBP domains containing a point mutation Phe<sup>36</sup> → Val (F<sub>V</sub>). AP20187 binds to F<sub>V</sub> with 1000× greater affinity than for endogenous FKBP (11). Cells expressing HA-JNK alone displayed a minimal response to AP20187 (Fig. 5C). The addition of the chemical dimerizer AP20187 to cells expressing both HA-F<sub>V</sub>-MEKK4 and HA-JNK resulted in significant induction of JNK activation as measured by *in vitro* phosphorylation of GST-c-Jun by HA-JNK (Fig. 5C). Together, these data demonstrate the ability of TRAF4 to induce the oligomerization of MEKK4 and promote MEKK4 activation and show that dimerization is sufficient to induce MEKK4-dependent JNK activation. Cumulatively, our results show that the TRAF4 domain of TRAF4 associates with the kinase domain of MEKK4 and promotes MEKK4 activation and signaling to JNK. The kinase-inactive MEKK4K<sub>K1361R</sub> behaves as a dominant negative inhibitory mutant in TRAF4 complexes, inhibiting the activation of wild-type MEKK4. Despite intensive investigation, upstream receptor pathways that regulate TRAF4 have not been identified. Several lines of evidence suggest that TRAF4 is an atypical member of the TRAF family. Unlike TRAF1 transgenics, and TRAF2, TRAF3, TRAF5 and TRAF6 knock-outs that each display immune deficiencies, TRAF4 knock-outs have normal immune systems (3, 4, 15). Instead, TRAF4 knock-outs display severe open neural tube and skeletal defects similar to the MEKK4 knock-out and the MEKK4K<sub>K1361R</sub> knock-in (4, 5, 8). Other TRAFs have been shown to bind several members of the TNF recep-
TRAF4 activates MEKK4 and JNK

**FIGURE 5. Activation of MEKK4 by dimerization.** A, TRAF4 promotes MEKK4 kinase activity. FLAG-MEKK4 was cotransfected with either empty vector or TRAF4. Lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were incubated with purified kinase-inactive His-MK66. Phosphorylation of His-MK66 was detected with anti-phospho-MK3/6 antibody. B, coprecipitation of HA-MEKK4wt with FLAG-MEKK4K1361R is enhanced by TRAF4. 293 cells were cotransfected with HA-MEKK4wt and TRAF4. Lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were incubated with anti-phospho-MKK3/6 antibody. C, dimerization of MEKK4 by AP20187 increases MEKK4-dependent JNK activation. 293 cells were cotransfected with HA-JNK and HA-FV-MEKK4 or empty vector. Cells were treated with either vehicle alone (ethanol) or 20 nM AP20187 for the indicated times. Immunoprecipitated HA-JNK was incubated with GST-c-Jun, and phosphorylation of substrate was determined using a phospho-specific anti-c-Jun antibody. Total levels of HA-JNK and HA-FV-MEKK4 were determined by immunoblotting with anti-HA antibody.

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