Synergistic Activation of the Wnt Signaling Pathway by Dvl and Casein Kinase Ie*

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Michiko Kishida‡, Shin-ichiro Hino‡, Tatsuo Michiue§, Hideki Yamamoto‡, Shosei Kishida‡‡, Akimasa Fukui‡, Makoto Asashima‡, and Akira Kikuchi‡¶

From the ‡Department of Biochemistry, Faculty of Medicine, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan, §PRESTO, Japan Science and Technology Corp., Hiroshima, Japan, and ¶Crest Project and Department of Life Science (Biology), University of Tokyo, 3-8-1, Komaba, Meguro-ku, Tokyo 153-8902, Japan

Although casein kinase Ie (CKI) has been shown to regulate the Wnt signaling pathway positively, its mode of action is not clear. In this study we show that CKI activates the Wnt signaling pathway in cooperation with Dvl. CKI and Axin associated with different sites of Dvl, and CKI and Dvl interacted with distinct regions on Axin. Therefore, these three proteins formed a ternary complex. Either low expression of Dvl or CKI regions on Axin. Therefore, these three proteins formed a homolog Axil/conductin), GSK-3 stabilized by a multiprotein complex containing Axin (or its maltose-binding protein; His 6, six histidine-tagged; rAxin, rat Axin; tAxin, Xenopus (2–5). In unstimulated cells, free cytoplasmic β-catenin is de-stabilized by a multiprotein complex containing Axin (or its homolog Axil/conductin), GSK-3β, and APC. Interaction of GSK-3β with Axin in the complex facilitates efficient phosphorylation of β-catenin by GSK-3β. Phosphorylated β-catenin forms a complex with Fbw1 (βTrCP/FWD1), a member of the F-box protein family, resulting in the degradation of β-catenin by the ubiquitin and proteasome pathways (14, 15). Because Axin inhibits Wnt-dependent accumulation of β-catenin and activation of Tcf/Lef, a transcription factor (10, 16), it is a negative regulator of the Wnt signaling pathway. In addition, APC and Axin are also phosphorylated by GSK-3β in the Axin complex. Phosphorylation of APC enhances its binding to β-catenin (17), and that of Axin stabilizes it, in contrast to phosphorylation of β-catenin (18).

When cells are stimulated by Wnt, a cytoplasmic protein, Dvl, antagonizes the action of GSK-3β. Although whether Dvl binds directly to Frizzled, the receptor for Wnt, or whether intermediary proteins are involved in the signal transmission between Frizzled and Dvl is not known at present, Dvl appears to bind to the Axin complex (19–21) and to inhibit GSK-3β-dependent phosphorylation of β-catenin, APC, and Axin (18, 20, 22). Once the phosphorylation of β-catenin is reduced, it dissociates from the Axin complex, and β-catenin is no longer degraded, resulting in its accumulation in the cytoplasm. Stabilized β-catenin is translocated into the nucleus, where it binds to Tcf/Lef (23–25) and serves as a coactivator of Tcf/Lef to stimulate transcription of the Wnt target genes including c-myc, fra, jun, cyclin D1, peroxisome proliferator-activated receptor δ, and matrilysin (26–31). Thus, the Wnt signal stabilizes β-catenin, thereby regulating various gene expression.

Three Dvl genes, Dvl-1, -2, and -3, have been isolated in mammals (32–34). Expression of Dvl in cells induces the accumulation of β-catenin and the activation of Tcf (20, 21, 35). Dvl homologs are conserved in Drosophila (dishevelled, Dsh) and Xenopus (Xenopus dishevelled, Xdsh) (36–38). All Dsh and Dvl family members contain three highly conserved domains (2–4): an N-terminal DIX domain, which is also found in the C terminus of Axin; a central PDZ domain, which has been shown to be a protein-protein interaction surface in several proteins; and a DEP domain, which is conserved in proteins that regulate GTP-binding proteins. The DIX domain is necessary for the Dvl activity to regulate the Wg and Wnt signaling pathways positively (20, 39–41). Disruption of the PDZ domains of Dsh and Dvl abolishes their activities in the Wg and Wnt signaling pathways and in the Xenopus secondary axis formation, suggesting that the PDZ domain is essential for the Wnt signaling pathway (20, 38, 42). Dvl antagonizes the ability of Axin to induce ventralization in Xenopus embryos (43), and the DIX and PDZ domains of Dvl are important for its complex formation with Axin (19–21). The DEP domain of Dsh has been found to be critical for rescue of the Drosophila Dsh planar cell polarity defect and for the activation of c-Jun N-terminal kinase but not essential for the Wg pathway (44, 45). The DEP...
domain of vertebrate Dvl is also necessary for c-Jun N-terminal kinase activation in mammals but not for the axis formation (39, 46). CKI comprises a large family of related gene products, α, β, γ, δ, and ε (47). Each shares at least 50% amino acid identity within the protein kinase catalytic domain. Distinct CKI family members are likely to show different tissue distributions and subcellular localization and to have distinct roles including the regulation of DNA repair, DNA replication, cell cycle progression, and circadian rhythm (47, 48). It has been reported that among CKI family, CKIκ and δ but not CKIα are involved in the Wnt signaling pathway (49, 50). Overexpression of CKIκ in Xenopus embryos induces the expression of siamois, a Wnt response gene, and axis duplication. These CKIκ-dependent responses are suppressed by Axin and GSK-3β, and Dvl-induced axis duplication is inhibited by CKIκ-I, a CKI inhibitor (49). Furthermore, CKIκ forms a complex with Dvl and Axin, and CKIκ activates Lef-1, which is inhibited by Axin in mammalian cells (50). These results suggest that CKIκ positively regulates the Wnt signaling pathway by functioning between Dvl and GSK-3β. However, the mode of action of CKIκ is not understood.

