MEKK4 Stimulation of p38 and JNK Activity Is Negatively Regulated by GSK3β*

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The MAPK kinase kinase MEKK4 is required for neurulation and skeletal patterning during mouse development. MEKK4 phosphorylates and activates MKK4/MK7 and MKK3/MK6 leading to the activation of JNK and p38, respectively. MEKK4 is believed to be auto-inhibited, and its interaction with other proteins controls its dimerization and activation. TRAF4, GADD45, and Axin each bind and activate MEKK4, with TRAF4 and Axin binding to the kinase domain and GADD45 binding within the N-terminal regulatory domain. Here we show that similar to the interaction with TRAF4 and Axin, the kinase domain of MEKK4 interacts with the multifunctional serine/threonine kinase GSK3β. GSK3β binding to MEKK4 blocks MEKK4 dimerization that is required for MEKK4 activation, effectively inhibiting MEKK4 stimulation of the JNK and p38 MAPK pathways. Inhibition of GSK3β kinase activity with SB216763 results in enhanced MEKK4 kinase activity and increased JNK and p38 activation, indicating that an active state of GSK3β is required for binding and inhibition of MEKK4 dimerization. Furthermore, GSK3β phosphorylates specific serines and threonines in the N terminus of MEKK4. Together, these findings demonstrate that GSK3β binds to the kinase domain of MEKK4 and regulates MEKK4 dimerization. However, unlike TRAF4, Axin, and GADD45, GSK3β inhibits MEKK4 activity and prevents its activation of JNK and p38. Thus, control of MEKK4 dimerization is regulated both positively and negatively by its interaction with specific proteins.

MEKK4 is a 180-kDa mitogen-activated-protein kinase kinase kinase (MAP3K)2 that functions in neurulation and skeletal patterning in the developing mouse embryo (1, 2). Mice harboring a kinase-inactive MEKK4 generated by knock-in mutation of the active site lysine K1361R are similar in phenotype to the knock-outs of TRAF4 and dishevelled-2 (1, 3, 4). TRAF4 and dishevelled-2 knock-out embryos and MEKK4 kinase-inactive knock-in embryos have significant defects in neural tube closure and skeletal patterning, including vertebral/rib malformations and scoliosis (3, 4). TRAF4 directly binds MEKK4 inducing MEKK4 dimerization and activation of MEKK4 kinase activity toward MKK4/7, resulting in the activation of JNK (5). Although the phenotypes of the dishevelled-2 knock-out and MEKK4 kinase-inactive knock-in are similar, there is no evidence that dishevelled-2 interacts with MEKK4. MEKK4 and dishevelled-2 are both binding partners of Axin, a scaffold protein involved in Wnt and Notch signaling (6). The binding of MEKK4 to Axin has been shown to regulate JNK activity (7), and here we also show that MEKK4 controls Axin-dependent activation of JNK and p38. Thus, dishevelled-2 and MEKK4 are both binding partners for Axin and genetically are in a common pathway based on their strongly overlapping phenotypes when deleted or mutated in the developing mouse embryo (1, 3, 6).

MEKK4 is predicted to exist in an auto-inhibited state with the N terminus folded over the C-terminal kinase domain (8). Binding to MEKK4 by activators, including TRAF4, GADD45α, -β, and -γ, and possibly Axin is thought to release MEKK4 from its auto-inhibited state and to promote MEKK4 homodimerization (5, 8, 9). The requirement of MEKK4 dimerization in promoting activation was demonstrated using a chemical inducer of dimerization to activate MEKK4 (5). The fact that MEKK4 and dishevelled-2 both bind Axin and appear genetically to be in the same pathway led us to search for other Axin-interacting proteins that would potentially regulate MEKK4 activity. Analysis of the MEKK4 primary sequence identified several putative GSK3 phosphorylation sites.

