The canonical Wnt-signaling pathway is critical for many aspects of development, and mutations in components of the Wnt pathway are carcinogenic. Recently, sufficiency tests identified casein kinase Iε (CKIε) as a positive component of the canonical Wnt/β-catenin pathway, and necessity tests showed that CKIε is required in vertebrates to transduce Wnt signals. In addition to CKIε, the CKI family includes several other isoforms (α, β, γ, and δ) and their role in Wnt sufficiency tests had not yet been clarified. However, in Caenorhabditis elegans studies, loss-of-function of a CKI isoform most similar to α produced the mom phenotype, indicative of loss-of-Wnt signaling. In this report, we examine the ability of the various CKI isoforms to activate Wnt signaling and find that all the wild-type CKI isoforms do so. Dishevelled (Dsh), another positive component of the Wnt pathway, becomes phosphorylated in response to Wnt signals. All the CKI isoforms, with the exception of γ, increase the phosphorylation of Dsh in vivo. In addition, CKI directly phosphorylates Dsh in vitro. Finally, we find that CKI is required in vivo for the Wnt-dependent phosphorylation of Dsh. These studies advance our understanding of the mechanism of Wnt action and suggest that more than one CKI isoform is capable of transducing Wnt signals in vivo.

Key Words: Casein Kinase I; Wnt; Dishevelled; signal transduction.

INTRODUCTION

The Wnt-signaling pathway is important for many aspects of development, including dorsal axis formation, tissue patterning, and establishment of cell polarity (Cadigan and Nusse, 1997; Moon et al., 1997; Rocheleau et al., 1997). In addition, mutations in components of the Wnt pathway lead to cancers, such as colon, liver, and skin cancer (Chan et al., 1999; Kinzler and Vogelstein, 1996; Koch et al., 1999; Morin et al., 1997). This signaling pathway has therefore been the focus of intense study, and many components of the pathway have been identified and their role in Wnt signaling characterized. In the canonical Wnt-signaling pathway, a secreted Wnt ligand binds to the seven-transmembrane frizzled receptor, which then transduces the signal, in an unknown manner, through Dishevelled (Dsh) (Bhanot et al., 1999; Moon et al., 1997; Xu et al., 1998; Yanagawa et al., 1995). Dishevelled, a PDZ-containing protein with no known enzymatic function, is thought to act as a scaffolding protein, bringing together several Wnt pathway components in a large multiprotein complex (Li et al., 1999). This complex has been shown to contain Dsh, Axin, GSK-3, and GBP (Li et al., 1999). In the absence of Wnt signaling, GSK-3, a negative regulator of the Wnt pathway, phosphorylates β-catenin, targeting it for degradation (Yost et al., 1996). In the presence of Wnt, GSK-3 activity is inhibited and β-catenin is stabilized; this allows β-catenin to accumulate, bind to the LEF/Tcf family of transcription factors, and activate transcription of target genes such as Xnr-3 and Siamois (Behrens et al., 1996; Brannon et al., 1997; McKendry et al., 1997; Molenaar et al., 1996).

One isoform of the CKI family of enzymes, CKIε, was recently identified as a positive component of the Wnt pathway (Peters et al., 1999; Sakana et al., 1999). CKIε, which was identified by expression cloning in Xenopus, was able to induce second axes, rescue UV-treated embryos, and induce transcription of the Wnt target genes Xnr-3 and Siamois (Peters et al., 1999; Sakana et al., 1999)—all hallmarks of a positive Wnt-pathway component. Gain-of-function studies in Drosophila cells and mammalian cell lines supported these data (Peters et al., 1999; Sakana et al., 1999). In addition, loss-of-function studies in Xenopus embryos and mammalian cell lines demonstrated that CKIε is a conserved component of the Wnt cascade (Peters et al., 1999; Sakana et al., 1999). By epistasis analysis, CKIε was
shown to act between Dsh and GSK-3 in the Wnt pathway. This placement was supported by yeast two-hybrid and coimmunoprecipitation data showing that CKIe interacts with Dsh as well as Axin (Peters et al., 1999; Sakanaka et al., 1999). Wnt signaling, in cell lines, and CKIe, in Xenopus oocytes, both increase the level of phosphorylation of Dsh (Lee et al., 1999; Peters et al., 1999; Yanagawa et al., 1995). Dsh contains a region, the PDZ domain, that is required for Dsh phosphorylation and for activation of the Wnt/β-catenin pathway (Sokol, 1996; Yanagawa et al., 1995). Of note, the PDZ domain of Dsh is necessary and sufficient for CKIe binding (Peters et al., 1999), suggesting that CKIe may be involved in the Wnt-dependent phosphorylation of Dsh.

