Casein Kinase I and Casein Kinase II Differentially Regulate Axin Function in Wnt and JNK Pathways*

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Yi Zhang‡, Wen-Jie Qi§, Siu Chiu Chan, Jiahuai Han†, Xi He¶, and Sheng-Cai Lin**

From the Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China, the Institute of Molecular and Cell Biology, Singapore 117609, Republic of Singapore, the Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, and the Division of Neuroscience, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Axin uses different combinations of functional domains in down-regulation of the Wnt pathway and activation of the MEKK1/JNK pathway. We are interested in the elucidation of the functional switch of Axin. In the present study, we show that the Wnt activator CKI, but not CKII, Frat1, LRP5, or LRP6, inhibited Axin-mediated JNK activation. We also found that both CKIα and CKIε interacted with Axin, whereas CKIIα did not bind to Axin and had no effect on Axin-mediated JNK activity even though CKIIα has also been suggested to be an activator for the Wnt pathway. The COOH-terminal region and the MEKK1-interacting domain of Axin are important for CKIα-Axin and CKIε-Axin interaction. We further demonstrated that CKIα and CKIε binding to Axin excluded MEKK1 binding, indicating that a competitive physical occupancy may underlie the inhibitory effect. Moreover, our data indicated that CKIε kinase activity plays an additive role in this effect. Taken together, we have demonstrated that CKI and CKII exhibit differential effects on Axin-MEKK1 interaction and Axin-mediated JNK activation. Furthermore, our data suggest that CKI may provide a possible switch mechanism for Axin function in the regulation of Wnt and JNK pathways.

Since its initial cloning from the analysis of the Fused locus, in which mutations cause defects in axis formation (1), Axin has turned out to be a multidomain scaffold protein that manifests pleiotropic functions in biological events including Wnt signaling. JNK mitogen-activated protein kinase signaling, and even tumorigenesis (2–8). Axin serves as an architectural protein on which many cellular factors have been identified including adenomatous polyposis coli (APC), β-catenin, glycogen synthase kinase-3β (GSK-3β), Dishevelled, protein phosphatase 2A (PP2A), and Axam (9–16). Factors frequently rearranged in activated T-cell (Frat1), as well as low-density lipoprotein receptor-related proteins LRP5 and LRP6 are also involved in transducing Wnt signaling (17–19). In the Wnt pathway, the Axin-based multimeric assembly acts to destabilize β-catenin in unstimulated cells (20–22). In response to Wnt signals, Frat1 and Dishevelled somehow prevent GSK-3β phosphorylation of β-catenin, resulting in elevated cellular levels of β-catenin (18, 23). Levels of β-catenin and its regulated transcriptional activities are assumed to be the common denominators for Wnt signaling, and presumably cell growth (24–27). In addition, we have recently shown that ectopic expression of Axin-induced stress-activated protein kinase JNK activation, and also found that Axin interacts with MEKK1 specifically on a domain flanked by the regulator of G protein signaling domain and the GSK-3β-binding site (5). The potential significance of the Axin-MEKK1 interaction is evident for the following reasons. First of all, Axin possesses a distinct binding domain for MEKK1, which we termed the MID domain. Second, MEKK1 binding per se does not suffice to activate JNK, as Axin also requires its C terminus for JNK activation. Furthermore, Axin activation of JNK is highly regulated by the Dishevelled protein (28), GSK-3β binding (29), and homodimerization (5). More importantly, Axin could cause apoptosis in certain cells, which requires its JNK activating activity (30). While biological models are being created to address the biological functions of the Axin-mediated JNK activation, we are interested in the functional switch of Axin for its dual function in the MEKK1/JNK pathway and the Wnt signaling pathway.

