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

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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, MEKK4^{K1361R}. 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 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.

MAP[2] 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 kinase (MAP3K) that phosphorylates and activates the MAPK kinases (MAP2K) MKK3, MKK4, MKK6, and MKK7. 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.

TRAF (TNF receptor-associated factor) family proteins play important roles in development and immunity (1, 2). TRAF proteins function in part as scaffolds to organize signaling complexes coupled to specific receptors at the plasma membrane. There are six proteins in mammalian genomes that encode TRAF domains near their C terminus that mediate oligomerization of TRAF proteins and their association with specific membrane proteins. The N terminus of TRAF proteins organize signaling complexes involved in the regulation of MAPK and NF-κB 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 MEKK4^{K1361R} knock-in mouse (4, 5).

TRAF4 is an atypical member of the TRAF family of proteins (1, 6). Its expression is strongest during development suggesting an important role for TRAF4 in the developing embryo (4, 7). Similar to TRAF4, MEKK4 is expressed at low levels in adult tissues but is expressed strongly during development (5, 8). TRAF4 has been shown to promote JNK activation, however the signaling pathways leading to JNK activation have not been defined (9). Therefore, we investigated the role of MEKK4 in TRAF4-mediated signaling. We have discovered that TRAF4 is a binding partner for MEKK4. Their association occurs through the kinase domain of MEKK4 and the TRAF domain of TRAF4. TRAF4 increases MEKK4 kinase activity by promoting the oligomerization of MEKK4 and enhances MEKK4 signaling to JNK.

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 SvEv 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, MEKK4^{K1361R}, was constructing 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 SalI sites on the wild-type MEKK4 kinase domain by PCR, and products were subcloned into pSH1/S_N-E-F_V′-F_{Vls}-E, a gift from David Spencer (Baylor) containing a point mutation Phe^{36} → Val (F_V) engineered in the FKBPs resulting in 1000× higher binding affinity of the synthetic dimerizer AP20187 compared with the wild-type FKBP (11). Two F_V 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 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 0.05 mM dithiothreitol, 1 μg/ml leupeptin, 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 $MgCl_2$, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 10 mM β-glycerphosphate, 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-MKK3/6 (Cell Signaling) antibodies. Chemical dimerization experiments using AP20187 provided by Ariad Pharmaceuticals (www.ariad.com/regulationkits) were performed as described previously (11).

Immunofluorescence—For TRAF4 and MEKK4 staining, COS-7 cells plated on coverslips were fixed for 10 mins in 3% paraformaldehyde containing 3% sucrose and phosphate-buffered saline pH 7.4 and were permeabilized for 6 min in 0.1% Triton 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 M 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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[2] The abbreviations used are: MAP, mitogen-activated protein; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; FKBP, FK506-binding protein; F_V, Phe^{36} → Val FKBP.

