Differential Molecular Assemblies Underlie the Dual Function of Axin in Modulating the Wnt and JNK Pathways*

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Yi Zhang‡, Wen-Jie Qiu‡, Dong-Xu Liu‡, Soek Ying Neo‡, Xi He§, and Sheng-Cai Lin‡‡

From the §Regulatory Biology Laboratory, Institute of Molecular and Cell Biology, Singapore 117609, Republic of Singapore and the ¶Division of Neuroscience, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Axin is a multidomain scaffold protein that exerts a dual function in the Wnt signaling and MEKK1/JNK pathways. This raises a critical question as to whether Axin-based differential molecular assemblies exist and how these may act to coordinate the two separate pathways. Here we show that both wild-type glycogen synthase kinase-3β (GSK-3β) and kinase-dead GSK-3β-K85M (capable of binding to Axin), but not GSK-3β-K85M (incapable of binding to Axin in mammalian cells), prevented MEKK1 binding to the Axin complex, thereby inhibiting JNK activation. We further show that casein kinase Iε also inhibited Axin-mediated JNK activation by competing against MEKK1 binding. In contrast, Axin functions in the negative regulation of Wnt signaling and activation of the JNK MAPK pathway.

The Wnt signaling pathway is one of the major development signaling pathways in embryonic induction, generation of cell polarity, and specification of cell fate (1–3). It also helps maintain cellular homeostasis in the adult (4). Intensive studies of Wnt signaling in a variety of organisms have provided a general understanding of the molecular machinery of the signaling pathway. Regulation of Wnt signaling is achieved at least in part by intracellular levels of β-catenin. In the absence of Wnt signals, there forms a large multimeric protein complex comprising Axin, adenomatous polyposis coli (APC),1 glycogen synthase kinase-3β (GSK-3β), and β-catenin, which enhances GSK-3β phosphorylation of β-catenin. Phosphorylation targets β-catenin for ubiquitination and subsequent degradation by a proteasome pathway, keeping the cytoplasmic levels of free β-catenin low (5, 6). Activation of the Wnt pathway through frizzled receptors (2, 7) inhibits GSK-3β by an unknown mechanism that involves Dishevelled protein (4, 8). This prevents β-catenin phosphorylation and its subsequent ubiquitination, resulting in the accumulation of cytoplasmic β-catenin that translocates into the nucleus, where it associates with transcription factors of the LEF families (9, 10) to activate the transcription of target genes such as c-jun, c-myc, fra-1, and cyclin D1 (11–13).

Clearly, GSK-3β is an essential player in the destruction complex of β-catenin. A serine/threonine kinase, GSK-3β phosphorylates one or more conserved serine/threonine residues in the N-terminal region of β-catenin. Phosphorylation at tyrosine 216 of GSK-3β is critical for its kinase activity (14). On the other hand, GSK-3β kinase activity can be inhibited through phosphorylation of its serine 9 residue by Akt or protein kinase A (15, 16). Overexpression of a kinase-dead GSK-3β mutant (GSK-3β-K85M) in which the conserved lysine residue in its ATP-binding domain is altered has been shown to stabilize and promote nuclear accumulation of β-catenin and to induce an ectopic axis in Xenopus (17–19). However, it has been observed that kinase-dead GSK-3β-K85M does not bind to Axin in mammalian cells (20). β-Catenin is one of the major targets for GSK-3β phosphorylation (20, 21). Mutation or deletion of the putative GSK-3β phosphorylation sites in β-catenin results in constitutively active forms of β-catenin that are highly stable (22–24). However, GSK-3β does not bind β-catenin directly and requires Axin and APC to facilitate its interaction with β-catenin (25, 26). Axin, which was first identified as the product of the fused locus in mice, strongly promotes the phosphorylation of β-catenin by GSK-3β (27). Axin hence serves as a scaffold protein, binding directly to APC, GSK-3β, β-catenin, protein phosphatase 2A (PP2A), and casein kinase to coordinate the regulation of β-catenin levels for Wnt signaling (20, 28–30). Mutations of Axin have been reported in hepatocellular carcinoma (31, 32), suggesting that Axin plays pleiotropic roles in processes such as tumorigenesis, in addition to embryonic axis formation. Consistent with its role in preventing tumorigenesis, reexpression of Axin into primary hepatocellular carcinoma cells or overexpression in Chinese hamster ovary cells leads to apoptosis (32, 33). APC can bind directly to β-catenin (34–36). The regulation of β-catenin signaling can also be achieved by the binding of the B56 subunit of protein phosphatase 2A to APC (37). In the Wnt pathway, the role of APC has recently been shown to be required for both degradation and β-catenin export out of the nucleus (38, 39).