Casein kinase Iε (CKIε) is a serine/threonine kinase involved in the regulation of diverse cellular processes ranging from DNA replication and repair to circadian rhythm (31–33). CKIε is regulated in part through inhibitory autophosphorylation at its carboxyl-terminal extensions (34, 35), and is maintained in active state by cellular protein phosphatases (36). Recently, through a functional cloning of factors that control axis formation in Xenopus, CKIε was identified to be an activator for Wnt signaling. Overexpression of CKIε mimics Wnt by stabilizing β-catenin, thereby increasing expression of β-catenin-dependent genes (37). Inhibition of endogenous CKIε attenuated gene transcription stimulated by Wnt, indicating that the kinase activity of CKIε is critical in transducing Wnt signal (37, 38). Based on a yeast two-hybrid screen, it was found to interact with Dishevelled (39), underscoring its biological significance in the Wnt pathway. Co-immunoprecipitation assays have confirmed that CKIε is present in the Dishevelled-Axin complex (37, 39). CKIε has also been shown to phosphorylate APC, in a manner that depends on Axin (40). One report also indicated that CKIα, another member of the CKI family, participates in transducing the Wnt signal (41). It has also been demonstrated that casein kinase II (CKII), which is involved in many prolif-
JNK activation. Moreover, we show that CKI activators Frat1, LRP5, and LRP6 had no effect on Axin-mediated JNK activation. In contrast, other Wnt signaling activation-related processes in the cell, potentiates Wnt/β-catenin signaling in mammary epithelial cells (42, 43).

The bifunctional nature of Axin suggested a switching mechanism might exist between the Wnt and JNK pathways. We previously found that GSK-3β binding to Axin prevents Axin activation of JNK, and that its kinase activity is not required for this inhibiting effect, indicating that other factors may also participate the functional switch mechanism. In our continuous effort to elucidate the molecular mechanism responsible for Axin functional switch, we first tested if CKI expression would affect JNK activation by Axin. Surprisingly, CKI dephosphorylation of FLAG-JNK1, plus 1 μg of vector, Myc-Frat1, LRP5, or LRP6, in the presence (dark column) or absence (light column) of 1 μg of HA-Axin. Total cell lysates were probed with anti-HA for the expression of CKI, CKIα, CKII, and CKII, and CKIα in the presence of GST-c-Jun as substrate. The amount of the kinase in each immunoprecipitate was quantified by immunoblotting. Data are expressed as fold kinase activation compared with vector-transfected cells. The values represent the mean ± S.E. from three separate experiments. B, Frat1, LRP5, or LRP6 has no effect on Axin-mediated JNK activation. Cells were transfected with 1 μg of FLAG-JNK1, plus 1 μg of vector, Myc-Frat1, LRP5, or LRP6, in the presence (dark column) or absence (light column) of 1 μg of HA-Axin. 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. Data are expressed as fold activation compared with the mean ± S.E. from three separate experiments.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—Plasmids of mouse Axin, its deletion mutants, and FLAG-tagged JNK1, were described previously (5). LRP5 and LRP6 were previously constructed (19). CKIα was NH2-terminal tagged with HA through the NcoI site in pBluescript-HA vector as was transferred to pCMV-FLAG vector through its EcoRI and XbaI sites. CKIα and CKIαK38R were HA-tagged and transferred into the pCMV5 vector via its EcoRI and BamHI sites. CKIαK38R was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene); the oligonucleotide sequences used to create the CKIαK38R mutant were 5′-GAAATTCATCAAGCTGGAATGTTGTTGAGTTC-3′ and 5′-CAGACTCTACGCTCTGCGCCTCC-3′. CKIαK38R was generated by ligation of the NH2-terminal NcoI/EcoRI fragment with the PCR-generated kinase domain region. The oligonucleotide sequences used for PCR were 5′-CTCTGGAAAGGCTTACCTCCTGAAATGAACTTGGTGCT-3′ and 5′-TTCTAGAGCCATGTCCATGGAATGACACG-3′. CKIα was generated by removal of the SallXbaI region of full-length CKI, and these two sites were ligated to create a stop codon. CKIαK2 was generated by ligation of the NH2-terminal NcoI/EcoRI fragment with the PCR-generated kinase domain region. The oligonucleotide sequences used for PCR were 5′-CTCTGGAAAGGCTTACCTCCTGAAATGAACTTGGTGCT-3′ and 5′-TTCTAGAGCCATGTCCATGGAATGACACG-3′. CKIα was generated by removal of the SallXbaI region of full-length CKI, and these two sites were ligated to create a stop codon. CKIαK2 was generated by ligation of the NH2-terminal NcoI/EcoRI fragment with the PCR-generated kinase domain region. The oligonucleotide sequences used for PCR were 5′-CTCTGGAAAGGCTTACCTCCTGAAATGAACTTGGTGCT-3′ and 5′-TTCTAGAGCCATGTCCATGGAATGACACG-3′. CKIα was generated by removal of the SallXbaI region of full-length CKI, and these two sites were ligated to create a stop codon.