GSK3 is a serine/threonine kinase that regulates multiple signaling pathways. There are two highly homologous isoforms, GSK3α and GSK3β. GSK3β is a constitutively active kinase that is regulated by phosphorylation, localization, and GSK3β-binding proteins (10, 11). GSK3β is activated by phosphorylation of tyrosine 216 and negatively regulated by phosphorylation of serine 9 (10, 11). GSK3β is a negative regulator of several signaling networks in cells. For example, GSK3 inhibits the canonical Wnt signaling pathway by promoting β-catenin degradation (10–12). In addition, GSK3β has been shown to negatively regulate JNK activation, but the mechanism of this regulation is undefined (13). Here we show that GSK3β binds the kinase domain of MEKK4, inhibiting dimerization and MEKK4 activity. The findings define MEKK4 as a MAP3K regulated by GSK3β for the negative regulation of JNK and p38 signaling.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Transfections—COS-7 cells were cultured in Dulbecco’s modified Eagle’s high glucose media supplemented with 10% fetal bovine serum, 1% penicillin.
and streptomyein. Transfection of COS-7 cells was performed in 35- and 60-mm dishes for 24 h using LipoFectamine Plus (Invitrogen) according to the manufacturer’s specifications. Trophoblast stem (TS) cells were isolated from blastocysts as described previously (14). Blastocysts were isolated at E3.5 from timed matings of 129 Svev mice according to university and federal guidelines for the use of animals. TS cells were cultured without feeders in 30% TS media (RPMI 1640, 20% heat-inactivated fetal bovine serum, 1% sodium pyruvate, 1% penicillin and streptomyein, 1% glucose, and 100 μM β-mercaptoethanol) and 70% conditioned media isolated from primary mouse embryonic fibroblasts (MEFs) cultured in TS media. Isolation of MEFs from littermate wild-type and homozygous MEKK4K1361R MEFs was described previously (1). For treatment with GSK3 inhibitor, cells were incubated with 30 μM SB216763 (Sigma).

**Plasmids**—Wild-type and K1361R FLAG-tagged MEKK4 were constructed as described previously (5). Full-length HA-tagged MEKK4 and HA-tagged MEKK4 kinase domain were as described previously (15). HA-tagged GSK3β wild-type and K85M constructs were kind gifts of Xianjun Fang (Virginia Commonwealth University). Potential serine/threonine phosphorylation sites were mutated to alanines using the QuikChange multisite-directed mutagenesis kit (Stratagene). The resulting four mutated fragments were verified by sequencing and reinserted into full-length FLAG-tagged MEKK4.

**Immunoprecipitations, Western Blot Analysis, Measurement of JNK and p38 Activity, and Kinase Assays**—For kinase assays using purified proteins, His-MEKK4 purified from Sf9 cells was incubated with His-GSK3β (Sigma) in 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 10 mM of [γ-32P]ATP for 20 min at 30 °C. Proteins were separated by SDS-PAGE, visualized by autoradiography, and quantitated using a PhosphorImager. For assays with immunoprecipitated proteins, 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, 1 mM sodium fluoride, 0.05 mM dithiothreitol, 1 μg/ml leupeptin, and 17 μg/ml aprotinin as described previously (1, 5). All chemicals were from Sigma. Lysates were immunoprecipitated, and blots were probed with anti-MEKK4 (1, 5), anti-FLAG (Sigma), anti-HA (12CA5), anti-GSK3β (Santa Cruz Biotechnology), anti-TRAF4 (Santa Cruz Biotechnology) antibodies, and phospho-specific antibodies, including anti-phospho-MKK3/6, anti-phospho-JNK, and anti-phospho-p38 (Cell Signaling). Kinase assays were performed as described previously (1). Briefly, immunoprecipitates were washed with cold kinase buffer described above. Reactions were incubated with kinase buffer containing kinase-inactive His-MKK6 and cold 0.5 mM ATP or 10 mCi of [γ-32P]ATP for 20 min at 30 °C. Cold kinase assays were probed with anti-phospho-MKK3/6 (Cell Signaling), and radioactive assays were exposed to film and quantitated with a PhosphorImager.