Casein kinase Iε (CKIε) has recently been found to be an activator of Wnt signaling in that microinjection of CKIε into Xenopus induces a secondary axis (29, 40). Overexpression of CKIε mimics Wnt signaling by stabilizing β-catenin, thereby increasing the expression of β-catenin-dependent genes. Inhi-
bition of CK1ε in Caenorhabditis elegans produced worms with a mom phenotype, which is indicative of a loss of Wnt signals. In addition, CK1ε can be detected in the Axin-GSK-3β complex (29). It binds to and increases the phosphorylation of Dishevelled, a known component of the Wnt pathway. However, the mechanism of CK1ε in regulating the stability of β-catenin as well as the JNK pathway remains elusive.

The multimeric nature of Axin complexes suggests that Axin may also be an important player in other cell signaling pathways, in addition to its role in Wnt signaling. In particular, we have demonstrated that Axin overexpression stimulates JNK/SAPK signaling (41, 42). Although structural analysis of Axin indicates that the binding domains for APC, GSK-3β, and β-catenin are not required for JNK activation, it is not known whether binding of these Wnt component proteins with Axin functionally affects the ability of Axin to activate JNK. Structurally, it is also not known whether there exist differential molecular assemblies based on Axin dual functions. Here we show that Axin activation of JNK in mammalian cells is inhibited by both GSK-3β and CK1ε, but not by β-catenin or APC. Whereas GSK-3β-K85M did not bind to Axin in mammalian cells and had no effect on Axin activation of JNK, GSK-3β-Y216F was capable of binding to Axin and inhibited Axin-mediated JNK activation as effectively as wild-type GSK-3β. This suggests that the inhibition of Axin-mediated JNK activation by GSK-3β depends on GSK-3β binding to Axin, but not its kinase activity. We further demonstrate that CK1ε also inhibits JNK activation by competing against MEKK1 binding to Axin. These observations indicate the existence of differential assemblies: a Wnt pathway Axin complex that includes GSK-3β and CK1ε and a JNK pathway Axin complex that includes MEKK1. On the other hand, β-catenin and APC do not affect the affinity of Axin for MEKK1, and they can exist in either of the complexes. Our results demonstrate that differential Axin-based molecular complexes unterlie the dual function of the scaffold protein Axin to down-regulate Wnt signaling or to facilitate JNK activation.

MATERIALS AND METHODS

Construction of Plasmids—The Axin, AxinARGS, AxinAMID, and MEKK1-Δ constructs were as previously described (41). AxinΔGSK-3β was generated by replacing the BamHI/SalI C-terminal portion of wild-type Axin with a polymerase chain reaction-generated fragment. The oligonucleotides used for the polymerase chain reaction were 5′-GGAG-1

RESULTS

Wild-type GSK-3β Inhibits Axin Activation of JNK—The scaffold protein Axin exerts a dual function in attenuating Wnt signaling by down-regulating the levels of β-catenin (20, 27) as well as activating the JNK MAPK pathway by binding to MEKK1 (33, 41). To understand the molecular mechanism of how this dual role is achieved and regulated, we tested for the effects on Axin activation of JNK and changes in Axin complex formation exerted by molecules that are known to interact with Axin, including GSK-3β, CK1ε, β-catenin, APC, and MEKK1. We began by examining the effect of GSK-3β on Axin activation of JNK. 293T cells were transfected with AxinΔK85M and GSK-3β alone had virtually no effect on the basal JNK activity, coexpression of Axin with wild-type GSK-3β resulted in a dramatic decrease in Axin activation of JNK. In a control experiment, we used AxinΔGSK, which is truncated for the GSK-3β-binding site. As shown in Fig. 1 (right panel), AxinΔGSK strongly activated JNK, and neither wild-type GSK-3β nor the GSK-3β mutants altered JNK activity induced by AxinΔGSK, indicating that GSK-3β inhibition acts through Axin binding.

