Axin Utilizes Distinct Regions for Competitive MEKK1 and MEKK4 Binding and JNK Activation*

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Axin is a multidomain protein that plays a critical role in Wnt signaling, serving as a scaffold for down-regulation of β-catenin. It also activates the JNK mitogen-activated protein kinase by binding to MEKK1. However, it is intriguing that Axin requires several additional elements for JNK activation, including a requirement for homodimerization, sumoylation at the extreme C-terminal sites, and a region in the protein phosphatase 2A-binding domain. In our present study, we have shown that another MEKK family member, MEKK4, also binds to Axin in vivo and mediates Axin-induced JNK activation. Surprisingly MEKK4 binds to a region distinct from the MEKK1-binding site. Dominant negative mutant of MEKK4 attenuates the JNK activation by Axin. Activation of JNK by Axin in MEKK1−/− mouse embryonic fibroblast cells supports the idea that another MEKK can mediate Axin-induced JNK activation. Expression of specific small interfering RNA against MEKK4 effectively attenuates JNK activation by the MEKK1 binding-defective Axin mutant in 293T cells and inhibits JNK activation by wild-type Axin in MEKK1−/− cells, confirming that MEKK4 is indeed another mitogen-activated protein kinase kinase kinase that is specifically involved in Axin-mediated JNK activation independently of MEKK1. We have also identified an additional domain between MEKK1- and MEKK4-binding sites as being required for JNK activation by Axin. MEKK1 and MEKK4 compete for Axin binding even though they bind to sites far apart, suggesting that Axin may selectively bind to MEKK1 or MEKK4 depending on distinct signals or cellular context. Our findings will provide new insights into how scaffold proteins mediate ultimate activation of different mitogen-activated protein kinase kinase kinases.

Axin is a highly modular protein, possessing numerous protein-binding domains that include adenomatous polyposis coli (APC),1 GSK-3β, β-catenin, casein kinases, Axam, PP2A, and

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§ The abbreviations used are: APC, adenomatous polyposis coli; JNK, c-Jun N-terminal kinase; DIX, Dishevelled/Axin homologous domain; MEK, mitogen-activated protein kinase; extracellular signal-regulated kinase kinase; MEKK, MEK kinase; MID, MEKK1-interacting domain; MAP, mitogen-activated protein; MAPK, MAP kinase; MAP3K, MAP kinase kinase; MAP2A, protein phosphatase 2A; HA, hemagglutinin; LRP, low density lipoprotein receptor-related protein; aa, amino acids; GST, glutathione S-transferase; GSK, glycogen synthase kinase.

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by either GSK-3β binding or casein kinase I binding to Axin (5, 16). We reasoned that further deciphering the molecular mechanism whereby Axin activates the JNK pathway would yield invaluable insights into how the scaffold protein Axin mediates MAP3K and subsequently JNK activation. Among them, we shall be able to understand the structural and biochemical basis for the differential complex formations based on Axin.

In the course of further delineating the binding region for MEKK1, we found that newly created deletion mutants of Axin, which could no longer bind to MEKK1, still fully effectively activated JNK. We wondered whether the previously created JNK activation-defective Axin, MID happened to adopt a conformation that dissallows Axin to activate JNK. To clarify this, we thoroughly examined all the critical Axin regions that could contribute to JNK activation. Through detailed analysis, we found that Axin also interacts with MEKK4 in addition to MEKK1. Surprisingly MEKK4 binds to a separate region inside the PP2A binding area far apart from the MEKK1-binding site. These findings argue for the complexity of the Axin-based differential complex formations.