**λ Protein Phosphatase Treatment**—2.5 μg of His-MEKK4 purified from Sf9 cells or immunoprecipitated HA-MEKK4 from transfected COS-7 cells was incubated for 45 min at 30 °C in the absence or presence of 800 units of λ protein phosphatase (New England Biolabs). Phosphatase reactions were terminated by washing twice with cold buffer A containing phosphatase...
**RESULTS**

**GSK3β Phosphorylates MEKK4**—The consensus sequence for GSK3β phosphorylation is (S/T)XXX(S/T)P (Fig. 1A) (11). The first serine or threonine in the consensus sequence is predicted to be phosphorylated by GSK3β. Although not absolutely required, GSK3β prefers substrates that are already phosphorylated on the second serine or threonine in the consensus sequence. This priming phosphorylation located four residues C-terminal to the GSK3β phosphorylation site is thought to increase the efficiency of GSK3β toward its substrates (11). Scanning of the MEKK4 sequence identified three consensus GSK3β phosphorylation sequences (Fig. 1A). All of the consensus sequences were located in the N terminus of MEKK4 (amino acids 1–1326), whereas none were found in the catalytic domain (amino acids 1327–1597). Two of the potential sites, Ser77 and Thr112, are located approximately 50 residues upstream of the GADD45 binding domain. The third site, Ser1237, is located within a putative dimerization domain located 90 residues upstream of the kinase domain (9). To test the hypothesis that MEKK4 was a substrate for GSK3β, purified recombinant GSK3β and MEKK4 were used. MEKK4 purified from S9 cells was incubated with increasing units of recombinant GSK3β. In *vitro* kinase assays were performed, and phosphorylation of MEKK4 was quantitated (Fig. 1B). Addition of 2 units of GSK3β resulted in a 31-fold increase in MEKK4 phosphorylation demonstrating that MEKK4 is a direct phosphorylation substrate for GSK3β. Similar results were obtained using immunoprecipitated HA-MEKK4 as a substrate for purified GSK3β (Fig. 1C). Kinase-inactive MEKK4 (HA-MEKK4K1361M) was also a phosphorylation substrate for GSK3β (Fig. 1C). However, the autophosphorylation of GSK3β was not affected by the expression of either wild-type or kinase-inactive MEKK4K1361M (Fig. 1C).

As described above, preferred substrates for GSK3β are generally primed by phosphorylation of a second site by another kinase (11). To test for a role of a second site priming phosphorylation on MEKK4, purified MEKK4 was treated with λ-phosphatase, a protein with activity toward phosphorylated serine, threonine, and tyrosine (Fig. 1D). Subsequent GSK3β *in vitro* kinase assays with control or phosphatase-treated MEKK4 demonstrated that phosphatase-treated MEKK4 was no longer a substrate for GSK3β, consistent with the known properties of previously defined GSK3β substrates (11). Identical results were obtained using λ-phosphatase-treated immunoprecipitated inhibitors and once with kinase buffer described above. Reactions were incubated with or without His-GSK3β in radioactive kinase buffer as described above.
HA-MEKK4 (data not shown). Cumulatively, the findings in Fig. 1 demonstrate that MEKK4 is a prototypical in vitro GSK3β phosphorylation substrate.

When expressed alone, transfected MEKK4 exhibits a modest basal level of phosphorylation and kinase activity (Fig. 2A), as predicted from studies suggesting that the N terminus of MEKK4 is auto-inhibitory (9). In vitro kinase assay of coimmunoprecipitated GSK3β and MEKK4 from cell lysates resulted in a 21-fold increase in the GSK3β-dependent phosphorylation of MEKK4 compared with immunoprecipitation of MEKK4 in the absence of GSK3β (Fig. 2A). The addition of purified, recombinant MKK6, a MEKK4 substrate (16), to the in vitro kinase assay resulted in the expected MEKK4-dependent phosphorylation of MKK6. Strikingly, coimmunoprecipitation of GSK3β with MEKK4 resulted in a marked inhibition of MKK6 phosphorylation (Fig. 2A). This phosphorylation of MKK6 is MEKK4-dependent as kinase-inactive MEKK4K1361M that is phosphorylated by GSK3β (Fig. 2B) was unable to phosphorylate MKK6 (Fig. 2A).