Here we demonstrate that by complex formation with Axin, CKIκ forms a complex with Dvl and Axin, and Dvl symmetrically accumulates β-catenin and activates Tcf in mammalian cells and that they also synergistically induce axis duplication in Xenopus embryos. Furthermore, we show that the binding of CKIκ to Dvl is necessary for their synergistic action. These results demonstrate that the binding of the complex of CKIκ and Dvl to Axin activates the Wnt signaling pathway.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The pSETB/human CKIκ (hCKIκ), pSETB/hCKIκ kinase negative (KN), pCEPH/hCKIκ, and pCEPH/hCKIκ (KN) were kindly provided by Dr. D. M. Virshup (University of Utah, Salt Lake City, UT) (51). TOPOFLASH and pOPFLASH were provided by Dr. H. Clevers (University Hospital, Utrecht, The Netherlands). The cDNA of human Dvl-1 and the anti-GST antibody were provided by Dr. H. Clevers (University Hospital, Utrecht, The Netherlands). The anti-Axin (for immunoprecipitation), anti-Dvl, anti-CKIκ, anti-FLAG (M2), anti-Axin, anti-GST-3β, anti-β-catenin antibodies to detect the protein expression levels. The same lysates (400 μg of protein) were immunoprecipitated with the normal rabbit serum, the anti-Dvl antibody, or the anti-Axin antibody, and then the immunoprecipitates were probed with the anti-Axin, anti-Dvl, anti-CKIκ, and anti-GSK-3β antibodies. To demonstrate the complex formation of CKIκ, rAxin, Dvl-1, APC, GSK-3β, and β-catenin in intact cells, COS cells (60-mm-diameter dish) transfected with pBMYc-, pBMYc-pEGFP, pCGN-, pDNAS-FLAG-, and pGFP-derived plasmids were lysed in 250 μl of the lysis buffer. The supernatant (20 μg of protein) was probed with the anti-Myc, anti-HA, anti-FLAG (M2), anti-CKIκ, anti-GSK-3β, and anti-β-catenin antibodies to determine the protein expression levels. The same lysates (200 μg of protein) were immunoprecipitated with the anti-Myc, anti-HA, or anti-GFP antibody, and then the immunoprecipitates were probed with the anti-Myc, anti-HA, anti-FLAG (M2), anti-CKIκ, anti-GSK-3β, and anti-β-catenin antibodies to detect the protein expression levels. The same lysates (200 μg of protein) were immunoprecipitated with the anti-Myc, anti-HA, or anti-GFP antibody, and then the immunoprecipitates were probed with the anti-Myc, anti-HA, anti-FLAG (M2), anti-CKIκ, anti-GSK-3β, and anti-β-catenin antibodies to detect the protein expression levels. The same lysates (200 μg of protein) were immunoprecipitated with the anti-Myc, anti-HA, or anti-GFP antibody, and then the immunoprecipitates were probed with the anti-Myc, anti-HA, anti-FLAG (M2), anti-CKIκ, anti-GSK-3β, and anti-β-catenin antibodies to detect the protein expression levels. The same lysates (200 μg of protein) were immunoprecipitated with the anti-Myc, anti-HA, or anti-GFP antibody, and then the immunoprecipitates were probed with the anti-Myc, anti-HA, anti-FLAG (M2), anti-CKIκ, anti-GSK-3β, and anti-β-catenin antibodies to detect the protein expression levels.

Immunocytochemistry—L cells grown on coverslips were fixed for 10 min in 4% paraformaldehyde. The cells were washed with PBS three times and then permeabilized with PBS containing 0.1% Triton X-100 and 2 mg/ml bovine serum albumin for 2 h. The cells were washed and incubated for 1 h with the anti-HA and anti-β-catenin antibodies. After being washed with PBS, they were further incubated for 1 h with Cy5-labeled anti-mouse IgG and Alexa 546-labeled anti-rabbit IgG. The coverslips were washed with PBS, mounted on glass slides, and viewed with a confocal laser-scanning microscope (LSM510, Carl-Zeiss, Jena, Germany).