In addition to CKIe, the CKI family includes the α, β, γ, and δ isoforms, all of which contain highly conserved kinase domains (Gross and Anderson, 1998). The isoforms are distinguished by amino and carboxyl extensions that flank the kinase domain (Fig. 1A). The α and β isoforms have short amino and carboxyl extensions. The δ and ε isoforms have short amino extensions and relatively long carboxyl-terminal tails that are of similar length and primary structure. Three highly related (>90% amino acid

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**FIG. 1.** The CKI isoforms activate the Wnt cascade. (A) Schematic of CKI isoforms; the catalytic kinase domains are hatched. (B, C) One-cell embryos were UV-irradiated, injected at the eight-cell stage, and tadpoles were photographed or scored with the dorso-anterior index (Kao and Elinson, 1988). Doses for α (175 pg), δ (350 pg), ε (350 pg), and ε CD (catalytic domain, 175 pg) were chosen for optimal rescue; for β (25 pg), γ3 (1.4 mg), and γ3 CD (1.4 mg), the maximum nontoxic dose was used. n > 100 in each group. (D) For the second axis assay, eight-cell embryos were injected and scored with the second axis index (no second axis = 0, partial duplications = 1, second axes with eyes and cement gland = 2). n > 75 for each group. (E) Embryos were injected at the one-cell stage with mRNA encoding CKIe(K→R) (350 pg), CKIα (175 pg), CKIβ (10 pg), CKIγ3 (700 pg), CKIδ (350 pg), CKIε (350 pg), or CKIε catalytic domain (175 pg). Animal cap explants were assayed at stage 10.5 for expression of the Wnt target genes Xnr-3 and Siamois (Siam) (Brannon et al., 1997; Darras et al., 1997; McKendry et al., 1997). EF-1α, a ubiquitously expressed transcript, serves as a loading control (Krieg et al., 1989).
identity in the kinase domain) γ isoforms are known: γ1, γ2, and γ3. The γ isoforms are distinguished from the rest of the CKI family by the presence of both carboxyl and amino extensions. It has been postulated that, for the CKI family, the amino- and carboxyl-terminal extensions are important for conferring differential function and regulation to the various isoforms (Graves and Roach, 1995; Gross and Anderson, 1998; Santos et al., 1996). The CKI family is thought to function in a variety of processes, including DNA repair, cell-cycle control, and circadian rhythm (Gross et al., 1997; Kloss et al., 1998; Lowrey et al., 2000; Santos et al., 1996). While gain-of-function studies in Xenopus embryos and mammalian cell lines have shown that CKIe and δ activate the canonical Wnt pathway, the ability of the other CKI isoforms to activate Wnt signaling is unknown (Peters et al., 1999; Sakanaka et al., 1999). Although it has been reported that the α isoform does not activate Wnt signaling in frogs or mammalian cell lines (Sakanaka et al., 1999), loss-of-function studies in worms suggest that the α isoform is required for Wnt signaling (Peters et al., 1999). This incongruity emphasizes the need for further studies to clarify which isoforms activate the Wnt cascade.

In this report, we examine the role of the other CKI isoforms in Wnt signaling and find that, in Xenopus embryos, all of the CKI isoforms activate the Wnt pathway as measured by UV rescue, formation of second axes, and induction of the Wnt markers Xnr-3 and Siamois. We also found that, in vitro, CKI directly phosphorylates Dsh, and that CKI is required for the Wnt-induced in vivo phosphorylation of Dsh. These studies demonstrate the sufficiency of all the CKI isoforms to activate Wnt signaling and demonstrate a necessity for CKI function in Wnt-dependent responses.