Full-length MEKK4, the N terminus of MEKK4 upstream of the kinase domain, or the kinase domain of MEKK4 was expressed in the absence or presence of GSK3β (Fig. 2B). Phosphorylation of full-length MEKK4 and the N terminus of MEKK4 in the presence of GSK3β was similar, suggesting that GSK3β phosphorylates the N terminus of MEKK4 (Fig. 2B). The kinase domain of MEKK4 is weakly autophosphorylated (Fig. 2, B and C), whereas the autophosphorylation of the kinase-inactive MEKK4K1361R kinase domain is completely inhibited (Fig. 2C). In the presence of GSK3β, autophosphorylation of the kinase domain of MEKK4 was significantly reduced (Fig. 2, B and C), even though the MEKK4 kinase domain does not appear to be a phosphorylation substrate for GSK3β (Fig. 2B). The kinase domain of MEKK4 is weakly autophosphorylated (Fig. 2, B and C), whereas the autophosphorylation of the kinase-inactive MEKK4K1361R kinase domain is completely inhibited (Fig. 2C). In the presence of GSK3β, autophosphorylation of the kinase domain of MEKK4 was significantly reduced (Fig. 2, B and C), even though the MEKK4 kinase domain does not appear to be a phosphorylation substrate for GSK3β (Fig. 2B). The kinase domain of MEKK4 is weakly autophosphorylated (Fig. 2, B and C), whereas the autophosphorylation of the kinase-inactive MEKK4K1361R kinase domain is completely inhibited (Fig. 2C). In the presence of GSK3β, autophosphorylation of the kinase domain of MEKK4 was significantly reduced (Fig. 2, B and C), even though the MEKK4 kinase domain does not appear to be a phosphorylation substrate for GSK3β (Fig. 2B). The kinase domain of MEKK4 is weakly autophosphorylated (Fig. 2, B and C), whereas the autophosphorylation of the kinase-inactive MEKK4K1361R kinase domain is completely inhibited (Fig. 2C). In the presence of GSK3β, autophosphorylation of the kinase domain of MEKK4 was significantly reduced (Fig. 2, B and C), even though the MEKK4 kinase domain does not appear to be a phosphorylation substrate for GSK3β (Fig. 2B).

Identification of MEKK4 Phosphorylation Sites for GSK3β—Fig. 1A identified three consensus sequences in MEKK4 as potential sites for GSK3β phosphorylation. Phosphatase treatment of MEKK4 inhibited GSK3β phosphorylation of MEKK4, suggesting a priming phosphorylation is required for GSK3β phosphorylation of MEKK4 (Fig. 1D). Both the consensus GSK3β phosphorylation site and the priming site were mutated in each of the three consensus sequences (Fig. 3A). Mutation of each GSK3β phosphorylation consensus sequence individually resulted in a partial loss of GSK3β-catalyzed phosphorylation of MEKK4 (Fig. 3B). Mutation of serine 1237 and 1241 in the third consensus sequence resulted in the greatest inhibition of MEKK4 phosphorylation (Fig. 3B). Mutation of multiple consensus sequences was generally additive with simultaneous mutation of all three sequences resulting in a very dramatic loss of MEKK4 phosphorylation catalyzed by GSK3β (Fig. 3C). These findings define three different sites of MEKK4 phosphorylation catalyzed by GSK3β.