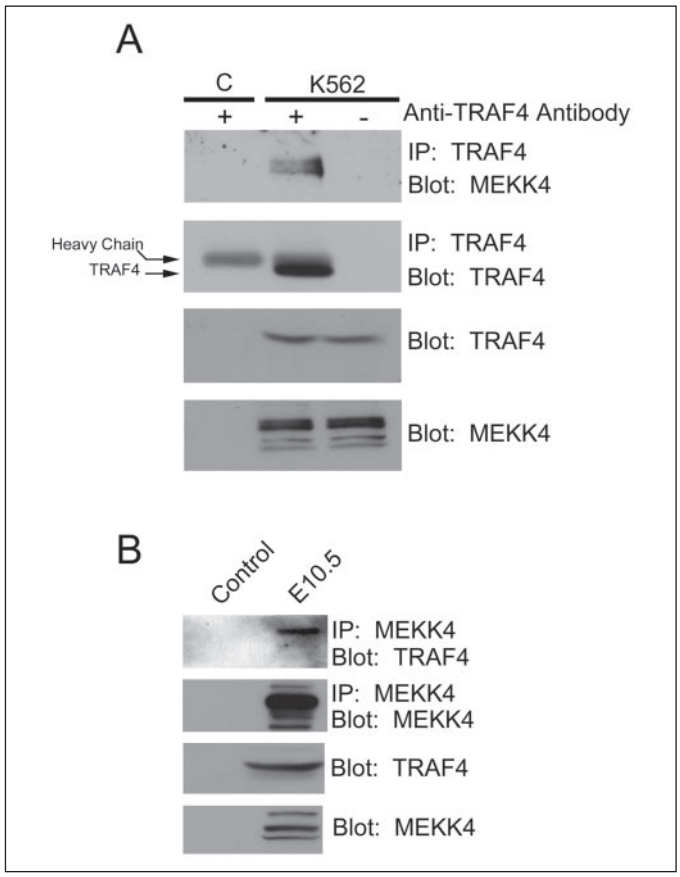

FIGURE 1. **Association of endogenous TRAF4 and MEKK4.** *A*, coprecipitation of endogenous MEKK4 with TRAF4 in K562 cells. Cells lysates (K562) or lysis buffer alone (*C*) was incubated with either an anti-TRAF4 antibody or no antibody and imunoblotted with antibodies to TRAF4 and MEKK4. MEKK4 specifically precipitated with TRAF4. *B*, 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 an anti-MEKK4 antibody and immunoblotted with antibodies to TRAF4 and MEKK4. MEKK4 is highly sensitive to proteolysis. The *lower bands* represent cleavage fragments of MEKK4.

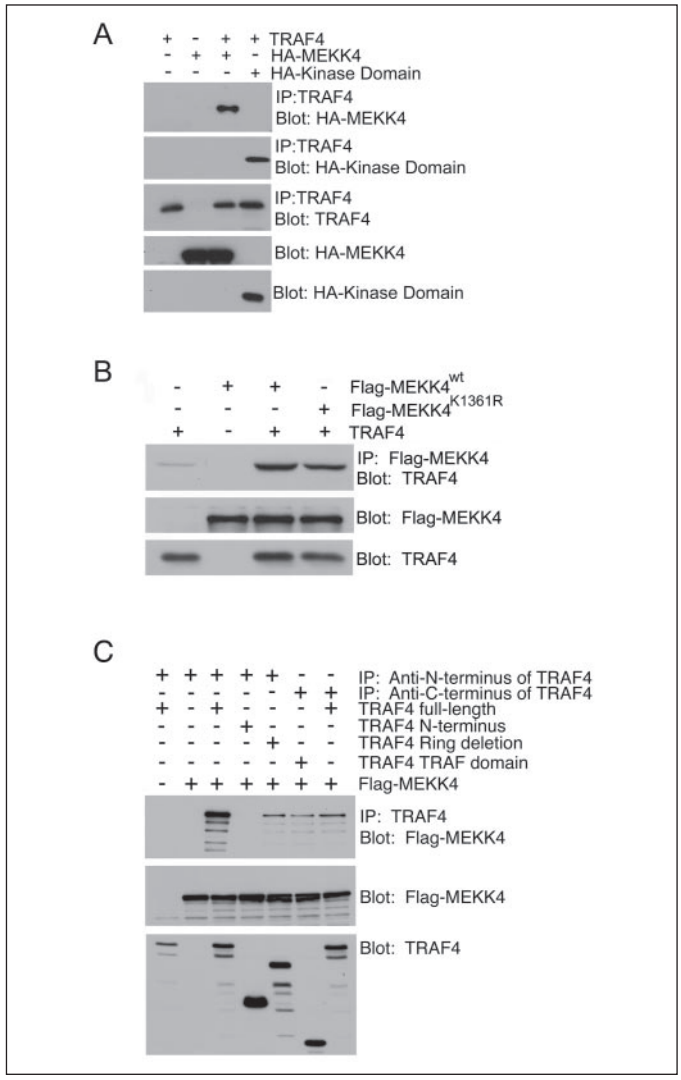

FIGURE 2. **TRAF4 binds to the kinase domain of MEKK4; however, this association does not require MEKK4 kinase activity.** *A*, the kinase domain of MEKK4 is sufficient for the association of MEKK4 with TRAF4. 293 cells were transiently cotransfected with TRAF4 and HA-tagged full-length MEKK4 or the kinase domain of MEKK4. Lysates were immunoprecipitated with an anti-TRAF4 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 TRAF4 and FLAG-MEKK4^{wt} or kinase-inactive FLAG-MEKK4^{K1361R}. Lysates were immunoprecipitated with an anti-FLAG antibody and then probed with antibodies to FLAG and TRAF4. *C*, coprecipitation of MEKK4 with the TRAF domain of TRAF4. 293 cells were transiently transfected 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 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.