MATERIALS AND METHODS

Constructs and RNA Synthesis

The CKI isoforms (bovine: α and β; human: δ, γ2, γ3, γ3 CD (amino acids 36–354)) were cloned into the plasmid pCS2+. All constructions and oligo sequences are available upon request. All other constructs and generation of synthetic, capped mRNA are as described (Peters et al., 1999).

Embryological Methods and RT-PCR Analysis

Embryos were obtained, microinjected, dissected, and cultured as described (Graff et al., 1994, 1996). Embryos were staged according to Nieuwkoop and Faber (1967). For the UV rescue, one-cell embryos were UV-irradiated, injected into one vegetal blastomere at the eight-cell stage, and tadpoles were assigned a DAI (Kao and Elinson, 1988). Doses for α, δ, ε, and γCD were chosen for optimal rescue for β, γ3, and γ3CD, the maximum nontoxic dose was used. In the second-axis assay, eight-cell embryos were injected into one ventral vegetal blastomere and scored between stages 25 and 30 for secondary axes as described (Peters et al., 1999). RT-PCR analysis has been described (Graff et al., 1994, 1996; LeSueur and Graff, 1999).
Phosphorylations

Oocytes. Oocytes were injected with 30 ng myc-Dsh mRNA and incubated for 24 h at 17°C. The same oocytes were then injected with 10 ng mRNA encoding CKIα, CKIβ, CKIγ, CKIδ, CKIε, or CKIεCD and transferred to medium containing 0.3 mCi/ml [32P]ATP for an additional 24 h at 17°C. Then, the oocytes were lysed in 50 mM Tris–HCl, 190 mM NaCl, 6 mM EDTA, 1% Triton X-100, and protease inhibitors. myc-Dsh was immunoprecipitated with 9E10, and the immunoprecipitates were subjected to SDS–PAGE and autoradiography. For the Dsh mobility-shift assay, oocytes were coinjected with 20 ng myc-Dsh and 5 ng CKI mRNA, incubated for 20 h at 15.5°C, lysed, immunoprecipitated, transferred, and probed with 9E10 antibody.

In vitro. Xdsh fragment 2 (amino acids 231–531) was cloned into the bacterial expression vector pET15b, produced in Escherichia coli, and purified with nickel beads. Purified fragment 2 was incubated with 1000 units of purified CKI (New England Biolabs) in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 200 μM ATP, 2 μCi [γ-32P]ATP at 18°C for the indicated periods of time, and phosphorylation evaluated by SDS–PAGE and autoradiography.

Cell culture. For the CKI upshift of Dsh, 2 × 10⁵ NIH-3T3 cells were seeded in 6-well dishes, grown to near confluency, and transfected (Lipofectamine) with 1.2 μg DNA total [0.6 μg of Dsh-myc (or Xdsh-myc) + 0.6 μg CKIε (or pCS2+)]. For the blockade of endogenous Dsh phosphorylation, 2 × 10⁵ cells were seeded in 6-well dishes. The next day, 600 μM CKI-7 (U.S. Biologics) or ethanol carrier was added for 1 h. Then, 100 μl Wnt3-A-conditioned media (Shibamoto et al., 1998) or control-CM was added to the appropriate wells and cells were incubated for an additional 3 h. For Dsh upshift assays, cells were lysed in 1% Triton X-100, 50 mM Tris—HCl, pH 8.0, and 150 mM NaCl with protease inhibitors, while, in the β-catenin mobility assay, the cells were lysed hypotonically (Lee et al., 1999). Then, the samples were subjected to SDS–PAGE, transferred to nitrocellulose, and probed with the appropriate antibodies: anti-myc (9E10), anti-β-catenin mAb (Transduction Labs), anti-CKIε mAb (Transduction Labs), anti-Grb2 mAb (Transduction Labs), and anti-Dvl1 antibody (gift of Karl Willert).