**Transient Transfection and Immunokinase Assays**—Human embryonic kidney 293T cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Transfections were performed in 60-mm dishes using Superfect according to the manufacturer’s instructions (Qiagen). The total amount of transfected DNA was adjusted to 4 μg with the empty vector pCMV5 where necessary. Cells were harvested at 40 h post-transfection and lysed in a lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 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 (5), followed by Western blotting using Phospho-c-Jun antibody (Cell Signaling) to examine the phosphorylation of c-Jun. Fold activation of the kinase was determined by an imaging analyzer (Molecular Dynamics model 425E) and normalized to their expression levels. Data are expressed as fold kinase activation compared with that in vector-transfected cells with the values representing the mean ± S.E. from three separate experiments.

**Coimmunoprecipitation and Western Blot Analysis**—Transiently transfected 293T cells in 60-mm dishes were lysed in the same lysis buffer as described above. Cell lysates were sonicated three times for 5 s each, and centrifuged at 13,000 rpm for 30 min at 4 °C. Axin, CKIα, CKIα, CKII, and MEKK1 proteins were immunoprecipitated from the cell lysates with anti-Myc (9E10), anti-HA (Roche Molecular Biochemicals), and anti-MEKK1 (C-22, Santa Cruz Biotechnology, Inc.) antibodies as indicated, and with protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Inc.) in 4 °C for 3 h. Immunoprecipitates or total cell lysates were analyzed by Western blotting as previously described (5). The boiled samples were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). After blocking with 5% skim milk in PBS-T (PBS with 0.1% Tween 20) for 1 h, the membranes were probed with anti-Myc (9E10), anti-MEKK1 (C-22), anti-HA, or anti-FLAG antibodies. Bound antibodies were visualized by enhanced chemiluminescence using horseradish peroxidase-conjugated antibodies (Amersham Biosciences).

**LEF1-luciferase Reporter Gene Assay**—293T cells were transfected with 0.1 μg of pGL3-5′-LEF-luciferase, 0.1 μg of pCMV-β-galactosidase, and 0.2 μg of vector, CKIα, or mutant CKIαK38R, using DOSPER according to the manufacturer’s instructions (Promega). Luciferase activities were measured as previously described (30). At 32 h post-transfection, cells were lysed and divided into two portions and measured for luciferase and β-galactosidase activities (Promega). The ratio of luciferase activity to β-galactosidase activity varied less than 10% among the samples. Data are presented as means from three separate experiments performed in triplicate.

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FIG. 2. Binding of CKIε or CKIα to Axin excludes MEKK1 from Axin complex. A, CKIε and CKIα, but not CKIIα, disrupts Axin-MEKK1 interaction. Cells were transfected with 1.5 μg of Myc-Axin, 1.5 μg of vector, HA-CKIε, HA-CKIα, or HA-CKIIα, in the absence or presence of 1.5 μg of MEKK1-C. Cell lysates were immunoprecipitated (IP) with anti-HA, anti-MEKK1, anti-Myc, or control IgG. The immunoprecipitates and cell lysates were then analyzed by immunoblotting using anti-Myc for Axin. B, Frat1, LRP5, and LRP6 do not affect Axin-MEKK1 binding. Cells were transfected with 1.5 μg of HA-Axin, 1.5 μg of MEKK1-C, and 1.5 μg of vector, Frat1, LRP5, or LRP6. Cell lysates were immunoprecipitated (IP) with anti-HA, anti-MEKK1, or control IgG. The immunoprecipitates and cell lysates were then analyzed by immunoblotting using anti-HA for Axin.