Luciferase Assay—To observe Tcf-4 activity, the indicated amounts of pCGN/Dvl-1 and/or pCEPH/hCKIκ were transfected into 293 cells (35-mm-diameter dish) with pTOPFLASH (0.5 μg), pBMYc-HA/Tcf-4 (0.5 μg) and pBMYc-pEGFP-C1 (0.5 μg). After 24 h, the cells were lysed, and luciferase activity was measured using a PicoGene (Toyo BENT-Co., Ltd., Tokyo, Japan) and luminesphometer TD4000 (Futaba Medical, Tokyo, Japan). To standardize the transfection efficiency, pME18S/lacZ carrying the SRE promoter linked to the coding sequence of the β-galactosidase gene was used as an internal control.
The lysates of L cells (400 μg of protein) were immunoprecipitated with the normal rabbit serum (lanes 2 and 5), the anti-Dvl antibody (lane 3), or anti-Axin antibody (lane 6), and then the immunoprecipitates were probed with the anti-Dvl, anti-CKIe, anti-Axin, and anti-GSK-3β antibodies. Lanes 1 and 4 show endogenous expression of Dvl, CKIe, Axin, and GSK-3β in the lysate (20 μg of protein) of the L cells. NRS, normal rabbit serum; IP, immunoprecipitation; Ab, antibody; Ig, immunoglobulin. The results shown are representative of four independent experiments.

**RESULTS**

**Complex Formation of CKIe with Dvl or Axin at Endogenous Level**—Deletion mutants of rAxin and Dvl-1 used in this study are shown in Fig. 1. CKIe was previously shown to form a complex with Dvl and Axin (49, 50). However, because these experiments were done with overexpression assays in mammalian cells and a yeast two-hybrid system, we first examined whether CKIe is phosphorylated Dvl and Axin (49, 50). However, because these experiments were done with overexpression assays in mammalian cells and a yeast two-hybrid system, we first examined whether CKIe associates with Dvl and Axin at endogenous level in intact cells. To this end, we generated the antibodies that immunoprecipitate Dvl and Axin. CKIe was detected slightly but reproducibly in the Dvl immune complex from L cells (Fig. 2, lanes 1–3). In the Axin complex immunoprecipitated from L cells, CKIe was observed in addition to GSK-3β (Fig. 2, lanes 4–6). Because endogenous Dvl was not detected in this Axin complex (data not shown), it is likely that the complex formation of CKIe with Axin is not mediated via Dvl in the condition without Wnt stimulation. These results indicate that CKIe at least forms a complex with Dvl or Axin at endogenous level in intact cells.

**Phosphorylation of Dvl and APC by CKIe**—To find substrates of CKIe in the Wnt signaling pathway, we examined whether Dvl, APC, Axin, β-catenin, and GSK-3β are phosphorylated by CKIe in intact cells and in vitro. When HA-Dvl-1 was co-expressed with GFP-CKIe in COS cells, it migrated more slowly on an SDS-polyacrylamide electrophoresis gel than when it was expressed alone (Fig. 3A, lanes 1 and 3). This mobility shift was reduced on treatment with alkaline phosphatase (Fig. 3A, lanes 4 and 5). Furthermore, GFP-CKIe (KN) did not affect the migration of HA-Dvl-1 (Fig. 3A, lane 2), suggesting that CKIe phosphorylates Dvl-1 in mammalian cells. These results are consist with previous observations that CKIe phosphorylates Dvl in Xenopus embryos (49). We showed previously that Axin binds to APC-(1211–2075) and enhances GSK-3β-dependent phosphorylation of APC (59). Myc-APC-(1211–2075) exhibited a lower mobility on an SDS-polyacrylamide electrophoresis gel by expression of CKIe but not of CKIe (KN) (Fig. 3A, lanes 6–8), suggesting that CKIe phosphorylates APC in intact cells. To show whether CKIe phosphorylates Dvl-1 and APC directly, these proteins were purified from E. coli and Spodoptera frugiperda 9 cells (Fig. 3B, lanes 1–6). Both MBP-Dvl-1 and GST-APC-(1211–2075) were phosphorylated by His6-CKIe, but MBP or GST was not (Fig. 3B, lanes 8 and 10–12). Mobility shifts of GST-Dvl-1 and GST-APC-(1211–2075) by the phosphorylation on an SDSL-polyacrylamide electrophoresis gel were also observed in the in vitro experiments (Fig. 3C, lanes 1–4). When GST-Dvl-1 and GST-APC-(1211–2075) were fully phosphorylated by His6-CKIe, ~2 and 5 mol of phosphates were incorporated into 1 mol of MBP-Dvl-1 and GST-APC-(1211–2075), respectively (data not shown). These results indicate that Dvl-1 and APC are substrates of CKIe in mammalian cells and that they have multiple phosphorylation sites for CKIe. Co-expression with CKIe did not affect the mobility of Axin (Fig. 3A, lanes 9–11) and β-catenin (data not shown) in COS cells, although they were phosphorylated by His6-CKIe in vitro (Fig. 3B, lanes 7 and 9). The mobility shift of MBP-rAxin by the phosphorylation in vitro was detected (Fig. 3C, lanes 5 and 6), but that of GST-β-catenin was not significant (Fig. 3C, lanes 7 and 8). GSK-3β was not phosphorylated by His6-CKIe (data not shown).