Endogenous GSK3β and MEKK4 Are Binding Partners—Cumulatively, the findings in Figs. 1–3 clearly demonstrate that MEKK4 is a GSK3β phosphorylation substrate. The findings in Fig. 2, A–C, and Fig. 3 are striking but seemed at first to be somewhat contradictory. First, GSK3β phosphorylates three sites N-terminal to the kinase domain and inhibits MEKK4 phosphorylation of MKK6. Second, GSK3β appears to inhibit the autophosphorylation activity of the MEKK4 kinase domain even though the MEKK4 kinase domain is not phosphorylated.

| A | MEKK4 Phosphorylation Site Mutants: |
|---|---|
| Mutant 1 (M1) S77A, T81A | + + + + + + + + |
| Mutant 2 (M2) T112A, S116A | + + + + + + + + |
| Mutant 3 (M3) S77A, T81A, T112A, S116A | + + + + + + + + |
| Mutant 4 (M4) S1237A, S1241A | + + + + + + + + |
| Mutant 5 (M5) S77A, T81A, T112A, S116A, S1237A, S1241A | + + + + + + + + |

**FIGURE 3. Identification of specific residues in the N terminus of MEKK4 that are phosphorylated by GSK3β.** A, description of MEKK4 phosphorylation mutants. B, phosphorylation of MEKK4 is reduced by mutation of specific serine and threonines in the N terminus of MEKK4. HA-GSK3β was transfected with either wild-type or phosphorylation mutant FLAG-MEKK4 constructs as indicated. HA-GSK3β and FLAG-MEKK4 were immunoprecipitated (IP) together with anti-HA and anti-FLAG antibodies, and immunoprecipitates were incubated for 20 min with [γ-32P]ATP. Autoradiograms (Autorad) show phosphorylation of MEKK4 and GSK3β. Western blotting with anti-FLAG and anti-HA antibodies shows equal expression of FLAG-MEKK4 and HA-GSK3β. Phosphorylation of MEKK4 was measured using a PhosphorImager. Percent phosphorylation of MEKK4 was calculated by dividing mutant MEKK4 phosphorylation in the presence of HA-GSK3β by the phosphorylation of MEKK4 in the absence of HA-GSK3β. A representative experiment from two independent experiments with similar results is shown. C, substitution of all putative GSK3β phosphorylation sites results in a 71% reduction in MEKK4 phosphorylation. Experiments were performed as described in B. The percent phosphorylation of MEKK4 phosphorylation mutants relative to wild-type MEKK4 obtained from two independent experiments expressed as the mean ± S.E. for M4 and M5 were the following: M1, 72.8 ± 2.1; M2, 82.9 ± 2.9; M3, 68.1 ± 3.4; M4, 46.9 ± 13.8; and M5, 22.6 ± 8.9.

*GSK3β Inhibits MEKK4 Signaling*
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by GSK3β. For these reasons, the interaction of endogenous MEKK4 and GSK3β was examined by coimmunoprecipitation (Fig. 4A). Unlike GSK3β that is expressed ubiquitously and strongly in most cell types, MEKK4 is expressed rather weakly in most adult cell types (1, 17). However, MEKK4 is expressed strongly during development in both embryonic and extraembryonic cells (1, 2). Therefore, the association of endogenous GSK3β and MEKK4 was examined using trophoblast stem (TS) cells, which are epithelial extraembryonic cells that differentiate to form the tissues of the placenta (14). Immunoprecipitation of TS cell lysates with anti-GSK3β antibody resulted in the specific coimmunoprecipitation of MEKK4 with GSK3β (Fig. 4A). MEKK4 did not coimmunoprecipitate with a nonspecific anti-rabbit antibody (Fig. 4A). These data show the specific association of endogenous GSK3β and MEKK4 in TS cells.

GSK3β Binds the Kinase Domain of MEKK4—Fig. 4B shows that FLAG-MEKK4 coimmunoprecipitated with HA-GSK3β. Although GSK3β phosphorylates the N terminus of MEKK4, the recognition of the MEKK4 phosphorylation sites by GSK3β within the N terminus of MEKK4 is not of sufficient affinity to allow coimmunoprecipitation of the two proteins (Fig. 4C). Instead, GSK3β binds to the kinase domain of MEKK4, as the kinase domain of MEKK4 strongly coimmunoprecipitated with GSK3β (Fig. 4D). Interestingly, the MEKK4 M5 mutant protein with alanine substitutions in each of the defined GSK3β consensus sequences (Fig. 3) retained the ability to coimmunoprecipitate with GSK3β (Fig. 4E). These findings demonstrate that endogenous MEKK4 and GSK3β are in a coimmunoprecipitating complex and that the kinase domain of MEKK4 is the primary site for the stable interaction of GSK3β and MEKK4.