RESULTS

CKI Isoforms Activate Wnt Signaling

The CKI family includes several different isoforms reported to have roles in various cell processes (Hoekstra et al., 1991; Price et al., 1998; Santos et al., 1996; Zhu et al., 1998). Recently, CKIε was identified as a positive component of the Wnt-signaling pathway (Peters et al., 1999; Sakanaka et al., 1999). We wanted to determine whether the other CKI isoforms (Fig. 1A) could also activate the canonical Wnt-signaling pathway. When Xenopus embryos...
are irradiated with ultraviolet (UV) light during the first cell cycle, dorsal axis formation is blocked and the embryos develop as ventralized balls of tissue (Fig. 1B) (Scharf and Gerhart, 1983). Positive regulators of the Wnt pathway, including CKIε, rescue this ventralized phenotype (Fig. 1B) (Peters et al., 1999; Smith and Harland, 1991). To determine whether the other CKI isoforms could also rescue the UV phenotype, we irradiated embryos and microinjected the various CKI isoforms into one vegetal blastomere at the eight-cell stage. Doses for α, δ, ε, and εCD were empirically chosen for optimal rescue; for β, γ3, and γ3CD, the maximum nontoxic dose was used. As a negative control, we expressed the inactive CKIε(K-R) mutant, and it did not generate any dorsal axes (Figs. 1B and 1C). In contrast, all of the wild-type CKI isoforms rescued UV-irradiated embryos (Figs. 1B and 1C). In this assay, one can quantitate the degree of rescue with the dorso-anterior index (DAI), in which normal embryos are a 5 and completely ventralized embryos are a 0 (Kao et al., 1986). Based on DAI results, all the isoforms rescued UV-irradiated embryos. Of note, the γ3 isofrom, which is the most divergent in primary structure (Figs. 1A and 1C) (Gross and Anderson, 1998), produced the lowest degree of rescue (lowest DAI). Similar results were obtained with the γ2 isofrom (data not shown). Elimination of the carboxyl-terminal tail is thought to increase the catalytic activity of CKI family members (Cegielska et al., 1998; Graves and Roach, 1995). So, we attempted to increase the activity of the γ3 isofrom by generating a isoform that contained only the catalytic domain (CD). We generated a similar form of CKIε as a control. In both cases, the isolated catalytic domains produced slightly better rescues than the wild-type versions (Figs. 1B and 1C).

As another test of the ability of the CKI isoforms to activate the Wnt pathway, we turned to the second-axis assay. Positively acting components of the Wnt pathway, including Wnt, Frizzled, Dsh, CKIε, β-catenin, and XTcf-3/LEF-1, induce formation of a second dorsal axis (Behrens et al., 1996; Deardoff et al., 1998; Funayama et al., 1995; Molenaar et al., 1996; Peters et al., 1999; Sakanaka et al., 1999; Smith and Harland, 1991; Sokol et al., 1995). mRNA encoding the various isoforms was microinjected into one ventral vegetal blastomere at the eight-cell stage, and the embryos were then scored for formation of second axes by using the second axis index (SAI) (Peters et al., 1999). In this scale, complete second axes are scored as a 2, partial axes are a 1, and no second axes are a 0. Uninjected embryos and embryos injected with the inactive CKIε(K-R) mutant had an SAI of 0 (Fig. 1D). In contrast, all the CKI isoforms generated second axes and, with the exception of γ, were equally effective (Fig. 1D). Again, γ3 and γ2 (data not shown) produced the least degree of axis formation and were slightly activated when the isolated kinase domain was expressed (Fig. 1D).