**Complex Formation of Dvl with CKIe**—HA-Dvl-1 was co-expressed with GFP-CKIe and GFP-CKIe (KN) in COS cells. When the lysates were immunoprecipitated with the anti-HA antibody, GFP-CKIe and GFP-CKIe (KN) were co-precipitated with HA-Dvl-1 with a similar efficiency (Fig. 4A), suggesting that kinase activity of CKIe is not required for its complex formation with Dvl. To examine which region of Dvl is responsible for the complex formation with CKIe, various deletion mutants of HA-Dvl-1 were expressed with GFP-CKIe in COS cells (Fig. 4B, lanes 1–8). When the lysates expressing GFP-CKIe alone were immunoprecipitated with the anti-HA antibody, GFP-CKIe was not observed in the immunoprecipitates (Fig. 4B, lane 9). The PDZ domain of Dvl-1 was shown to be
With GFP-CKI–deletion mutants, HA-Dvl-1-(1519) and HA-Dvl-1-(140–33150) but it might be due to the difference of assays. Among other important for its interaction with CKI by the yeast two-hybrid assay (49). However, GFP-CKI was immunoprecipitated with HA-Dvl-1ΔPDZ as well as HA-Dvl-1 but not with HA-Dvl-1 (201–371), that contains the PDZ domain (Fig. 4B, lanes 10–12). The reasons for this discrepancy are not known at present, but it might be due to the difference of assays. Among other deletion mutants, HA-Dvl-1-(1–519) and HA-Dvl-1-(140–670) but not HA-Dvl-1-(1–378) or HA-Dvl-1-(1–432) formed a complex with GFP-CKI (Fig. 4B, lanes 13–16). These results suggest that the N-terminal region of Dvl-1 including the DIX and PDZ domains is not important but that the entire DEP domain is necessary for its complex formation with CKI. Taken together with the observations that the N-terminal region of Dvl-1 including the DIX and PDZ domains binds to Axin (20, 54, 60), these results suggest that the binding sites of Dvl-1 for Axin and CKI are distinct. To determine that the interaction of Dvl with CKI is direct, GST-Dvl-1 was incubated with MBP-CKI. GST-Dvl-1 bound to MBP-CKI (Fig. 4C). In this experiment, equal amounts (20 pmol) of GST-Dvl-1 and MBP-CKI were used. Although this is not a saturated condition, it seems that ~10–20% of GST-Dvl-1 bound to MBP-CKI, an estimation from the density of a band in the Western blotting. When the kinase activity of CKI was measured with casein as a substrate, co-expression with HA-Dvl-1 did not affect the CKI activity (data not shown), suggesting that the interaction with Dvl-1 does not regulate the kinase activity of CKI.

**Complex Formation of Axin with CKI—**Myc-rAxin was co-expressed with GFP-CKI and GFP-CKI (KN) in COS cells. When the lysates were immunoprecipitated with the anti-Myc antibody, GFP-CKI and GFP-CKI (KN) were co-precipitated with Myc-rAxin with a similar efficiency (Fig. 5A). This suggests that kinase activity of CKI is not required for its complex formation with Axin. To examine which region of Axin is important for the complex formation with CKI, various deletion mutants of Myc-rAxin were expressed with GFP-CKI in COS cells (Fig. 5B, lanes 1–9). When the lysates expressing GFP-CKI alone were immunoprecipitated with the anti-Myc anti-

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**FIG. 3. Phosphorylation of Dvl and APC by CKI.** A, phosphorylation of Dvl-1 by CKI in intact cells. HA-Dvl-1 (lanes 1–5), Myc-APC (1211–2075) (lanes 6–8), and Myc-rAxin (lanes 9–11) were expressed with GFP-CKI (lanes 1, 4–6, and 9) or GFP-CKI (KN) (lanes 2, 7, and 10) in COS cells. The lysates of COS cells (20 μg of protein) were probed with the anti-HA antibody, the anti-Myc antibody, and anti-CKI (lanes 1–5, 6–11) antibodies. The lysates (200 μg of protein) of COS cells expressing HA-Dvl-1 and GFP-CKI (lanes 4 and 5) were immunoprecipitated with the anti-HA antibody, and the immunoprecipitates were incubated with (lane 6) or without (lane 5) His6-CKI (0.5 μg of protein) or Myc-rAxin (20 μg of protein) were stained with Coomassie Brilliant Blue. The results shown are representative of four independent experiments.