RESULTS

CKIε and CKIα, but Not CKIIα, Frat1, LRP5, or LRP6, Inhibit JNK Activation by Axin—We previously made an interesting finding that Axin uses distinct combinations of functional domains in its role as a bifunctional protein in the Wnt signaling and MEKK1/JNK pathways (5). It was not clear how these two Axin-modulated pathways may be coordinated. As part of our efforts to address the switching mechanism, we tested whether the Wnt activators CKIε, Frat1, LRP5, or LRP6 had any effect on Axin activation of JNK. We set out to address this question by expressing these Wnt signaling activators in 293T cells and monitoring the changes in Axin-mediated JNK activation using JNK immunokinase assay. As shown in Fig. 1, A and B, expression of Axin alone robustly activated JNK (~11-fold) as seen previously (5, 28, 29, 44). Expression of CKIε, Frat1, LRP5, or LRP6 alone did not affect JNK activity in the immunokinase assay. Coexpression of Frat1, LRP5, or LRP6 with Axin had no effect on Axin-mediated JNK activation (Fig. 1B). However, coexpression of CKIε with Axin significantly diminished the Axin-induced JNK activity (Fig. 1A). Interestingly, another member of the casein kinase I family, CKIα, also dramatically inhibited Axin-mediated JNK activation (Fig. 1A). In contrast, expression of the casein kinase II family member CKIIα, either wild type or its kinase-dead mutant CKIIα-KM, had no effect on Axin-mediated JNK activation (Fig. 1A). Thus, Axin-mediated JNK activation is specifically inhibited by casein kinase I family members CKIε and CKIα.

CKIε and CKIα, but Not CKIIα, Directly Bind to Axin and Exhibit a Competitive Binding with MEKK1 on Axin—Previous studies had implied the presence of CKIε and CKII in Axin-based complexes. Yeast two-hybrid screening and coimmunoprecipitation assays revealed that CKIε and CKII can bind to Dishevelled (38). To elucidate the molecular mechanism by which CKIε and CKIα blocked Axin-JNK activation, we first tested whether CKIε and CKIα were physically associated with Axin. Immunoprecipitation assays using proteins tagged with different epitopes, coupled with Western blot analysis, revealed that Myc-Axin co-precipitated with CKIε or CKIα (Figs. 2A and 3). This interaction was confirmed by in vitro binding assay using bacterially expressed CKI protein and in vitro TNT-generated 35S-labeled Axin protein (data not shown). These data from the protein interaction assays are in agreement with a recent report (45). In contrast, CKIIα did not interact with Axin in the same immunoprecipitation assay (Fig. 2A).

We then included MEKK1 in the coimmunoprecipitation assays to determine whether the inhibitory effect on JNK activation observed with the casein kinase members was due to their interference with MEKK1 binding to Axin. When MEKK1 was included in these co-transfection and immunoprecipitation assays, Myc-Axin was only detected in MEKK1 immunoprecipitates from cells transfected with either the control vector or HA-CKIα, which did not bind to Axin; but not from cells transfected with CKIε or CKIα, which bound to Axin (Fig. 2A). These results suggest that CKIε or CKIα binding to Axin excludes MEKK1 from binding to Axin. In contrast, co-expression of CKIα, Frat1, LRP5, or LRP6 did not affect Axin-MEKK1 interaction (Fig. 2B).

CKIα and CKIε do Not Affect Axin Homodimerization—We have previously demonstrated that dimerization of Axin is required for its JNK activation. Specifically, Axin mutants that are still capable of binding to MEKK1 but lack of the dimerization domain failed to activate JNK. Intriguingly, MEKK1 also has to be present on both of the dimerized Axin proteins, as the Axin mutant Axin-MID, which retains the dimerization domain but lacks the MEKK1-binding domain, effectively inhibits Axin-induced JNK activation (5, 28). To further address the mechanism whereby CKI members inhibit Axin-induced JNK activation, we asked if binding of CKIε or CKIα to Axin affects Axin homodimerization. HA- and Myc-tagged Axin proteins were coexpressed in the presence or absence of FLAG-tagged CKIε, and lysates were immunoprecipitated with anti-HA or anti-Myc antibody. As shown in Fig. 3, HA-Axin was detected in anti-Myc immunoprecipitates, and vice versa. In the presence of FLAG-CKIε, anti-FLAG antibody could precipitate both HA- and Myc-tagged Axin (Fig. 3). Moreover, HA-Axin can still be detected in anti-Myc immunoprecipitates, indicating that Axin homodimerization is not affected by CKIε (Fig. 3) or CKIα binding (data not shown).
The MID Domain of Axin Is Also Required for CKI-Axin Interaction