Our current data also suggest that Axin may receive distinct signals via MEKK1 or MEKK4 to activate JNK.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney 293T cells were maintained in RPMI 1640 medium as described previously (13). Mouse embryonic MEKK1−/− fibroblast cells, a gift from M. Karin (University of California, San Diego), were generated as described previously (25) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 IU of penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Construction of Expression Plasmids—Expression vectors for wild-type mouse HA-Axin, Myc-Axin, Axin M5, C2, HA-MEKK1, and FLAG-tagged JNK1 were generated as described previously (23). Wild-type full-length human MEKK2 and mouse MEKK3 were generated from fragments by polymerase chain reaction using primers 5′-catggtagat-cagcagcttgaac-3′ and 5′-tatcagtagataagtcaagacacaggtgc-3′ for MEKK2 and 5′-catggtagacagcagcattgcag-3′ and 5′-tctagactacatctagctggctg-3′ for MEKK3. The cDNA sequence for MEKK4-C starts from the BamHI site to the 3′-end derived from Clone KIAA0213 (courtesy of Kazusa DNA Research Institute) encoding aa 755–1605. All PCR products were verified by sequencing. MEKK2, MEKK3, and MEKK4 were fused in-frame with HA and Myc tag and cloned into the ClaI and XbaI sites of the mammalian expression vector pCMV5. Axin deletion mutants D4, D19, DR2, D20, M4, M6, M8, M9, M10, and M11 were created by subcloning PCR-generated fragments that contained convenient restriction sites followed by fusing the respective deletion mutant fragments with their appropriate flanking wild-type cDNA. Axin deletion mutant D19/M8 was created by fusing the Clal-Xbal fragment of Axin D19 with the Xbal-BamHI fragment of Axin M8 followed by cloning into the Clal and BamHI sites of pCMV5. Detailed information is available upon request.

Transient Transfection and Immunokinase Assays—Transfections were performed in 60-mm dishes using D sper Liposomal Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions. The total amount of transfected DNA of each plate was adjusted with the empty vector pCMV5 where necessary. Cells were harvested at 48 h posttransfection and lysed in a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). FLAG-tagged JNK1 was immunoprecipitated using mouse monoclonal anti-FLAG M2 beads (Sigma); the JNK activity was determined as described previously using 1 μg of GST-c-Jun (1–79) (Stratagene) as substrate (13) followed by Western blotting using phospho-c-Jun antibody (Cell Signaling Inc.) to examine the phosphorylation of c-Jun. Fold activation of the kinase was determined by an imaging analyzer (Amersham Biosciences, Model 425E) and normalized to the total amount of the JNK protein. Data are expressed as fold kinase activation compared with basal kinase activity in vector-transfected cells with the values representing the mean ± S.E. from three separate experiments.
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**RESULTS**

Axin Mutants Lacking the MEKK1-binding Site Retain the Ability to Activate JNK—It was previously shown that MEKK1, but not TAK or ASK, functionally interacted with Axin on a domain termed MID (aa 210–352) (13). We have also shown that GSK-3β or casein kinase Iα and Iε binding to Axin excludes MEKK1 binding (16), suggesting that JNK activation by Axin is at least in part regulated by conformational changes of the Axin protein. As part of efforts to address the structural basis underlying JNK activation by Axin, we fine mapped the region required for MEKK1 binding (Fig. 1, lower left panel). Deletion mutant D4 had aa 210–352 removed and was intact in MEKK4 activation (16), whereas mutants D19 and D20 were lacking the binding to Axin MID domain (aa 210–352). The MID domain mutants D19 and D20 were tested for their ability to activate JNK, and for transfection, 293T cells were transiently transfected with 1 μg of Myc-tagged wild-type Axin plus 2 μg of HA-MEKK1-C, HA-MEKK2, HA-MEKK3, or HA-MEKK4-C. Cell lysates were immunoprecipitated (IP) with anti-Myc. The immunoprecipititates and cell lysates were then analyzed by immunoblotting (IB) separately using anti-Myc for Axin and anti-HA for MEKK proteins. Schematic representation indicates Axin mutants with different deletions in the PP2A-binding region that were used in transient transfection of 293T cells for Axin/MEKK4 co-immunoprecipitation assay. 293T cells were transiently transfected with HA-Axin (2 μg each) together with Myc-MEKK4-C (2 μg). After transfection, cell lysates were immunoprecipitated with anti-HA; immunoprecipitates were analyzed for co-immunoprecipitation of MEKK4 using anti-Myc. The abilities of the Axin proteins to interact with MEKK1 or MEKK4 are summarized as + for positive interaction and − for no interaction. The ability of Axin D19 to interact with MEKK4 is at least in part regulated by conformational changes of the Axin protein. As part of efforts to address the structural basis underlying JNK activation by Axin, we fine mapped the region required for MEKK1 binding (Fig. 1, lower left panel). Deletion mutant D4 had aa 210–352 removed and was intact in MEKK4 activation (16), whereas mutants D19 and D20 were lacking the binding to Axin MID domain (aa 210–352). The MID domain mutants D19 and D20 were tested for their ability to activate JNK, and for transfection, 293T cells were transiently transfected with 1 μg of Myc-tagged wild-type Axin plus 2 μg of HA-MEKK1-C, HA-MEKK2, HA-MEKK3, or HA-MEKK4-C. Cell lysates were immunoprecipitated (IP) with anti-Myc. The immunoprecipititates and cell lysates were then analyzed by immunoblotting (IB) separately using anti-Myc for Axin and anti-HA for MEKK proteins. Schematic representation indicates Axin mutants with different deletions in the PP2A-binding region that were used in transient transfection of 293T cells for Axin/MEKK4 co-immunoprecipitation assay. 293T cells were transiently transfected with HA-Axin (2 μg each) together with Myc-MEKK4-C (2 μg). After transfection, cell lysates were immunoprecipitated with anti-HA; immunoprecipitates were analyzed for co-immunoprecipitation of MEKK4 using anti-Myc. The abilities of the Axin proteins to interact with MEKK1 or MEKK4 are summarized as + for positive interaction and − for no interaction.