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**FIG. 4. Complex formation of Dvl with CKI.** A, interaction of Dvl-1 with CKI (KN) in intact cells. The lysates (200 μg of protein) of COS cells expressing the indicated proteins were immunoprecipitated with the anti-HA antibody, and the immunoprecipitates were probed with the anti-HA and anti-CKI antibodies. IP, immunoprecipitation; Ab, antibody; WT, wild type. B, interaction of the deletion mutants of Dvl-1 with CKI in COS cells. The lysates (20 μg of protein) of COS cells expressing the indicated proteins were probed with the anti-HA and anti-CKI antibodies (lanes 1–8). The same lysates (200 μg of protein) were immunoprecipitated with the anti-HA antibody, and then the immunoprecipitates were probed with the anti-HA and anti-CKI antibodies (lanes 9–16). C, direct interaction of Dvl-1 with CKI. After GST-Dvl-1 (0.2 μm) was incubated with 20 pmol of MBP-CKI (lane 1) or MBP (lane 2) immobilized on amylase resin, MBP fusion proteins were precipitated by centrifugation, and the precipitates were probed with the anti-GST antibody (upper panel). 10% of GST-Dvl-1 used in this experiment was probed with the anti-GST antibody (lower panel). The results shown are representative of four independent experiments.
FIG. 5. Complex formation of Axin with CKI. A, interaction of rAxin with CKI (KN). The lysates (20 μg of protein) of COS cells expressing the indicated proteins were probed with the anti-Myc and anti-CKI antibodies (lanes 1 and 2). The same lysates (200 μg of protein) were immunoprecipitated with the anti-Myc antibody, and the immunoprecipitates were probed with the anti-Myc and anti-CKI antibodies (lanes 3 and 4). IP, immunoprecipitation; Ab, antibody; WT, wild type. B, interaction of the deletion mutants of rAxin with CKI in COS cells. The lysates (20 μg of protein) of COS cells expressing the indicated proteins were probed with the anti-Myc and anti-CKI antibodies (lanes 1–9). The same lysates (200 μg of protein) were immunoprecipitated with the anti-Myc antibody, and the immunoprecipitates were probed with the anti-Myc and anti-CKI antibodies (lanes 10–18). The results shown are representative of four independent experiments.

body, GFP-CKI was not observed in the immunoprecipitates (Fig. 5B, lane 10). Among various deletion mutants of rAxin, GFP-CKI was immunoprecipitated with Myc-rAxin-(1–713) as well as Myc-rAxin (Fig. 5B, lanes 11 and 16). GFP-CKI was also complexed with Myc-rAxin-(298–713) and Myc-rAxin-(1–529) (Fig. 5B, lanes 12 and 13) but not with Myc-rAxin-(1–437), Myc-rAxin-(1–229), Myc-rAxin-(298–506), and Myc-rAxin-(508–832) (Fig. 5B, lanes 14, 15, 17, and 18). These results suggest that the region containing amino acid residues 438–529 is important for the complex formation with CKI. Because this region is different from the Dvl binding site (rAxin-(508–620)) (22), CKI and Dvl-1 could form a complex with Axin through its different regions. To examine the direct interaction of Axin with CKI, GST-rAxin-(298–832) and MBP-CKI were purified from E. coli. MBP-CKI did not bind to GST-rAxin-(298–832) (data not shown). Therefore, it seems that CKI forms a complex with Axin via another protein. Although rAxin-(438–529) contains the β-catenin binding site, β-catenin did not bind to CKI directly (data not shown). It is likely that CKI forms a complex with Axin via a protein other than β-catenin. When the kinase activity of CKI was measured with casein as a substrate, co-expression with Myc-rAxin did not influence the CKI activity (data not shown), suggesting that the complex formation with Axin does not regulate the kinase activity of CKI.

Ternary Complex Formation of Axin, Dvl, and CKI—The results above demonstrated that Axin and CKI bind to the distinct regions of Dvl-1 and that Dvl-1 and CKI form a complex with the different sites of Axin, suggesting that these three molecules form a ternary complex. To clarify this possibility, various combinations of rAxin, Dvl-1, and CKI were expressed in COS cells. When the lysates expressing Myc-rAxin alone were immunoprecipitated with the anti-Myc antibody, GSK-3β was detected in the Myc-rAxin immune complex (Fig. 6, lane 1). Co-expression with either HA-Dvl-1, GFP-CKI, or GFP-CKI (KN) did not affect the complex formation of Myc-rAxin with GSK-3β (Fig. 6, lanes 2, 5, and 6). When Myc-rAxin was co-expressed with both HA-Dvl-1 and GFP-CKI, these two proteins did not compete with each other for their binding to Myc-rAxin (Fig. 6, lane 3). Furthermore, the interaction of Myc-rAxin with both HA-Dvl-1 and GFP-CKI did not influence its complex formation with GSK-3β. Co-expression with HA-Dvl-1 and GFP-CKI (KN) showed the same results (Fig. 6, lane 4).