Inhibition of Axin-mediated JNK Activation by GSK-3β Depends on GSK-3β Binding to Axin, but Not Its Kinase Activity—To determine whether GSK-3β inhibition of Axin JNK activity was due to the kinase activity of GSK-3β, we assayed the level of JNK activation by Axin in the presence of either the kinase-dead GSK-3β mutants, GSK-3β-K85M and GSK-3β-Y216F. These GSK-3β mutants decreased GSK-3β kinase activity compared with wild-type GSK-3β (data not shown). As shown in Fig. 1 (left panel), coexpression of Axin with GSK-3β-K85M had no effect on Axin JNK activity. However, GSK-3β-Y216F inhibited JNK activation by Axin as effectively as wild-type GSK-3β, suggesting that GSK-3β inhibition of JNK activation is irrespective of its kinase activity. To further elucidate whether the inhibition is due to the binding of

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FIG. 2. GSK-3β binds to Axin, but not its kinase activity, is required for its inhibitory effect on Axin-mediated JNK activation by preventing MEKK1 interaction. Cells were transiently transfected with 1.5 μg of FLAG-JNK1 plus 2 μg of HA-GSK-3β, HA-GSK-3β-Y216F, or HA-GSK-3β-R85M in the presence (black bars) or absence (white bars) of 1 μg of Axin (left panel) or in the presence (hatched bars) or absence (white bars) of 1 μg of AxinΔGSK (right panel). Following immunoprecipitation of FLAG-JNK1, their kinase activities were assayed using GST-c-Jun as substrate. The amount of the kinase in each immunoprecipitate was quantified by immunoblotting (IB). Data are expressed as fold kinase activation compared with vector-transfected cells. The values represent the means ± S.E. from three separate experiments. Total cell lysates were probed with anti-HA antibody to detect the expression of HA-GSK-3β (lanes 3 and 4), HA-GSK-3β-Y216F (lanes 5 and 6), and HA-GSK-3β-R85M (lanes 7 and 8) in the presence (lanes 4, 6, and 8) or absence (lanes 3, 5, and 7) of Axin (left panel) or AxinΔGSK (right panel). WT, wild-type; β-Cat., β-catenin; PP2A, protein phosphatase 2A.

FIG. 1. GSK-3β inhibits JNK activation by Axin, which requires the GSK-3β-binding site in Axin. Cells were transiently transfected with 1 μg of FLAG-JNK1 plus 2 μg of HA-GSK-3β, HA-GSK-3β-Y216F, or HA-GSK-3β-R85M in the presence (black bars) or absence (white bars) of 1 μg of Axin (left panel) or in the presence (hatched bars) or absence (white bars) of 1 μg of AxinΔGSK (right panel). Following immunoprecipitation of FLAG-JNK1, their kinase activities were assayed using GST-c-Jun as substrate. The amount of the kinase in each immunoprecipitate was quantified by immunoblotting (IB). Data are expressed as fold kinase activation compared with vector-transfected cells. The values represent the means ± S.E. from three separate experiments. Total cell lysates were probed with anti-HA antibody to detect the expression of HA-GSK-3β (lanes 3 and 4), HA-GSK-3β-Y216F (lanes 5 and 6), and HA-GSK-3β-R85M (lanes 7 and 8) in the presence (lanes 4, 6, and 8) or absence (lanes 3, 5, and 7) of Axin (left panel) or AxinΔGSK (right panel). WT, wild-type; β-Cat., β-catenin; PP2A, protein phosphatase 2A.
We have previously reported that binding of Axin with GSK-3β reduced the activity of GSK-3β/H9252 and HA-GSK-3β/K85M. As shown in Fig. 2 (A and C), Myc-Axin coprecipitated with wild-type HA-GSK-3β and HA-GSK-3β/Y216F, but not with HA-GSK-3β/K85M. Reciprocal co-immunoprecipitation studies revealed a similar result. Wild-type HA-GSK-3β and HA-GSK-3β/Y216F were detected in Myc-Axin immunoprecipitates, whereas HA-GSK-3β/K85M was not detected. These results suggest that inhibition of Axin-mediated JNK activation by GSK-3β depends on GSK-3β binding to Axin, but not its kinase activity. To examine this point further, we cotransfected Axin and GSK-3β with Akt, which can phosphorylate and inhibit GSK-3β (15). Although coexpression of Akt reduced the activity of GSK-3β to phosphorylate its substrate peptide in an immunokinase assay, Akt had virtually no effect on the Axin-GSK-3β assembly and did not affect Axin-mediated JNK activation (data not shown).