**Fig. 2.** MEKK4 interacts with Axin via a novel domain distinct from the MEKK1-binding site. A, MEKK4 interacts with Axin. 293T cells were transiently transfected with 1 μg of Myc-tagged wild-type Axin plus 2 μg of HA-MEKK1-C, HA-MEKK2, HA-MEKK3, or HA-MEKK4-C. Cell lysates were immunoprecipitated (IP) with anti-Myc. The immunoprecipititates and cell lysates were then analyzed by immunoblotting (IB) separately using anti-Myc for Axin and anti-HA for MEKK proteins. Schematic representation indicates Axin mutants with different deletions in the PP2A-binding region that were used in transient transfection of 293T cells for Axin/MEKK4 co-immunoprecipitation assay. 293T cells were transiently transfected with HA-Axin (2 μg each) together with Myc-MEKK4-C (2 μg). After transfection, cell lysates were immunoprecipitated with anti-HA; immunoprecipitates were analyzed for co-immunoprecipitation of MEKK4 using anti-Myc. The abilities of the Axin proteins to interact with MEKK1 or MEKK4 are summarized as + for positive interaction and − for no interaction.

**Fig. 3.** Axin interacts with MEKK1 and MEKK4 in their endogenous concentrations. Left panel, 293T cells were lysed with lysis buffer. Endogenous Axin, MEKK1, and MEKK4 were immunoprecipitated from the total cell lysate using anti-Axin, anti-MEKK1, and anti-MEKK4, respectively. Protein A/G Plus agarose beads with no antibody linked were used in immunoprecipitation (IP) as a negative control. Immunoprecipitates were analyzed by immunoblotting (IB) using anti-Axin, anti-MEKK1, and anti-MEKK4 antibodies for Axin, MEKK1, and MEKK4, respectively. Middle panel, 293T cells were transfected with 1 μg of HA-Axin D19 that lacks the MEKK1-binding site. Cell lysates were subjected to immunoprecipitation using anti-Axin, anti-MEKK1, or anti-MEKK4. Immunoprecipitates were then analyzed by immunoblotting as described above. Right panel, 293T cells were transfected with 1 μg of HA-Axin M8 that lacks the MEKK4-binding site. Immunoprecipitation and immunoblotting were carried out as above.