GSK-3β was observed in the HA-Dvl-1 immune complex via Myc-rAxin (Fig. 6, lanes 8 and 9). It is notable that GSK-3β was present in the Dvl-Axin complex (Fig. 6, lanes 7 and 9), because it was reported that the Dvl-ARP (Axin-related protein) complex in Xenopus embryos does not contain GSK-3β (61). Further expression of GFP-CKI or GFP-CKI (KN) did not affect the complex formation of HA-Dvl-1 with Myc-rAxin and GSK-3β (Fig. 6, lanes 10 and 11). GSK-3β was observed in the GFP-CKI or GFP-CKI (KN) immune complex via Myc-rAxin (Fig. 6, lanes 12–16). Further expression of HA-Dvl-1 did not affect the complex formation of GFP-CKI or GFP-CKI (KN) with Myc-rAxin and GSK-3β (lanes 17 and 18). Taken together, these results indicate that Axin, Dvl-1, and CKI form a ternary complex and that Dvl-1 and CKI do not affect the interaction of Axin with GSK-3β in the complex.

Effects of Phosphorylation of APC by CKI on Its Complex Formation with Axin—As shown in Fig. 3, APC was phosphorylated by CKI in intact cells and in vitro. Because APC directly binds to Axin (9, 15), we examined whether the phosphorylation of APC by CKI influences its complex formation with Axin in intact cells. Because full-length APC was not expressed ectopically, APC-(1211–2075), which can bind to Axin and down-regulate β-catenin (59), was used. Expression of GFP-CKI or GFP-CKI (KN) did not affect the complex formation of Myc-APC-(1211–2075) with FLAG-rAxin (Fig. 7, lanes 2, 7, and 8). GSK-3β was observed in the Myc-APC-(1211–2075) immune complex via FLAG-rAxin (Fig. 7, lanes 1 and 2). The complex formation of GFP-CKI or HA-Dvl-1 with Myc-APC-(1211–2075) was enhanced by expression of FLAG-rAxin (Fig. 7, lanes 3–10), suggesting that CKI or Dvl forms a complex with APC via Axin. However, expression of GFP-CKI or HA-Dvl-1 did not influence the complex formation of GSK-3β with Myc-APC-(1211–2075) via FLAG-rAxin (Fig. 7, lanes 2, 7–10). These results suggest that the phosphorylation of APC by CKI does not influence its complex formation with Axin, Dvl, and GSK-3β.

β-Catenin was observed in the Myc-APC-(1211–2075) immune complex (Fig. 7, lane 1). Expression of FLAG-rAxin de-
increased the complex formation of β-catenin with Myc-APC-(1211–2075) slightly (Fig. 7, lanes 1 and 2). Although this would reflect the down-regulation of β-catenin by Axin, a further decrease in the amount of β-catenin in the APC-Axin complex by Axin may be small because β-catenin complexed with APC is degraded efficiently. In contrast, expression of GFP-CKIe slightly increased the level of β-catenin complexed with Myc-APC-(1211–2075), but that of GFP-CKIe (KN) did not (Fig. 7, lanes 3 and 4). Further expression of HA-Dvl-1 increased the amount of β-catenin associated with Myc-APC-(1211–2075) (Fig. 7, lane 5). These results suggest that Dvl-1 and CKIe may co-operate to increase β-catenin.

Synergistic Effects of Dvl and CKIe on Wnt Signaling—Previously we showed that transient overexpression of Dvl-1 in L cells induces the nuclear accumulation of β-catenin (20). However, accumulation of β-catenin was not observed in L cells stably expressing Dvl-1 (L/Dvl cells) (Fig. 8, B and D). This result suggests that a low expression level of Dvl-1 in L/Dvl cells is not sufficient for the stabilization of β-catenin. Because CKIe may enhance Dvl-1 stimulation of the Wnt signaling pathway (observations in Fig. 7), we examined the effects of combination of CKIe and Dvl-1 on the accumulation of β-catenin. Although expression of CKIe in wild-type L cells did not accumulate β-catenin (Fig. 8, A and B), its expression in L/Dvl cells increased the level of β-catenin in the nucleus (Fig. 8, C–E). Expression of CKIe (KN) in L/Dvl cells did not induce the accumulation of β-catenin (Fig. 8, F–J). These results show that Dvl-1 and CKIe co-operate to accumulate β-catenin and that the kinase activity of CKIe is necessary for this synergistic effect.