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. 1*A*). 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. 1*B*). These data demonstrate an *in vivo* association of TRAF4 and MEKK4 and suggests 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. 2*A*). 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 (MEKK4^{K1361R}) (Fig. 2*B*). The interaction domains of TRAF4 with full-length MEKK4 were similarly examined. In contrast to the N terminus of TRAF4 that failed to bind MEKK4, the TRAF domain of TRAF4 was necessary and sufficient for binding to MEKK4 (Fig. 2*C*). Consistent with the lack of binding of MEKK4 to the N terminus of TRAF4, MEKK4 retained the ability to bind a mutant TRAF4 wherein the RING domain in the N terminus was deleted (Fig. 2*C*). Together, these data show the specific interaction of the TRAF domain of TRAF4 with the kinase domain 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 localized to similar regions within the cell, COS-7 cells were cotransfected with TRAF4 and MEKK4, and the localization of these proteins was examined by the immunofluroescence of deconvolved 0.1 μM sections. As shown in Fig. 3, MEKK4 and TRAF4 colocalized in the cytoplasm of COS-7 cells with pronounced staining in the perinuclear region of the cell. Immunostaining for TRAF4 and MEKK4 was not detected in the nuclei of cells as assessed by lack of colocalization with the nuclear stain DAPI (Fig. 3). The

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 co-expression of MEKK4^{K1361R} (Fig. 4*C*). Additionally, the TRAF domain of TRAF4, the site that binds MEKK4, was able to inhibit activation of JNK by MEKK4 (Fig. 4*D*). 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).

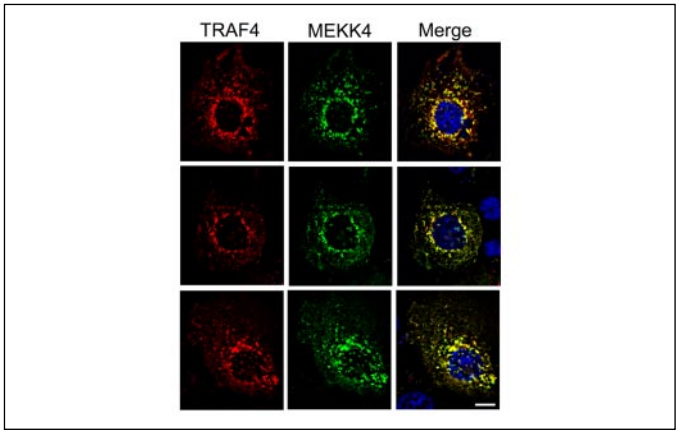

FIGURE 3. **Colocalization of TRAF4 and MEKK4 in the cytoplasm of COS-7 cells.** COS-7 cells were transiently cotransfected with constructs encoding wild-type FLAG-tagged MEKK4 and full-length TRAF4. Cells were immunostained with anti-FLAG (*green*) and anti-TRAF4 (*red*) antibodies. Three independent examples are shown. Nuclei were stained with DAPI. *Bar* equals 10 μM.

TRAF4 Increases MEKK4 Kinase Activity by Promoting Dimerization of MEKK4—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. 5*A*). 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 MEKK4 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 MEKK4^{K1361R} and found that wild-type MEKK4 coprecipitated weakly with the kinase-inactive MEKK4^{K1361R} and that coprecipitation of wild-type and kinase-inactive MEKK4^{K1361R} is significantly enhanced by the presence of TRAF4 (Fig. 5*B*). 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$^{36} \rightarrow$ Val (F_V). AP20187 binds to F_V with 1000× greater affinity than for endogenous FKBP (11). Cells expressing HA-JNK alone displayed a minimal response to AP20187 (Fig. 5*C*). The addition of the chemical dimerizer AP20187 to cells expressing both HA-F_V-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. 5*C*). 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 TRAF domain of TRAF4 associates with the kinase domain of MEKK4 and promotes MEKK4 activation and signaling to JNK. The kinase-inactive MEKK4^{K1361R} 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 MEKK4^{K1361R} knock-in (4, 5, 8). Other TRAFs have been shown to bind several members of the TNF recep-