The finding that CKI-H9251 and CKI-H9280 directly interact with Axin prompted us to define the CKIe- and CKIα-binding sites on Axin. The schematic diagrams in Fig. 4A depict the series of Myc-Axin mutant constructs used in co-transfection with HA-CKIe or HA-CKIα. Cell lysates were immunoprecipitated with anti-Myc and anti-HA antibodies for Axin and CKI, respectively. As shown in Fig. 4, B and C, wild type Axin as well as the Axin deletion mutant N1 were detected in CKIα (Fig. 4B) and CKIe (Fig. 4C) immunoprecipitates and vice versa, whereas the Axin mutant C1 lacking the COOH-terminal region was not co-precipitated with CKIα or CKIe, indicating that the COOH-terminal region of Axin is crucial for interaction with CKIα and CKIe. However, the COOH-terminal region alone (N3) is not sufficient for binding to either CKIα (Fig. 4B)

**Fig. 3.** CKIe has no effect on Axin homodimerization. Cells were transfected with 1 μg of HA-Axin and Myc-Axin in the presence or absence of 1 μg of FLAG-CKIe. Cell lysates were immunoprecipitated (IP) with anti-HA, anti-Myc, anti-FLAG, or control IgG. The immunoprecipitates and cell lysates were then analyzed by immunoblotting separately using anti-HA, anti-Myc, and anti-FLAG for Axin and CKIe proteins, respectively.

**Fig. 4.** Mapping of CKIe- and CKIα-binding sites on Axin. A, schematic diagrams depict different Axin deletion constructs used in the domain mapping experiments. B, mapping of CKIα-binding sites in Axin. Cells were transfected with 2 μg of HA-CKIe and 2 μg of Myc-tagged Axin, N1, M2, C1, N3, or M3. Cell lysates were immunoprecipitated (IP) with anti-HA, anti-Myc, or control IgG. The immunoprecipitates and cell lysates were then analyzed by immunoblotting separately using anti-HA, anti-Myc, and anti-FLAG for Axin and CKIα proteins, respectively.

**Fig. 5.** Mapping of CKIe for CKIe-Axin interaction. A, schematic diagrams depict different CKIe deletion constructs used in the experiments. B, region of amino acids 248 to 295 in CKIe is important for CKIe-Axin interaction. Cells were transfected with 2 μg of HA-Axin and either of 2 μg of Myc-tagged CKIe, CKIeC1, CKIeC2, or CKIeC3. Cell lysates were immunoprecipitated (IP) with anti-HA, anti-Myc, or control IgG. The immunoprecipitates and cell lysates were then analyzed by immunoblotting separately using anti-HA for Axin and anti-HA for CKIe.
or CKIe (Fig. 4C). Interestingly, when the COOH-terminal of Axin was linked to the MID domain, this M3 Axin mutant restored the ability to interact both CKIa (Fig. 4B) and CKIe (Fig. 4C). Although the M2 Axin mutant lacking the MID domain could still bind to CKIa (Fig. 4B), it showed significantly less affinity toward CKIe (Fig. 4C). These data indicate that the binding of CKIe to Axin obviously requires both the COOH-terminal sequence and the MID domain on Axin, whereas CKIa primarily depends on the COOH-terminal region of Axin for interaction. Since the Axin COOH-terminal alone does not suffice for CKI binding, the interaction between CKI and Axin may elicit conformational changes.

Region of Amino Acids 248 to 295 in CKIe Is Important for CKIe-Axin Interaction—Next we determined the structural requirements of CKIe interaction with Axin. The schematic diagrams in Fig. 5A depict Myc-CKIe constructs used in co-transfection with HA-Axin. Cell lysates were immunoprecipitated with anti-Myc and anti-HA for CKIe and Axin, respectively. Results from the Western blots shown in Fig. 5B demonstrate that HA-Axin was detected in wild type Myc-CKIe, deletion mutants Myc-CKIeC1 and Myc-CKIeC2 immunoprecipitates, but not in the deletion mutant Myc-CKIeC3 immunoprecipitates, indicating that the region of amino acids 248 to 295 in the kinase domain of CKIe is important for Axin-CKIe interaction (Fig. 5B). Consequently, when MEKK1 was included in these co-transfection and co-immunoprecipitation assays, HA-Axin was only detected in MEKK1 immunoprecipitates from samples co-transfected with vector or Myc-CKIeC3. MEKK1 immunoprecipitates from cells co-transfected with HA-Axin and wild type Myc-CKIe, Myc-CKIeC1, or Myc-CKIeC2, however, did not contain Axin (Fig. 5C).