GSK-3β inhibited Axin activation of JNK (Fig. 1), we asked if wild-type GSK-3β would affect the binding of MEKK1 with Axin. To address this, we transfected cells with full-length Myc-Axin and MEKK1-C in the presence or absence of HA-GSK-3β and performed co-immunoprecipitation assays. As expected, when Myc-Axin was expressed with either HA-GSK-3β or MEKK1-C, Myc-Axin was detected in the HA-GSK-3β and MEKK1-C immunoprecipitates, respectively (Fig. 2A). In reciprocal co-immunoprecipitation experiments, similar results were obtained. However, when HA-GSK-3β was coexpressed with Myc-Axin and MEKK1-C, we noted a significant reduction of Myc-Axin in the MEKK1-C immunoprecipitate. This suggests that binding of Axin with MEKK1-C and with GSK-3β is mutually exclusive and that Axin preferentially binds GSK-3β over MEKK1-C. In a control experiment using Myc-Axin\_AGSK, which has lost its ability to bind HA-GSK-3β, MEKK1-C was detected in the immunoprecipitate even in the presence of HA-GSK-3β (Fig. 2B).

We further proved that GSK-3β binding to Axin is an event prior to the inhibition of Axin activation of JNK in the experiments using two kinase-dead GSK-3β mutants, GSK-3β-K85M and GSK-3β-Y216F, which exhibit different characteristics in Axin binding. We took advantage of the finding that GSK-3β-Y216F is capable of binding to Axin, whereas GSK-3β-K85M loses its ability to interact with Axin in mammalian cells. We tested if these two mutants would affect the Axin-MEKK1 interaction by performing similar co-immunoprecipitation assays. 293T cells transfected with different combinations of Myc-Axin, HA-GSK-3β, and Ha-\_MEKK1-C in the presence or absence of HA-GSK-3β/K85M, reciprocal co-immunoprecipitation studies revealed a similar result. In the presence of MEKK1-C, GSK-3β-Y216F could be detected in the Axin immunoprecipitate. In contrast, MEKK1-C was detected in the Axin immunoprecipitate even in the presence of GSK-3β-K85M, which could not bind to Axin. These data confirm that Axin-GSK-3β and Axin-MEKK1-C are separate complexes. Axin preferentially binds GSK-3β over MEKK1-C.

CKIe Also Inhibits JNK Activation by Competing against MEKK1 Binding—The observation that GSK-3β and MEKK1 are mutually exclusive in the complex formation with Axin prompted us to further investigate if CKIe, a Wnt signaling activator, can modulate the differential assemblies of the Axin complex. It has been shown that CKIe is in the Dvl:Axin complex; however, it is not clear if CKIe directly interacts with Axin independent of Dvl. As shown in Fig. 3A, in 293T cells cotransfected with Myc-tagged CKIe together with HA-tagged Axin, we found that CKIe was present in the Axin immunoprecipitate. Reciprocally, in the CKIe immunoprecipitate, Axin was similarly detected. Although Axin was detected in the MEKK1 precipitate in the absence of CKIe, when CKIe was cotransfected with Axin and MEKK1, MEKK1 was no longer co-immunoprecipitated with Axin (Fig. 3B). In accordance with the binding results, CKIe significantly inhibited Axin-induced JNK activation in the immunokinase assay (Fig. 3C).