MEKK4 Dimerization Is Disrupted by the Binding of GSK3β—MEKK4 forms homodimers that have been shown to activate MEKK4 kinase activity (5, 9). TRAF4 and GADD45α, -β, and -γ have each been shown to induce MEKK4 dimerization resulting in increased MEKK4 kinase activity (5, 9). Dimerization induced by a chemical dimerizer leads to activation of MEKK4 and the stimulation of JNK, a downstream pathway controlled by MEKK4 (5).

Coexpressed HA-MEKK4 and FLAG-MEKK4 form a stable association as measured by the coimmunoprecipitation of HA-MEKK4 with FLAG-MEKK4, consistent with an oligomeric MEKK4 protein complex (Fig. 5). Coexpression of GSK3β with HA-MEKK4 and FLAG-MEKK4 completely abrogates this HA-MEKK4-FLAG-MEKK4 complex (Fig. 5). In addition, HA-GSK3β is strongly coimmunoprecipitated with FLAG-MEKK4 using an anti-FLAG antibody (Fig. 5). These data clearly show that GSK3β binds MEKK4 and blocks MEKK4 dimerization, which is required for MEKK4 activation (5, 9).

GSK3β Inhibits the Kinase Activity of MEKK4—Coexpression of GSK3β with full-length MEKK4 inhibits MEKK4 kinase
activity (Fig. 2A and Fig. 6A). Although the phosphorylation sites for GSK3β lie in the N terminus of MEKK4, GSK3β retains the ability to inhibit the kinase activity of the MEKK4 kinase domain in the absence of the MEKK4 N terminus (Fig. 6A). These findings demonstrate that GSK3β inhibits MEKK4 kinase activity through the direct binding to the kinase domain of MEKK4.

Activation of MEKK4 results in the activation of both the JNK and p38 MAPK pathways (1, 15). Consistent with the inhibition of MEKK4 kinase activity in the presence of GSK3β, MEKK4 activation of both p38 (Fig. 6B) and JNK (Fig. 6C) is inhibited by GSK3β. The MEKK4 M5 mutant with all three GSK3β phosphorylation sites mutated retains the ability to activate both JNK and p38, and this activity is inhibited by GSK3β (Fig. 6, D and E). This finding is consistent with the retention of GSK3β binding to the MEKK4 M5 phosphorylation mutant (Fig. 4E).

Inhibition of GSK3 by SB216763 Enhances MEKK4 Activation of JNK and p38—For reasons that are unclear, mutation of the GSK3β active site lysine (GSK3βK85M) impedes the binding of GSK3β with MEKK4 (not shown). For this reason, we tested whether an active site inhibitor of GSK3 (GSKα and β), SB216763 (18), would alter GSK3β regulation of MEKK4 activity. Cells coexpressing HA-MEKK4 and HA-JNK that were treated with SB216763 exhibited enhanced JNK phosphorylation compared with cells treated with carrier alone (Fig. 7A). SB216763 had no effect on JNK activation by the related MAP3K MEK1 (Fig. 7B). SB216763 treatment had a more modest effect on MEKK4 activation of p38, as compared with MEKK4 activation of JNK. MEKK4 activation of p38 was not enhanced by 2.5 h of SB216763 treatment. However, prolonged treatment for 16 h resulted in a 50% increase in MEKK4 activation of p38 (Fig. 7C).