**Table 1.** MEKK4 Mediates JNK Activation by Axin.

| MEKK4 Interaction | Axin D19 | Axin M8 |
|------------------|---------|---------|
| Axin D19         | +       | −       |
| Axin M8          | −       | −       |

**Fig. 4.** MEKK4 interacts with Axin via a novel domain distinct from the MEKK1-binding site. A, MEKK4 interacts with Axin. 293T cells were transiently transfected with 1 μg of Myc-tagged wild-type Axin plus 2 μg of HA-MEKK1-C, HA-MEKK2, HA-MEKK3, or HA-MEKK4-C. Cell lysates were immunoprecipitated (IP) with anti-Myc. The immunoprecipititates and cell lysates were then analyzed by immunoblotting (IB) separately using anti-Myc for Axin and anti-HA for MEKK proteins. Schematic representation indicates Axin mutants with different deletions in the PP2A-binding region that were used in transient transfection of 293T cells for Axin/MEKK4 co-immunoprecipitation assay. 293T cells were transiently transfected with HA-Axin (2 μg each) together with Myc-MEKK4-C (2 μg). After transfection, cell lysates were immunoprecipitated with anti-HA; immunoprecipitates were analyzed for co-immunoprecipitation of MEKK4 using anti-Myc. The abilities of the Axin proteins to interact with MEKK1 or MEKK4 are summarized as + for positive interaction and − for no interaction.
MEKK4 Interacts with Axin via a Novel Domain Distinct from the MEKK1-binding Site—As MEKK2, -3, and -4 are structurally related to MEKK1, we first tested whether they also interacted with Axin, which might account for the JNK-activating function of the Axin mutants defective in MEKK1 binding. Myc-tagged Axin was co-transfected with HA-tagged MEKK1-C, -2, -3, or -4. Immunoprecipitation was carried out reciprocally with anti-Myc and anti-HA; the immunoprecipitates were analyzed by Western blotting with anti-HA for MEKKs and anti-Myc for Axin. As shown previously, the C-terminal fragment of MEKK1 containing the kinase domain bound to wild-type Axin (Fig. 2A, Ref. 13). Interestingly MEKK4-C also strongly interacted with Axin, whereas MEKK2 or MEKK3 was not co-immunoprecipitated with Axin (Fig. 2A).

To map the region of Axin for MEKK4 binding, a series of Axin deletion mutants were created, including the ones indicated on Fig. 2B, top. We separately co-transfected different HA-tagged Axin mutants with Myc-MEKK4-C and performed co-immunoprecipitation. Results revealed that a domain, distinct from the MEKK1-binding region, binds to MEKK4, which is located around aa 679–746 as deduced from MEKK4 binding assays with constructs M6 and C2 (Fig. 2B). Further deletion of aa 507–672 (M6) or removal of the C-terminal aa 747–832 (C2) did not affect the ability of Axin to bind MEKK4, whereas further removal of aa 673–705 (M5) rendered Axin incapable to bind MEKK4. Consistently deletion of the 33 aa from 679 to 711 (M8) abolished Axin binding to MEKK4. This MEKK4-binding site is inside the region previously known also to interact with PP2A (10). It is interesting to note that the two regions for MEKK1 and MEKK4 binding are far apart.

To verify the Axin-MEKK4 interaction, we tested whether the interaction occurred in their physiologic concentrations. We lysed untransfected 293T cells and pulled down endogenous Axin, MEKK1, or MEKK4 by using anti-Axin, anti-MEKK1, or anti-MEKK4 polyclonal antibodies, respectively. Axin was detected in the anti-MEKK1 and anti-MEKK4 immunoprecipitates; MEKK1 and MEKK4 were both detected in the anti-Axin immunoprecipitated complexes. Interestingly MEKK1 was not detected in the immunocomplexes pulled down by the anti-MEKK4 antibody. Similarly MEKK4 was not present in the anti-MEKK1 immunocomplexes (Fig. 3, left panel). The mutually exclusive presence of MEKK1 and MEKK4 in the Axin complexes is consistent with our finding that they compete against each other in binding to Axin (see below). In parallel, we also performed single transfection experiments to test Axin site dependence for MEKK binding. Axin deletion mutants that lack the binding site of either MEKK1 (D19) or MEKK4 (M8) were transfected into 293T cells followed by immunoprecipitation using antibodies against Axin, MEKK1, or MEKK4. Immunoblotting analysis of the immunoprecipitates showed that Axin D19 could no longer interact with the endogenous MEKK1 and that Axin M8 could not interact with the endogenous MEKK4.