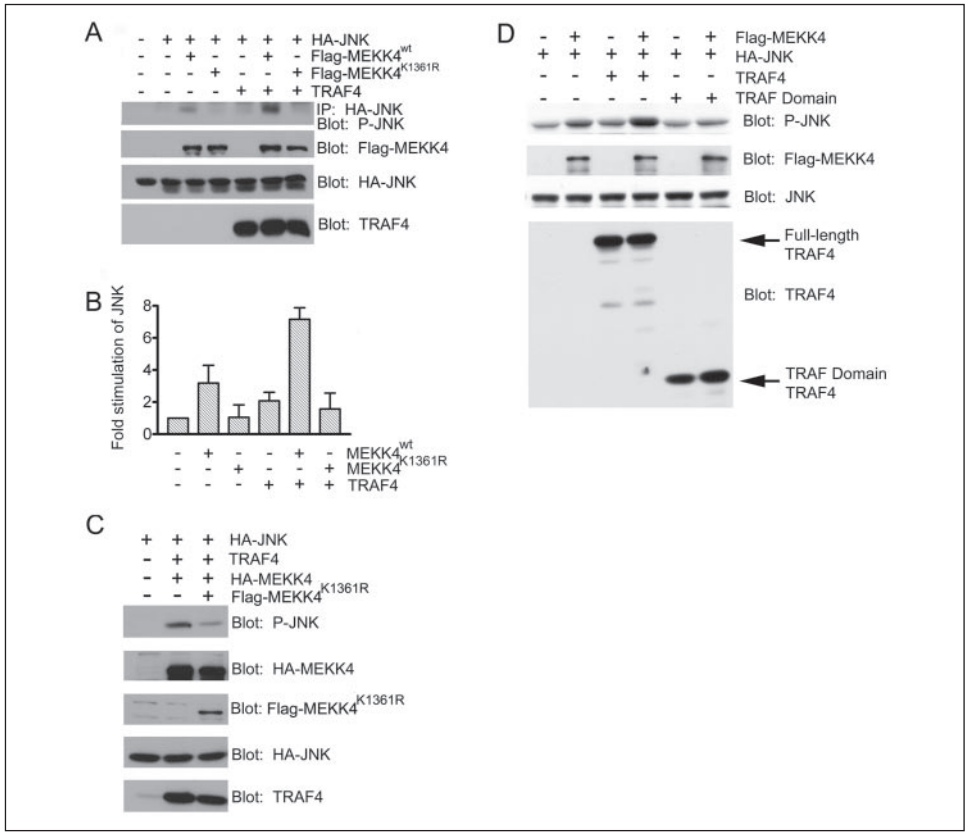

FIGURE 4. **Regulation of JNK by TRAF4 and MEKK4.** *A*, coexpression of MEKK4 with TRAF4 results in the synergistic activation of JNK. 293 cells were cotransfected with constructs encoding HA-JNK, TRAF4, and FLAG-MEKK4^{wt} or FLAG-MEKK4^{K1361R}. HA-JNK was immunoprecipitated with an anti-HA antibody and probed with an antibody to phospho-JNK. *B*, quantitation of JNK activation from *A*. P-JNK was normalized to total HA-JNK. Data represent the mean ± range from two independent experiments. *C*, synergistic activation of JNK by MEKK4 and TRAF4 is blocked by co-expression of MEKK4^{K1361R}. 293 cells were cotransfected with constructs encoding HA-JNK, TRAF4, HA-MEKK4, and FLAG-MEKK4^{K1361R}. HA-JNK was immunoprecipitated with an anti-HA antibody and probed with an antibody to phospho-JNK. *D*, JNK activation by MEKK4 is inhibited by the TRAF domain of TRAF4. Cells were cotransfected with constructs encoding HA-JNK, FLAG-MEKK4, and either full-length TRAF4 or the TRAF domain only of TRAF4 and treated as described above.