Effects of Kinase Activity of CKIe on Axin-mediated JNK Activation—Kinase activity of CKIe is crucial for its role in Wnt signaling in that inhibition of endogenous CKIe activity by the kinase-defective CKIeK38R or a CKIe antisense oligonucleotide attenuated gene transcription stimulated by Wnt-1 (37). We wondered if the kinase activity of CKIe is also required for inhibiting Axin-mediated JNK activation. Kinase-defective mutant CKIeK38R was generated, and coexpressed in the cells with FLAG-JNK1 and Axin. As shown in Fig. 6A, expression of Axin alone induced JNK activation by 11-fold as observed earlier (Fig. 1A). Expression of the wild type CKIe alone did not alter the already low basal JNK activity. However, the kinase-defective mutant CKIeK38R alone moderately enhanced basal JNK activity (Fig. 6A). This was confirmed in the experiments using the casein kinase I inhibitor CK7, N-2-aminoethyl-5-chloroisoxoquinoline-8-sulfonamide (Seikagaku Corp., Japan). Treatment of cells with 160 μM of the inhibitor CK7 for 10 h led to more than 2-fold enhancement of basal JNK activation. Consistently, expression of CKIe or CKIa in the presence of CK7 only partially attenuated Axin-mediated JNK activation (Fig. 6A). In contrast, expression of CKIIa had no effect on Axin-mediated JNK activation (Figs. 1A and 6A). These results suggest that the kinase activity of CKIe also contributes to the regulation of JNK activation by Axin. CKIeK38R bound to Axin on the same region as wild type CKIe binding to Axin (Fig. 6B, and data not shown), and excluded MEKK1 from the Axin complex (Fig. 6C), suggesting physical binding is an a priori condition for the CKI inhibitory effect. However, the binding affinity of CKIeK38R to Axin is less than that of wild type CKIe to Axin. That may help to explain the partial loss of the inhibitory effect of CKIeK38R on Axin-mediated JNK activation. This is also in agreement with the observation that the binding affinity of CKI to Axin is reduced upon treatment with casein kinase I inhibitor CK7 (data not shown). Compared with the partial requirement of the CKI kinase activity in the regulation of the Axin-JNK pathway, CKI kinase activity is fully required for the Wnt pathway (Fig 6D).
DISCUSSION

Biological signals are often transduced via multimeric complex formations of intracellular proteins, which are coordinated by architectural proteins referred to as scaffold proteins. Axin has become one of the most studied such scaffold proteins, and it is known to interact with many proteins including APC, MEKK1, β-catenin, GSK-3β, Dishevelled, PP2A, and Axam (5, 9–16). Most perplexing is that the same scaffold proteins can form different complexes in response to different signals. Our previous work has shown that Axin forms different complexes in its bifunctional roles. When GSK-3β binds to its cognate site in Axin, MEKK1 cannot bind to the MBD domain, although the two binding sites are physically separated. However, APC and β-catenin are present in both of the complexes either for Wt signaling or the Axin/MEKK1 pathway, suggesting the default function of Axin is to down-regulate the function of β-catenin (29). As a continuous effort to understand how Axin exerts its diverse roles, we have extended our studies to other factors that participate in the Wnt pathway, and thoroughly analyzed how casein kinases may modulate Axin function in the Axin-regulated JNK pathway. In this study, we have demonstrated that CKI can inhibit Axin-mediated JNK activation, but that CKII, Fct1, LRP5, and LRP6 cannot, although they all activate Wnt-dependent transcription. These results indicate that the regulation of Axin activated JNK by CKI is not directly linked to Wnt signaling itself. This is particularly true given that the kinase activity of CKI is partially involved in its inhibitory effect on the JNK pathway, whereas it is fully critical for transducing Wnt signaling.