**Dominant Negative MEKK4 Diminished Axin-mediated JNK Activation**—To test whether MEKK4 could indeed mediate the Axin-induced JNK activation, we substituted the conserved...
lysine (K) residue in MEKKs with methionine (M) to create their respective dominant negative forms, MEKK-K/Ms. As expected, Axin alone activated JNK to greater than 10-fold. In contrast, when Axin was co-transfected with MEKK4-C-K/M, the JNK activation was drastically diminished as in the cells co-transfected with MEKK1-C-K/M. In contrast, MEKK2-K/M or MEKK3-K/M failed to attenuate the Axin-induced JNK activation (Fig. 4). These observations are in accordance with their binding activity toward Axin.

**MEKK1 and MEKK4 Require a Common Region of Axin in Addition to Their Respective Binding Sites for JNK Activation**—During the course of fine deletion mapping for both MEKK1 and MEKK4 interaction regions in Axin, we found another region of Axin that is required by both MEKK1 and MEKK4 for JNK activation (Fig. 5). M6 (∆aa 507–672) could bind to both MEKK1 and MEKK4 but failed to activate JNK (Fig. 5), indicating that the region of aa 507–672 is required by both MEKK1 and MEKK4 for JNK activation. The requirement for this novel domain is further supported by observations that other mutants such as M4 (∆aa 507–730), in which part of the region of aa 507–672 is deleted, could bind to MEKK1 as strongly as did the wild-type Axin but failed to activate JNK (Fig. 2 and data not shown). In contrast, M8 (∆aa 679–711) that retains the region of aa 507–672 activated JNK well. To further define this important region of Axin for JNK activation, we generated another three deletions, M9, M10, and M11, in the region of aa 610–720 as schematically diagrammed in Fig. 4. JNK assay and MEKK4 binding experiment were carried out with these mutants. The results showed that the region encompassing aa 642–673 N-terminal to the MEKK4-binding site is indispensable for Axin to activate JNK (Fig. 5), suggesting that MEKK1 and MEKK4 independently require this novel domain for JNK activation. As the Axin regions of aa 210–338 and aa 679–711 are required for MEKK1 and MEKK4 binding respectively, we constructed an Axin deletion mutant, D19/M8, that removes both of the two MEKK-binding sites and tested whether it could activate JNK. The result in Fig. 5 (right panel) shows that AxinD19/M8 cannot activate JNK.

**MEKK4 Competes against MEKK1 for Binding to Axin, and Its Binding in Axin but Not Its Kinase Activity Is Required for This Competition**—We previously showed that Axin forms different complexes in regulating the Wnt pathway and JNK pathways. GSK-3β and casein kinase Ie binding abolished MEKK1 binding to Axin (5, 16). We therefore asked whether MEKK1 and MEKK4 could mutually affect their binding to Axin. As dimerization-defective Axin is fully capable of binding to both MEKK1 and MEKK4 (∆aa 642–711), MEKK1 and MEKK4 independently require their binding site is indispensable for Axin to activate JNK (Fig. 5), suggesting that MEKK1 and MEKK4 independently require this novel domain for JNK activation. As the Axin regions of aa 210–338 and aa 679–711 are required for MEKK1 and MEKK4 binding respectively, we constructed an Axin deletion mutant, D19/M8, that removes both of the two MEKK-binding sites and tested whether it could activate JNK. The result in Fig. 5 (right panel) shows that AxinD19/M8 cannot activate JNK.