We also examined the effects of combination of CKIe and Dvl-1 on the activation of Tcf-4 that is dependent on β-catenin. Expression of Dvl-1 alone in 293 cells activated Tcf-4 in a dose-dependent manner as described (Fig. 9A) (21). CKIe alone increased the activity of Tcf-4 slightly (Fig. 9A). When a low dose of CKIe that is not sufficient for the activation of Tcf-4 was expressed with Dvl-1, CKIe promoted the activity of Dvl-1 to stimulate Tcf-4 (Fig. 9A). Dvl-1 also enhanced the activity of
CKI to activate Tcf-4 greatly (Fig. 9A). These experiments were performed with TOPFLASH as a reporter gene. When FOPFLASH, a negative control of TOPFLASH, was used, Tcf-4 activity was not detected (data not shown). These results suggest that Dvl-1 and CKI activate Tcf-4 synergistically. Which regions of Dvl-1 are important for the synergistic action with CKI was examined. Dvl-1-(1–519) but not Dvl-1-(1–432) bound to CKI, although both associated with Axin (Fig. 4) (20, 60). Either Dvl-1-(1–432) or Dvl-1-(1–519) alone activated Tcf-4, but the activity was less than full-length Dvl-1 (Fig. 9B). Dvl-1-(1–519) synergized with CKI to increase the activity of Tcf-4 as well as full-length Dvl-1, but Dvl-1-(1–432) did not (Fig. 9B). Dvl-1-PDZ bound to CKI and Axin (Fig. 4) (60, 61). Dvl-1-(140–670) bound to CKI but weakly to Axin (Fig. 4) (60, 61). Neither Dvl-1-PDZ nor Dvl-1-(140–670) had the activity to stimulate Tcf-4. Furthermore, they did not show the synergistic action with CKI (Fig. 9B). Therefore, these results suggest that the interaction of CKI with Dvl is not essential for the Wnt signaling pathway and that binding of Dvl to Axin is necessary but not sufficient. Furthermore, the binding of CKI to Dvl is necessary for the synergistic action to stimulate the Wnt signaling pathway.

**Synergistic Action of CKI and Dvl on Axis Duplication**—To confirm the mode of action of CKI and Dvl, we examined their effects on the Wnt signaling pathway using *Xenopus* embryos. The Wnt signaling pathway regulates axis formation of *Xenopus* embryos (62). Ventral injection of mRNAs of Xwnt-8, Dvl, and β-catenin has been shown to induce the formation of a secondary dorsal axis (38, 62–64). Consistent with previous observations (49, 50), ventral injection of a high dose (200 pg) of CKI mRNA into embryos resulted in dorsalization of phenotypes such as axis duplication (Fig. 10B). Embryos injected ventrally with either low doses (25 or 50 pg) of Dvl-1 or CKI mRNA did not exhibit significant abnormalities (Fig. 10, A and B). However, co-injection of low doses of Dvl-1 and CKI mRNA induced axis duplication greatly (Fig. 10, A and B). These results demonstrate that CKI synergistically functions with Dvl-1 to regulate axis formation and are consistent with the results observed in mammalian cells.

**DISCUSSION**

In this study we found that CKI and Dvl form a complex with the different regions of Axin and that CKI and Axin associate with distinct sites of Dvl. Although the binding of Dvl to CKI was direct, that of Axin to CKI was not. Furthermore, our results demonstrated that the simultaneous binding of Dvl and CKI to Axin induces the maximal activation of the Wnt
that Frat, which was identified as a GSK-3 signaling pathway is not understood, it has been suggested the Wnt signaling pathway.

Although another protein to Axin is important for the activation of Dvl to Axin, it is not sufficient for the binding of Dvl to Axin. We do not know the exact reasons, but it might be due to the differences between yeast two-hybrid and mammalian intact cell assays.

Our results showed that CKIe and Dvl-1 co-operate to stimulate β-catenin accumulation and activate Tcf in mammalian cells and to induce axis duplication in Xenopus embryos. These synergistic activities between CKIe and Dvl-1 were observed when Dvl-1-(1–519) but not Dvl-1-(1–432) was used. Since Dvl-1-(1–519) but not Dvl-1-(1–432) forms a complex with CKIe, these results suggest that the entire DEP domain of Dvl-1 is necessary for its binding to and synergistic action with CKIe. Dvl-1-(1–40)–670), which binds to CKIe but weakly to Axin, did not activate Tcf, suggesting that the binding of CKIe to Dvl-1 is not sufficient for the activation of the Wnt pathway, but that the binding of the DIX domain of Dvl-1 to Axin is necessary. Furthermore, Dvl-1ΔPDZ, which binds to both CKIe and Axin, did not activate Tcf, suggesting that the binding of Dvl to Axin is not sufficient for the activation of the Wnt signaling pathway. Therefore, the protein that binds to the PDZ domain of Dvl-1 and activates the Wnt signaling pathway remains to be clarified. Taken together, these results indicate that the binding of the complex including Dvl-1, CKIe, and at least one more protein to Axin is important for the activation of the Wnt signaling pathway.