β-Catenin Does Not Affect Axin Activation of JNK and Is Co-present in the Axin-MEKK1 Complex—A critical aspect of Axin function is to regulate free β-catenin at proper levels. We therefore examined if Axin still binds to β-catenin when it is engaged in MEKK1 binding. We used the degradation-resistant N-terminal deleted form of β-catenin, β-catenin\_ΔN, in the co-immunoprecipitation and immunokinase assays. 293T cells were transfected with different combinations of Myc-Axin, MEKK1-C, and HA-tagged β-catenin\_ΔN. As shown in Fig. 4B, when Myc-Axin was coexpressed with HA-β-catenin\_ΔN in the absence of MEKK1-C, Myc-Axin was detected in the HA-β-catenin\_ΔN immunoprecipitate and vice versa. MEKK1-C itself...
did not bind to HA-β-cateninN in the absence of Axin. When all three proteins were expressed together, MEKK1-C was coprecipitated in the β-cateninN immunoprecipitate as well as in the Axin immunoprecipitate. Reciprocal co-immunoprecipitation studies indicated that HA-β-cateninN was coprecipitated with MEKK1-C and Myc-Axin, suggesting that binding of Axin with β-catenin does not disrupt Axin-MEKK1 complex formation. We next examined the effect of β-catenin on Axin activation of JNK. 293T cells were transfected with JNK1 and Axin in the presence or absence of HA-β-cateninN and as-
sayed for JNK activation. Fig. 4A shows that expression of β-cateninΔN alone had no effect on JNK activity. The JNK activity induced by Axin was unaffected even in the presence of degradation-resistant β-cateninΔN (Fig. 4A), in contrast to the inhibition by wild-type GSK-3β observed earlier (Figs. 1 and 4A). Similar results were obtained in the experiments using full-length β-catenin, although its protein levels became much lower in those samples coexpressed with wild-type Axin (data not shown).

**APC Is A Component in the Axin-MEKK1-β-Catenin Complex—**APC is a critical modulator of β-catenin function in that APC is needed for ubiquitin-mediated degradation as well as for shuffling β-catenin between the cytoplasmic components and the nucleus. We then asked if APC is with Axin when MEKK1 is bound. As shown in Fig. 5B, when Myc-Axin was coexpressed with APC in the absence of MEKK1-C, Myc-Axin was detected in the APC immunoprecipitate; and reciprocally, APC was present in the Axin complex. When MEKK1-C was coexpressed, it did not change the Axin-APC complex formation. APC is therefore co-present in the Axin-MEKK1 complex (Fig. 5B). We next examined the effect of APC on Axin activation of JNK. 293T cells were transfected with JNK1 and Axin in the presence or absence of APC and assayed for JNK activation. Fig. 5A shows that the high level of JNK activity induced by Axin was unaffected in the presence APC.

To further examine whether APC, Axin, β-catenin, and MEKK1 could coexist in the same complex, we transfected 293T cells with Myc-Axin, HA-β-cateninΔN, and APC in the absence or presence of MEKK1-C. As shown in Fig. 5C, when Myc-Axin was coexpressed with HA-β-cateninΔN and APC in the absence of MEKK1-C, Axin was detected in both β-cateninΔN and APC immunoprecipitates. MEKK1-C did not bind to β-cateninΔN and APC in the absence of Axin, as shown in Figs. 4B and 5B. When these four proteins were expressed together, MEKK1-C did not interfere with Axin:β-catenin-APC complex formation; instead, it was found to coexist in this complex (Fig. 5C). Thus, the MEKK1-containing Axin assembly involves both β-catenin and APC.