It is interesting that CKI binds directly to both Dishevelled and Axin in the Dishevelled-Axin complex (37, 39, 45), whereas CKII binds to only Dishevelled (46). The importance of both CKI and CKII binding to Dishevelled in the Wnt signaling is demonstrated by the findings that both the CKI (39) and CKII (46) can phosphorylate and activate Dishevelled. CKI binding to Axin has been shown to be important for its phosphorylation of the APC protein (40). Our results in this study further suggest that the importance of the direct binding of CKI to Axin is to engage Axin functionality in the Wnt pathway. Although the specific triggers of CKI to either activate Wnt or regulate MEKK1/JNK pathway is at present unclear, it is evident that CKI binding to Axin is required for its inhibitory effect on Axin activation of JNK. More importantly, it excludes MEKK1 binding on Axin, which is a prerequisite for Axin-mediated JNK activation. Recent reports showed that CKI binds to the COOH-terminal Axin region overlapping with the PP2A binding area (44, 45). Our results here demonstrated that the COOH-terminal of Axin is required for its interaction with CKI, however, the COOH-terminal region alone does not suffice for CKI binding. These results indicate that binding of CKI to Axin may cause conformational changes in Axin, which may lead to the exclusion of MEKK1 binding to Axin. Indeed, our results here further demonstrated that the MBD domain is also important for CKI binding, particularly for CKII. The observed lesser inhibitory effect by the kinase-dead mutant CKI-K38R is mirrored by its diminished affinity for Axin, and is consistent with a requirement of its kinase domain for Axin binding. It is important to point out that the reduced binding affinity is not due to any protein structural change derived from the amino acid substitution, because experiments conducted with the casein kinase inhibitor CK7 also indicate that CKI kinase activity plays a role in the inhibition of Axin-mediated JNK activation.

The kinase activity of CKIε is crucial in its role as an activator of the Wnt signaling pathway (37, 39). It was recently demonstrated that it activates the Wnt pathway, presumably by phosphorylating Dishevelled (38, 39). It has also been reported that the kinase activity of CKIε is important for function (40). The kinase activity of CKIε is also necessary in its function to regulate circadian rhythm, in that CKIε controls the nuclear entry of the circadian regulator mPer1 by phosphorylation of mPer1 (32, 47). Our results in this study showed that the kinase activity of CKIε also contributes to the inhibitory effect on Axin-mediated JNK activation; unlike GSK-3β, in which the kinase activity of GSK-3β is fully dispensable for its inhibitory effect on Axin-mediated JNK activation (29). Consistent with our results here, it is reported that inhibition of the kinase activity of CKI leads to JNK activation (44). As GSK-3β plays a central role in transducing Wnt signaling, it is possible that CKI is not initiating factor for Wnt signaling as in the case of Akt regulation of the Wnt pathway (48). This leads us to speculate that CKI may serve to fine tune Axin function in the balance between the two distinct pathways, Wnt and JNK.

The kinase activity of CKIε can be regulated, and maintained in an active state, by cellular protein phosphatases such as PP2A (36). Given the fact that PP2A can bind to Axin through its catalytic domain (15) as well as bind to tumor suppressor APC through its B56 regulatory subunit (20), it is worth investigating the mutual regulation of PP2A and CKIε activation in the Wnt signaling pathway. As CKIε exhibits different functions, the specificity of the regulation of its kinase activities may require factors other than PP2A. Moreover, the present finding that the kinase activity of CKIε also partially contributes to its inhibitory effect on the JNK pathway further highlights the diversity of the function and regulation of this important kinase.

In summary, we have demonstrated that CKI kinases bind directly to Axin and exclude Axin-MEKK1 interaction in vivo, thereby inhibiting Axin-mediated JNK activation. Moreover, our data have indicated that CKI kinase activity also contributed to this effect. Unlike CKI, CKII does not bind to Axin, and affects neither Axin-based molecular assemblies nor JNK activation. Thus, we have demonstrated that CKI and CKII exhibit differential effects on Axin-mediated JNK activation, and suggest that CKI may provide a possible switch mechanism for Axin function in the regulation of Wnt and JNK pathways.

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