**MEKK4 Mediates JNK Activation by Axin**

![Fig. 6. MEKK1 and MEKK4 compete against each other for Axin binding.](image-url)
MEKK4 Mediates JNK Activation by Axin

Small Interfering RNA of MEKK4 Attenuates JNK Activation by MEKK1 Binding-defective Axin Mutant—As shown above, when the MEKK1-binding domain of Axin is removed (D19 and D20, Fig. 1), the Axin mutant could utilize MEKK4 to activate JNK. We reasoned that, in the absence of MEKK1, a decrease of the endogenous MEKK4 should result in reduced JNK activation by Axin. In particular, it would indicate whether MAP3Ks other than MEKK1 and MEKK4 could also play a role in the Axin-mediated JNK activation. The small interfering RNA technique has been utilized to successfully knock down a variety of genes in mammalian cells (26–32). We therefore constructed a pSUPER vector that contains a human MEKK4-specific 19-mer oligonucleotide to knock down the expression of MEKK4 in 293T cell (Fig. 8A) and tested whether it could diminish JNK activation. Axin D19 co-transfected with the blank pSUPER vector activated JNK as usual; however, when co-transfected with pSUPER-MEKK4, Axin D19 virtually lost its ability to activate JNK (Fig. 8B). As expected, pSUPER-MEKK4 only slightly reduced JNK activation by wild-type Axin (data not shown). We also co-transfected wild-type Axin and pSUPER-MEKK4 into MEKK1+/− cells. The result showed that JNK activation of Axin was drastically reduced by specific knock-down of MEKK4 in MEKK1+/− cells (Fig. 8C), confirming that MEKK4 can mediate Axin activation of JNK independently of MEKK1.

**DISCUSSION**

MAP kinases are instrumental in integrating numerous signals to biological processes including embryogenesis, cell differentiation, cell proliferation, and cell death (33–37). It is generally known that a MAP kinase cascade contains at least three components, namely MAP3K, MAPKK, and MAP kinase. However, relatively little is known about how MAP3Ks are activated by upstream signals. In the present study, through detailed mapping by creating a series of deletion mutants of Axin and utilizing MEKK1+/− cells and RNA interference, we have demonstrated that MEKK4 also plays a role in Axin-induced JNK activation. We have found several peculiar aspects in the Axin-mediated JNK activation. First of all, it was found that MEKK4 interacts with Axin on a distinct domain that is far apart from the site for MEKK1 binding despite the fact that MEKK1 and MEKK4 share significant similarity with each other, −55% in the catalytic domain (38). Second, like MEKK1, the C-terminal region of MEKK4 containing its kinase domain is involved in Axin binding instead of its long N-terminal regulatory regions that have been known to interact with multiple proteins (38, 39). Third, as previously shown, Axin-mediated JNK activation requires homodimerization via the DIX domain and sumoylation at the C terminus (23, 24). Fourth, in addition to their respective binding domains, both MEKK1 and MEKK4 require an additional common domain, operationally termed domain X (Fig. 9) outside of their binding sites to trigger JNK activation.

It is unclear why the originally generated MEKK1 binding-defective deletion mutant AxinΔMID (M2 removing aa 210–352) is inactive in JNK activation as tested on numerous occasions. AxinΔMID is intact in binding to MEKK4, homodimerization, and sumoylation. It should be pointed out that it is unlikely that any other factor may bind to the regions flanking the MEKK1-binding site as other deletion mutants that combine to remove the region of aa 210–352 are all capable of activating JNK (data not shown). In fact, larger deletions to both sides that flank the originally defined MID domain did not lose their ability to activate JNK. It is therefore likely that the original deletion AxinΔMID M2 based on two convenient restriction sites happened to create a conformation that disallows Axin to activate JNK. This is especially true given that
different Axin complex formations seem to be controlled by its conformational changes (16).