**Axin-MEKK1 Assembly Retains the Role of Sequestration of β-Catenin through Axin and APC—**Since the Axin-MEKK1 assembly still contains APC and β-catenin, two important components of Wnt signaling, we were interested in examining whether this assembly would play a role in the regulation of Wnt-dependent transcription. First, we determined the structural requirements of Axin in the regulation of LEF1-luciferase activity using different Axin deletion mutants. We transfected 293T cells with each of the various Axin constructs and the
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reporter LEF1-luciferase plasmid together with full-length β-catenin or degradation-resistant β-cateninΔN and measured the LEF1-luciferase activities. As shown in Fig. 6A, expression of β-catenin with LEF1-luciferase in the absence of Axin resulted in high luciferase activity. Coexpression with wild-type Axin significantly reduced LEF1-luciferase activity by 40% (Fig. 6A), in agreement with its role as a negative regulator of Wnt signaling. Coexpression with AxinΔGSK did not inhibit LEF1 as effectively as wild-type Axin, consistent with the crucial role of GSK-3β in destabilization of wild-type β-catenin. Furthermore, AxinΔβ-catenin did not affect the ability of full-length β-catenin to induce LEF1-luciferase activity, indicating the importance of the β-catenin-binding site in Axin in regulating free β-catenin levels for Wnt signal transduction. In parallel, when degradation-resistant β-cateninΔN was coexpressed with the LEF1-luciferase reporter, higher luciferase activity than that of wild-type β-catenin was detected, consistent with the role of degradation as an important means for regulating β-catenin levels. Interestingly, wild-type Axin, AxinΔGSK, and AxinARGS, which all retain the β-catenin-binding site, still had some effect on lowering the LEF1-luciferase activities, possibly by sequestration of degradation-resistant β-cateninΔN. Consistent with this, coexpression with AxinΔβ-catenin, which has lost the β-catenin-binding site, had no effect on β-cateninΔN-induced LEF1-luciferase activity. These results suggest that in addition to the regulation of β-catenin degradation mediated by GSK-3β, Axin also participates in the down-regulation of Wnt-dependent transcription by sequestration of β-catenin. This process may be significant in preventing over-activity of degradation-resistant mutant forms of β-catenin frequently found in tumor cells (43–45).

We next examined the role of APC, another component in the Axin-MEKK1 assembly, in the regulation of LEF1-luciferase activity. We cotransfected 293T cells the same way as described above in the presence of APC with each of the various Axin constructs together with full-length β-catenin or degradation-resistant β-cateninΔN and measured the LEF1-luciferase activity. As shown in Fig. 6A, expression of APC reduced both degradation-resistant β-cateninΔN- and full-length β-catenin-induced LEF1-luciferase activities. Moreover, APC could be synergistic with Axin in the inhibition of Wnt-dependent transcriptions. It is noteworthy that APC seemed to be more effective in sequestration of degradation-resistant β-cateninΔN, in agreement with its role in the nuclear export of β-catenin (38, 46). Thus, our results demonstrate that the role of sequestration of β-catenin by the Axin-MEKK1 assembly demands the presence of APC.

In contrast, MEKK1 does not seem to play a role in modulating Wnt signaling. As shown in Fig. 6B, AxinΔMID, in which the MEKK1-binding site is deleted, still inhibited β-catenin-induced LEF1-luciferase activity as effectively as wild-type Axin. In addition, coexpression of MEKK1 had no effect on LEF1 regulation, suggesting that MEKK1-mediated JNK activation by Axin is separate from Wnt signaling.

DISCUSSION

Axin is a multidomain protein that binds Dishevelled, GSK-3β, β-catenin, APC, protein phosphatase 2A, and CK1ε to coordinate Wnt signaling. It also binds to MEKK1 to activate JNK MAPK. It therefore has become imperative to understand how Axin complex formation is regulated. In this study, we examined if binding of some of the Wnt signaling components (GSK-3β, CK1ε, β-catenin, and APC) with Axin functionally affects Axin activation of JNK. We showed that GSK-3β and CK1ε, but not β-catenin or APC, inhibit JNK activation by Axin. Moreover, we found that GSK-3β and CK1ε inhibit Axin-mediated JNK activation by preventing MEKK1 binding to Axin.

Our results suggest that Axin appears to differentially assemble to form a Wnt signaling complex containing GSK-3β and CK1ε, among others, and a separate JNK activation complex with MEKK1 (Fig. 7). β-Catenin and APC do not affect the affinity of Axin for MEKK1. In either case, Axin can still function to keep free β-catenin levels in check by degradation or sequestration of the cytosolic portion of β-catenin.

The role of GSK-3β as a down-regulator of Wnt signaling has been extensively investigated. In Xenopus, dorsoventral axis formation established in the early blastula depends on signaling events that inhibit GSK-3β. The dominant-negative mutant GSK-3β-K85M induces the duplication of the dorsal axis in the ventral side of Xenopus embryos. However, GSK-3β-K85M does not bind to Axin in the mammalian system. The regulation of GSK-3β is now envisioned as part of an integrated circuit that processes specific upstream signals, scaffolding complexes, and differential target substrates in the inhibitory network in various cellular systems rather than as a simple on-off switch. In the metabolic pathway for glycogen synthase, inhibition of GSK-3β via phosphorylation of serine 9 by Akt is differentially regulated by insulin, whereas the developmental process is modulated by Wnt ligands (47). Akt inhibition of GSK-3β is also distinct from the Axin-mediated JNK activation event in this study. Moreover, results from this study suggest that GSK-3β inhibition of Axin-mediated JNK activation is irrespective of its kinase activity; rather, it is dictated by the Axin complex assembly event, pointing to the complexity and diversity of GSK-3β regulation. Thus, our results provide more evidence that GSK-3β is a versatile switch that can be selective in target discrimination as well as in pathway insulation in the course of cell fate specification and tumorigenesis.