Enhanced MEKK4 Kinase Activity with Inhibition of GSK3 by SB216763—Fig. 7D shows that continuous exposure of cells expressing wild-type HA-MEKK4 to SB216763 over a 16-h time period resulted in enhanced MEKK4 kinase activity. HA-MEKK4 immunoprecipitated from cells treated for increasing time with SB216763 was subjected to in vitro kinase assays using kinase-inactive MKK6 as a substrate. A time-dependent increase in MEKK4 kinase activity was observed with SB216763 treatment demonstrating that inhibition of GSK3 enhanced MEKK4 kinase activity (Fig. 7D). The activity measured in the in vitro kinase assay was dependent on MEKK4, because kinase-inactive HA-MEKK4K1361M did not have any kinase activity toward MKK6 in the assay (Fig. 7D). In addition to enhancing the kinase activity of overexpressed MEKK4, treatment with GSK3 inhibitor also increased the kinase activity of endogenous MEKK4. Endogenous MEKK4 immunoprecipitated from MEFs treated with SB216763 exhibited a 34 ± 0.02% increase in kinase activity relative to treatment with carrier alone. The activation of MEKK4 resulting from inhibition of GSK3 kinase activity further demonstrates that GSK3 is a negative regulator of MEKK4 signaling. The loss of GSK3β-mediated inhibition of MEKK4 kinase activity with SB216763 treatment is consistent with the observation that kinase-inactive GSK3βK85M binds poorly to MEKK4, suggesting that the interaction of GSK3β with MEKK4 involves residues within the kinase domain of GSK3β.

MEKK4 Mediates Axin-dependent Activation of JNK and p38—It has been demonstrated that TRAF4 and GADD45 proteins both activate MEKK4 (5, 9). Fig. 8A and the work of others (7) show that the scaffolding protein, Axin, binds the kinase domain of MEKK4. Kinase-inactive MEKK4 (MEKK4K1361R) strongly abrogates the activation of both JNK and p38 in response to Axin expression (Fig. 8, B and C). These results show that Axin binds the kinase domain of MEKK4 and are the first to show that a kinase-inactive full-length MEKK4 inhibits Axin-mediated activation of both JNK and p38, suggesting that MEKK4 regulates Axin-dependent control of JNK and p38. GSK3β also binds Axin (19), demonstrating that MEKK4 and GSK3β are not only in a complex in cells but also interact with a common scaffold protein.

DISCUSSION

We have shown previously that chemical induction of MEKK4 dimerization activates MEKK4 kinase activity (5). In addition, we have shown that through its TRAF domain TRAF4 also induces oligomerization of MEKK4 and activates its kinase activity (5). Activation of MEKK4 by two independent mechanisms, the FKBP dimerization system and TRAF4-induced oligomerization, clearly shows that like other protein kinases MEKK4 is activated by oligomerization and transphosphorylation in the kinase domain (5). It has also been proposed that GADD45 proteins induce dimerization required for activation of MEKK4 (9). It is unclear exactly how Axin activates MEKK4, but Axin is a dimer and could potentially activate MEKK4 in a manner similar to TRAF4 and GADD45 proteins (7, 20). Whereas Axin and TRAF4 bind to the C-terminal kinase domain of MEKK4, GADD45 proteins bind to an N-terminal region; thus there are multiple mechanisms to regulate MEKK4 dimerization. Our current findings demonstrate that endogenous MEKK4 and GSK3β are binding partners in cells, and like TRAF4 and Axin, GSK3β binds to the C-terminal region that
encodes the kinase domain of MEKK4. The binding of GSK3β to the MEKK4 C-terminal kinase domain inhibits MEKK4 kinase activity by inhibiting MEKK4 dimerization. Fig. 9A shows a schematic model of the known effectors that control MEKK4 activity. It is interesting that multiple proteins that directly bind MEKK4 regulate its activity by controlling its dimerization.