In addition to identification of MEKK4 as a new MAP3K mediating Axin activation of JNK, we found that MEKK1 and -4 are mutually exclusive in binding to Axin based on the following observations. First of all, using Axin C-250 that is not able to form dimmers with increasing concentrations of MEKK4, MEKK1 binding is gradually abolished from binding to the Axin protein. Conversely increasing amounts of MEKK1 abolished MEKK4 binding to Axin. These results indicate that MEKK1 and -4 could not bind to the same molecule of Axin. Furthermore, from co-immunoprecipitation assays for endogenous MEKK-Axin interactions, we found that anti-MEKK1 could not co-immunoprecipitate MEKK4 and vice versa. That anti-Axin could pull out both MEKK1 and MEKK4 is because the anti-Axin antibody could precipitate the whole pool of Axin-MEKK1/4 complexes. Moreover, although MEKK1 or MEKK4 alone seems to be sufficient for Axin-mediated JNK activation, introduction of either MEKK1-K/M or MEKK4-K/M alone blocked virtually the entire JNK activation by Axin in 293T cells. The data are also consistent with the notion that pre-occupation of Axin by either MEKK prevents the other MEKK from binding to Axin. These observations that MEKK1 and MEKK4 bind to two distinct regions of Axin and that they are competitive in binding to Axin raise an interesting possibility that they may participate in different signaling pathways. Through targeted gene disruption experiments, MEKK1 is known to be required for JNK activation in response to microtubule disruption, viral infection, and double-stranded RNA (25, 40, 41). MEKK1 is also essential for induction of embryonic cell migration by serum factors (25, 41). However MEKK1 is not required for tumor necrosis factor- or interleukin-1 regulation of JNK or NF-κB activation in macrophages or fibroblasts. Relatively little is known about MEKK4 except that it can also activate JNK (42). Nevertheless it is evident that MEKK1 and MEKK4 may have functional specificity as well as functional redundancy depending on cell types. MEKK1 is associated with the plasma membrane, while MEKK4 is localized to the perinuclear membrane and vesicular compartments (38, 43). In addition, they contain diverse N-terminal regions and functional domains (38, 44, 45). The possibility that MEKK1 and -4 may have distinct functions is also supported by our observations that they compete for Axin binding and that in MEKK1-null cells Axin activated JNK equally well as in 293T cells. Furthermore 14-3-3 and 14-3-3 were found to interact with MEKK1, -2, and -3 but not MEKK4 (46).

Another interesting feature of Axin in JNK activation is that there seems to be another domain critical for Axin-mediated
JNK activation, which is located between the MEKK1- and MEKK4-binding sites. Removal of either the MEKK1-binding site or MEKK4-binding site did not affect JNK activation by Axin. However, removal of a small region encompassing aa 642–673, N-terminal to the MEKK4-binding site, completely abolished Axin activation of JNK, although these mutants are intact in MEKK1 or MEKK4 binding. As it was formally possible that removal of this novel domain could cause a conformational change that somehow renders Axin incapable of activating JNK as seen with AxinΔMID, we created multiple deletion mutants of this region, including M10 and M11. M10, M11, and several other mutants not shown failed to activate JNK even though they all bound to MEKK1 well. These data indicate that MEKK binding alone does not suffice to activate JNK. This is reminiscent of a requirement of the extreme C-terminal six amino acid residues that comprise two sumoylation sites as well as homodimerization via its DIX domain in Axin-mediated JNK activation. This observation adds a new complexity to the mechanism by which Axin activates JNK. It remains an interesting issue as to how and why Axin-mediated JNK activation requires this region. Based on our current and previous findings, we summarize domain requirements for the Axin/JNK pathway in Fig. 9. Specifically either MEKK1- or MEKK4-binding domain combines with the newly defined domain X (aa 642–673), DIX homodimerization domain, and the C-terminal sumoylation motifs to activate JNK. Implied in the model is also that MEKK1- and MEKK4-binding sites each independently combine with the common domains for JNK activation. In support of this notion are the observations that, when either the MEKK1- or MEKK4-binding site is removed, Axin is fully capable of activating JNK, whereas removal of any of the other domains abolishes JNK activation. How these domains coordinate with one another in the Axin/JNK pathway requires further investigation.

A number of scaffold proteins for different MAP3Ks have been identified, including Ste5p, JIPs, and MIP-1 (47, 67). Many of these proteins have pulled out from yeast two-hybrid screens, Axin is found to directly (13). Moreover, from the new interacting factors we have identified, including Ste5p, JIPs, and MIP-1, Axin is fully capable of activating JNK, whereas removal of any of the other domains abolishes JNK activation. How these domains coordinate with one another in the Axin/JNK pathway requires further investigation.

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2 W. W. Ng and S.-C. Lin, unpublished data.