Although the mechanism by which Dvl activates the Wnt signaling pathway is not understood, it has been suggested that Frat, which was identified as a GSK-3β-binding protein (65), forms a complex with the PDZ domain of Dvl and that this complex induces the dissociation of GSK-3β from Axin in response to Wnt (66). Furthermore, it has been shown that expression of Dvl induces the displacement of GSK-3β from the ARF (Axin-related protein) and that the PDZ domain is necessary for this Dvl activity (61). In addition to Frat, CKIe (67) and protein phosphatase 2C (68) have been reported to form a complex with the PDZ domain of Dvl. Since none of these proteins contains the (S/T)XV sequence in their C termini (69), the PDZ domain of Dvl may have different properties from that of other known PDZ domains regarding protein-protein interactions. CKII associates with and phosphorylates Dvl (67). Although the interaction with CKII requires the region containing the PDZ domain, whether this interaction is direct is not known, and its significance in the Wnt signaling pathway is not yet clear. Protein phosphatase 2C has been isolated by the yeast two-hybrid assay using the PDZ domain as bait (68). Expression of protein phosphatase 2C in COS cells dephosphorylates and down-regulates Axin and stimulates the transcriptional activity of Lef-1. These results suggest that protein phosphatase 2C works as a positive regulator of the Wnt signaling pathway by inhibiting phosphorylation in the Axin complex.

We have recently identified a novel Dvl-binding protein, Idax, that suppresses the Wnt signaling (54). Idax interacts with the PDZ domain of Dvl and inhibits the complex formation of Dvl and Axin. It inhibits Wnt-dependent accumulation of β-catenin in mammalian cells and Wnt-dependent axis duplication in Xenopus embryos. Therefore, Idax negatively regulates the Wnt signaling pathway probably by inhibiting the binding of Axin to Dvl or by competing with other PDZ domain-binding proteins for their interaction with Dvl. It is necessary to examine whether CKIe affects the complex formation of Frat, CKII, protein phosphatase 2C, or Idax with Dvl and regulates their functions.

How does CKIe activate the Wnt signaling pathway? To accumulate β-catenin in the cells, the phosphorylation of β-catenin must be reduced. The simple model is that CKIe induces the dissociation of GSK-3β from Axin by phosphorylating substrates in the Axin complex or that CKIe inhibits the GSK-3β activity. Dvl-1, APC, Axin, and β-catenin were good substrates of CKIe in vitro, and at least Dvl-1 and APC were phosphorylated by CKIe in intact cells. However, CKIe did not affect the interaction of Dvl or APC with Axin. The CKIe complex contained GSK-3β via Axin, and CKIe did not dissociate GSK-3β from Axin. We also examined whether CKIe regulates GSK-3β activity. CKIe did not phosphorylate GSK-3β directly. The activity of GSK-3β complexed with CKIe via Axin was the same as that complexed with CKIe (KA) (data not shown). Dvl did not affect the activity of GSK-3β complexed with CKIe, either (data not shown). Therefore, it is unlikely that CKIe regulates the localization and activity of GSK-3β in the Axin complex. Another possibility is that an unknown protein might be phosphorylated by CKIe and that it may prevent the ubiquitination of β-catenin.

The complex formations of CKIe and Dvl or CKIe and Axin at endogenous levels were small. Furthermore, we could not detect the endogenous association of Dvl with Axin. Therefore, it is likely that the complexes of Axin, Dvl, and CKIe are less stable than that of Axin and GSK-3β. This is reasonable, because β-catenin is usually degraded in the Axin-GSK-3β complex and accumulated in response to Wnt. The more stable complex of Axin, Dvl, and CKIe may be formed once Wnt stimulates the cells. It is possible that the interactions we observed with overexpressed proteins are representative of those in the cells activated by Wnt. We are examining the possibility that Wnt stabilizes the complex of Axin, Dvl, and CKIe, thereby accumulating β-catenin.

In summary, CKIe and Dvl synergistically activate the Wnt signaling pathway by their complex formation with Axin, and the binding of CKIe to Dvl is important for their synergistic effects. In addition to them, one more protein that binds to the PDZ domain of Dvl could be essential for the activation of the Wnt signaling pathway. CKIe could be located to the proper position via Axin and Dvl and phosphorylate substrates in the Axin complex, resulting in the accumulation of β-catenin. Further studies will be necessary for understanding the whole picture of the mechanism by which Dvl and CKIe regulate the Wnt signaling pathway positively.

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REFERENCES
1. Wodarz, A., and Nusse, R. (1998) Annu. Rev. Cell Dev. Biol. 14, 59–88
2. Cadigan, K. M., and Nusse, R. (1997) Genes Dev. 11, 3286–3305
3. Dale, T. C. (1998) Biochem. J. 329, 209–223
4. Miller, J. R., Hocking, A. M., Brown, J. D., and Moon, R. T. (1999) Oncogene 18, 7860–7872
5. Hanelson, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., and Birchmeier, W. (2000) J. Cell Biol. 148, 567–578
6. Kikuchi, A. (1999) Cell. Signal. 11, 777–788
7. Ikeda, S., Kishioka, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A.
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Michiko Kishida, Shin-ichiro Hino, Tatsuo Michiue, Hideki Yamamoto, Shosei Kishida, Akimasa Fukui, Makoto Asashima and Akira Kikuchi

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