GSK3β has been shown previously to be a negative regulator of JNK signaling (13). However, the mechanisms whereby GSK3β decreases JNK signaling were unknown. Here we show that GSK3β inhibits MEKK4 kinase activity and MEKK4 activation of JNK. The exact mechanism of how GSK3β inhibits MEKK4 activity is unclear, but several findings suggest that the active site of GSK3β might bind MEKK4. The GSK3β inhibitor, SB216763, binds to the active site of GSK3β and blocks GSK3β inhibition of MEKK4. Mutation of the kinase domain active site lysine in GSK3β (K85M) causes a marked decrease in the stable interaction of GSK3β and MEKK4, suggesting a functional GSK3β active site is required for MEKK4 binding. This hypothesis is consistent with the finding that GSK3β binding to the MEKK4 kinase domain inhibits MEKK4 kinase activity. However, the kinase domain of MEKK4 does not appear to be a substrate for GSK3β-catalyzed phosphorylation, so the inhibition of MEKK4 kinase activity by GSK3β appears to be directly due to protein-protein interaction and not phosphorylation. The phosphorylation of the MEKK4 N-terminal residues defined in Figs. 1–3 does not have a measurable function in controlling MEKK4 inhibition by GSK3β, and so far we have been unable to define a function for GSK3β phosphorylation of these sites in the MEKK4 N terminus. It is possible that phosphorylation of these sites controls the interaction of MEKK4 with other proteins such as GADD45 or Axin, but we have no evidence of this, and additional studies will be required to answer this difficult question. The MEKK4 M5 mutant with substitution of all three GSK3β consensus phosphorylation sites still retains a low level of GSK3β-dependent phosphorylation. The presence of this residual phosphorylation suggests that there may be additional GSK3β phospho-

3 A. N. Abell, D. A. Granger, and G. L. Johnson, unpublished observations.
there is a complex interaction of Axin, GSK3, MEKK4, and TRAF4, all of which are critical in early embryonic development (1, 2, 4, 21, 22), with MEKK4 acting as a signaling hub to positively control JNK and p38 activity in response to TRAF4 and Axin and negatively regulate these pathways in response to GSK3β (Fig. 9B). In the connections map proposed in Fig. 9B, MEKK4 is a central MAP3K for the control of signaling in the Axin-regulated Wnt signaling pathway. In addition, literature
GSK3β Inhibits MEKK4 Signaling

![Diagram of MEKK4 signaling](Image)

**FIGURE 9. Regulation of MEKK4 activation of JNK and p38.** A schematic diagram of the regulation of MEKK4 signaling to JNK and p38. GADD45 stimulates MEKK4 activation by binding the N terminus of MEKK4. TRAF4, Axin, and GSK3β all bind to the kinase domain of MEKK4. Unlike TRAF4 and Axin that activate MEKK4, GSK3β inhibits MEKK4 signaling to JNK and p38. The location of Ser77, Thr112, and Ser1237 that are phosphorylated by GSK3β and GSK3βδ that activate MEKK4, GSK3β, and Axin. MEKK4 function is clearly critical in this signaling network, and its kinase activity is both positively and negatively regulated by proteins within the network for control of JNK and p38. See text for discussion.

searches show that DTRAF1, the *Drosophila* TRAF most similar to mammalian TRAF4, interacts with Misshapen, the mammalian homolog of Nck-interacting kinase (23), which indirectly may connect TRAF4 to dishevelled and Axin. Interestingly, dishevelled 1/2, Axin, TRAF4, and JNK1/2 gene knock-outs and the MEKK4 kinase-inactive gene knock-in all display similar neurulation defects during embryonic mouse development (1, 3, 4, 21, 24, 25). In addition, TRAF4, dishevelled 1/2, and conditional c-Jun knock-outs and the MEKK4 kinase-inactive knock-in also have skeletal malformations (1, 3, 4, 26). The GSK3β knock-out is lethal between E13.5 and E14.5 because of liver apoptosis (22). Redundant signaling by GSK3α is thought to compensate for the loss of GSK3β, except in the liver (22, 27). Loss of both GSK3α and GSK3β is early embryonic lethal, so it is unclear how GSK3 function influences neurulation and vertebral/rib patterning (27). Therefore, our studies begin to provide a biochemical explanation for the genetic pathway that has been mapped by overlapping knock-out and knock-in phenotypes for MEKK4, TRAF4, dishevelled 1/2, GSK3β, and Axin. MEKK4 function is clearly critical in this signaling network, and its kinase activity is both positively and negatively regulated by proteins within the network for control of JNK and p38.

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