Wnt transduces its signal by generating a complex between β-catenin and the transcription factor XTcf/LEF-1, which binds directly to the promoter of certain genes, such as Xnr-3 and Siamois, and activates their transcription 

![FIG. 4.](Image 360x439 to 510x709)

FIG. 4. The Wnt-dependent phosphorylation of endogenous Dsh requires CKI. (A) Western blot of total cell lysates from NIH-3T3 cells probed with an anti-β-catenin monoclonal antibody. Wnt3a-conditioned media induced β-catenin stabilization in NIH-3T3 cells. This stabilization was decreased by CKI-7, a CKI inhibitor. Grb2 serves as a loading control. (B) Wnt3a-conditioned media was added to NIH-3T3 cells to induce the phosphorylation of endogenous Dsh, assessed by mobility shift with an anti-Dsh antibody. The Dsh phosphorylation was blocked by CKI-7. Results of two independent experiments are shown.
CKI Binds to the C Terminus of Axin

A previous report demonstrated that CKI binds communoprecipitated with Axin and suggested that the C-terminal domain of CKI was required for this interaction (Sakanaka et al., 1999). The C-terminal tail of CKI was also proposed to be required for Wnt signaling (Sakanaka et al., 1999). However, we found that forms of CKI—CKIα, CKIβ, and CKIγ—lacking the C-terminal tail were able to rescue UV embryos, generate second axes, and induce the expression of Wnt-specific targets (Fig. 1). The incongruity between the Xenopus activity and the communoprecipitations might suggest that the interaction with Axin is not physiologically relevant. We therefore tested the requirement of the C-terminal tail of CKI for binding to Axin. Using a directed yeast two-hybrid assay, we expressed CKIα, the C-terminal tail of CKIα, and the catalytic domain of CKIα (CKIγCD) and quantitated binding to Axin with a liquid β-galactosidase assay (Fig. 2A) (Gietz et al., 1997). We found that, while the kinase domain alone bound strongly to Axin, the C-terminal tail did not (Fig. 2A). The C-terminal tail of CKIα is therefore neither sufficient nor necessary for binding to Axin in yeast.

To delineate the region of Axin that bound to CKI, we expressed different fragments of Axin in yeast with CKIα as bait. CKIα did not bind fragments of Axin encoding amino acids 1–420 or 819 to the stop (Fig. 2B). However, CKIα did bind to portions of Axin that encompass amino acids 653–839 (Fig. 2B). This corresponds to a region of Axin required for the activation of the JNK cascade (Zhang et al., 1999).

CKI Directly Phosphorylates Dsh

Wnt signaling induces the phosphorylation of Dsh in intact cells, and CKI increases the phosphorylation of Dsh in Xenopus oocytes (Lee et al., 1999; Peters et al., 1999; Yanagawa et al., 1995). To extend our analysis of the CKI isoforms, we tested whether all the isoforms could induce this posttranslational modification. To that end, we injected Xenopus oocytes with mRNA encoding the various CKI isoforms, and we tested whether all the isoforms could induce this posttranslational modification. To that end, we injected Xenopus oocytes with mRNA encoding the several fragments of Axin in yeast with CKIα as bait. CKIα did not bind fragments of Axin encoding amino acids 1–420 or 819 to the stop (Fig. 2B). However, CKIα did bind to portions of Axin that encompass amino acids 653–839 (Fig. 2B). This corresponds to a region of Axin required for the activation of the JNK cascade (Zhang et al., 1999).

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Western blot analysis of Wnt signaling showed that addition of Wnt increased the phosphorylation and decreased the mobility of Dsh (Lee et al., 1999; Yanagawa et al., 1995). As these amino acids are necessary and sufficient for CKI binding (Peters et al., 1999), it seemed plausible that CKI was involved in the Wnt-dependent phosphorylation of Dsh observed in intact cells. If so, one would predict that CKI should increase the phosphorylation of wild-type Dsh in cell lines, but should not alter the migration of Xdd1. To evaluate this, we transfected 293 cells with Dsh or Xdd1 in the presence or absence of CKI and evaluated the mobility of the Dsh constructs with Western blots. CKI upshifted Dsh but not Xdd1 in the presence of CKI (Fig. 3C) (Yanagawa et al., 1995).

In Xenopus oocytes, CKI phosphorylates a central portion of Dsh termed Dsh fragment 2 (Peters et al., 1999). To determine whether CKI directly phosphorylates Dsh, we performed an in vitro phosphorylation assay, testing the ability of bacterially expressed and purified CKI to phosphorylate a bacterially expressed and purified Dsh fragment 2. Control lysates, from bacteria that did not express Dsh fragment 2, were not phosphorylated by purified CKI (Fig. 3D). In contrast, CKI directly phosphorylated the middle fragment of dishevelled in a time-dependent fashion (Fig. 3D). In addition, the mobility of Dsh fragment 2 decreased upon phosphorylation, mirroring the Dsh upshifts observed in Xenopus oocytes and cell lines (Figs. 3B and 3C). This is consistent with the notion that the CKI-dependent phosphorylation of Dsh detected in vivo is direct.