JNK MAPKs play a role in numerous physiological processes, including tissue morphogenesis, cell proliferation, and cytochrome c release for cell death in response to stress (48, 49). In particular, we previously found that Axin-mediated JNK activation facilitates apoptosis in certain cells (33). The biological significance of Axin-mediated apoptosis is best illustrated by a recent report that Axin mutations could lead to tumorigenesis due to a possible lack of apoptosis (31, 32). Reactivation of wild-type Axin via an adenovirus delivery system into hepatocellular and colorectal cancer cells induces considerable apoptosis. Apoptosis induced by wild-type Axin is due to the activation of JNK (33) and degradation or cytoplasmic sequestration of β-catenin or its degradation-resistant mutant forms as shown in the case of hepatocarcinoma HepG2 cells (32). We found that expression of Axin in HepG2 cells still activated JNK despite the high abundance of degradation-resistant mutant β-catenin (data not shown), further confirming our finding that β-catenin had no effect on Axin-mediated JNK activation. In addition, the finding of the preferential binding of Axin with GSK-3β is consistent with the observations that in cells in the normal state, Axin is bound to GSK-3β and that JNK activity is low. On the other hand, gene knockout experiments revealed that GSK-3β plays an important role in preventing cell death (50). Our finding that GSK-3β inhibition of Axin activated JNK is in accordance with its role in cell survival, although it was found that the null mutation of GSK-3β led to a defect in the nuclear factor-κB signaling pathway. It is therefore of great interest to identify the common target(s) of nuclear factor-κB signaling and JNK activation in modulating apoptosis.

Axin complex formation seems to be controlled by conformational changes. It is interesting to note that the binding sites of GSK-3β and MEKK1 in Axin are far apart; the exclusion of

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MEKK1 from Axin by GSK-3β binding suggests that upon binding to GSK-3β, Axin assumes a conformation that does not allow MEKK1 binding. It is equally possible that GSK-3β binding may recruit other factors into the complex, rendering the MİD inaccessible to MEKK1. As for the CKI competition for MEKK1 binding, fine mapping experiments showed that CKI occupies the same region in Axin for MEKK1 binding. As CKI has been shown to be co-present with GSK-3β in the Axin complex (29), it is of interest to understand the structural basis as to why GSK-3β binding can accommodate CKI, but not MEKK1, in the same complex.

We previously demonstrated that Axin-induced apoptosis depends on the extent of its JNK activation and its ability to down-regulate β-catenin levels (33). Our results here show that in the Axin-MEK11 complex, β-catenin and APC are co-present. Moreover, binding of β-catenin and APC to Axin in the Axin-MEK11 complex still down-regulates LEF activity by cytoplasmic sequestration or nuclear export of free β-catenin. Thus, JNK activity is effectively achieved, whereas the β-catenin signal is still in check in the Axin-MEK11:β-catenin:APC complex, facilitating apoptosis. However, there is increasing evidence to suggest that the down-regulation of β-catenin may not be the sole biological function of APC. APC has been reported to bind to microtubules (51), and APC binding to β-catenin and Axin may be involved in microtubule organization during formation of membrane extensions (46, 52). Together, we have demonstrated in this study that distinct complex formations govern Axin either to serve as a scaffold for β-catenin degradation or to bind to MEKK1 for JNK activation. It is fascinating to note that in either functional state, Axin constantly keeps β-catenin levels in check. Our results thus provide a biochemical mechanism to support the notion that differentially localized Axin complexes interplay between signaling pathways and cytoskeletal structures during development and tumorigenesis. We further suggest that in addition to its role in cell fate determination, the central role of Axin is to down-regulate cell growth and help sustain cellular homeostasis.

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