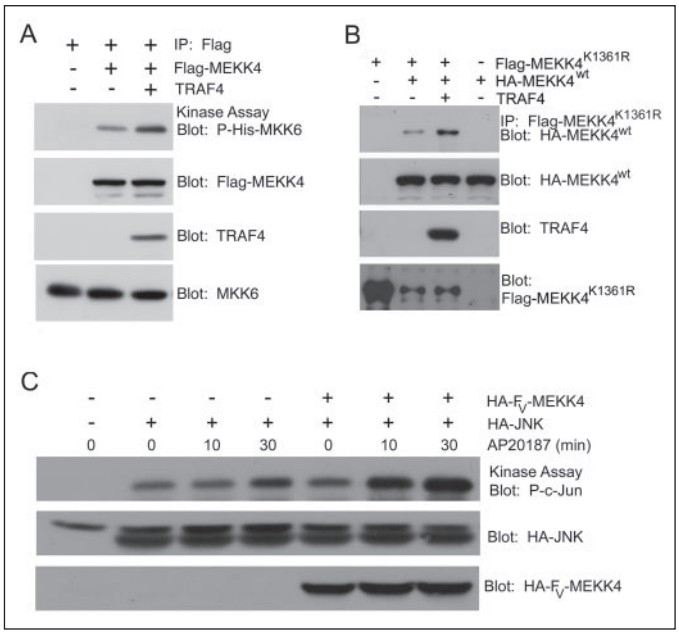

FIGURE 5. **Activation of MEKK4 by dimerization.** *A*, TRAF4 promotes MEKK4 kinase activity. FLAG-MEKK4 was cotransfected with either empty vector or TRAF4. Lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were incubated with purified kinase-inactive His-MKK6. Phosphorylation of His-MKK6 was detected with anti-phospho-MKK3/6 antibody. *B*, coprecipitation of HA-MEKK4^{wt} with FLAG-MEKK4^{K1361R} is enhanced by TRAF4. 293 cells were cotransfected with HA-MEKK4^{wt} and FLAG-MEKK4^{K1361R} in the absence or presence of TRAF4. Lysates were immunoprecipitated with an anti-FLAG antibody and probed with an anti-HA antibody. *C*, dimerization of MEKK4 by AP20187 increases MEKK4-dependent JNK activation. 293 cells were cotransfected with HA-JNK and HA-F_V-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-F_V-MEKK4 were determined by immunoblotting with anti-HA antibody.

tor superfamily (3). *In vitro* binding experiments have shown that TRAF4 interacts weakly with the LTβR and p75 nerve growth factor receptor but not at all with TNFR1, TNFR2, Fas, or CD40 (16–18). Furthermore, TRAF4 shows highest homology with the Drosophila TRAF (DTRAF1) with 45% amino acid identity, suggesting that TRAF4 may represent an archaic member of the TRAF family (1). Both DTRAF1 and TRAF4 have seven zinc fingers (as compared with five in TRAF2, TRAF3, TRAF5, and TRAF6) and have a truncated coiled-coil domain (1, 19). DTRAF1 has also been shown to activate JNK via a mechanism involving binding to the MAP4K Misshapen (19). The similarity of TRAF4 to DTRAF1 and the significant differences from other mammalian TRAF family members suggest that regulation of TRAF4, the non-classical mammalian TRAF, may be functionally different from other TRAF proteins (6). Although we do not know the upstream stimuli for initiating TRAF4 activation of MEKK4 during development, we show that MEKK4 and TRAF4 associate in the developing embryo. The existence of an endogenous complex, the coregulation of the JNK signaling pathway, and the overlapping phenotypes of the TRAF4 knock-out and the MEKK4 knock-out and MEKK4^{K1361R} knock-in demonstrate the relevance of the TRAF4/MEKK4 interaction and suggest that TRAF4 and MEKK4 are in a common pathway (4, 8, 20). TRAF4 effectors have been elusive, and our work defines MEKK4 as the first signaling protein shown to be an effector for TRAF4 regulation of its kinase activity.

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