CKI Is Required for the Wnt-Dependent Phosphorylation of Dsh

Previous work in a variety of cell lines showed that addition of Wnt increased the phosphorylation and decreased the mobility of Dsh (Lee et al., 1999; Yanagawa et al., 1995). As these amino acids are necessary and sufficient for CKI binding (Peters et al., 1999), it seemed plausible that CKI was involved in the Wnt-dependent phosphorylation of Dsh observed in intact cells. If so, one would predict that CKI should increase the phosphorylation of wild-type Dsh in cell lines, but should not alter the migration of Xdd1. To evaluate this, we transfected 293 cells with Dsh or Xdd1 in the presence or absence of CKI and evaluated the mobility of the Dsh constructs with Western blots. CKI upshifted Dsh but not Xdd1 in the presence of CKI (Fig. 3C) (Yanagawa et al., 1995).

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β-catenin levels evaluated by Western blots probed with an anti-β-catenin monoclonal antibody. In cells treated with control conditioned media or with CKI-7 alone, no stabilization of β-catenin was observed (Fig. 4A). Wnt3a-conditioned media markedly increased β-catenin levels, and this stabilization was decreased by CKI-7 (Fig. 4A). A Western blot of these same samples probed with a monoclonal antibody against Grb2 showed that roughly equal levels of protein were present in all samples. These data demonstrate that the Wnt3a-conditioned media activates the Wnt cascade and that CKI-7 can block CKI function in intact cells. To determine whether CKI was necessary for the Wnt-induced phosphorylation of Dsh, we added control-conditioned media and Wnt3a-conditioned media with or without CKI-7 to NIH-3T3 cells and evaluated Dsh phosphorylation. While the Wnt3a mediated induced phosphorylation and upshift of Dsh, addition of CKI-7 blocked this effect (Fig. 4B). We performed the identical assay in another cell line, C57MG, with equivalent results (not shown). Taken together, these data demonstrate that CKI is required for the Wnt-dependent phosphorylation of Dsh.

DISCUSSION

CKIε is a recently identified component of the β-catenin-independent Wnt cascade (Peters et al., 1999; Sakanaaka et al., 1999). In Xenopus, CKIε induces second axes, rescues UV-treated embryos, and induces expression of Wnt-signaling markers (Peters et al., 1999; Sakanaaka et al., 1999). In cell lines, CKIε activates transcription of a Lef1 reporter gene, and depletion of CKIε abolishes Wnt-1-induced Lef-1 reporter activity (Sakanaaka et al., 1999). These data demonstrate a role for CKIε as a positive component of the Wnt pathway. In addition to CKIε, other CKI isoforms exist, including α, β, γ, and δ. While these isoforms differ due to variable amino- and carboxy-terminal extensions, all the isoforms contain a highly conserved kinase domain, suggesting a similar substrate specificity (Gross and Anderson, 1998). Previous studies have shown various roles for these isoforms, including DNA repair, circadian rhythm in flies and Drosophila, and a role in the vertebrate Wnt signaling process (Sakanaka et al., 1999). These data demonstrate that many isoforms of the CKI family can activate the Wnt signaling pathway.

How many isoforms of CKI participate in Wnt signaling endogenously? Previous reports demonstrate that CKIε is a required component of the vertebrate Wnt pathway (Peters et al., 1999). The sufficiency tests presented here demonstrate that many isoforms of the CKI family can activate the Wnt pathway. C. elegans also contains several isoforms of CKI. However, loss-of-function of one isoform, kin-19, which is most similar to the vertebrate α, produced a Wnt mutant phenotype (Peters et al., 1999). This suggests that the α isoforms also transduce Wnt signals. Loss-of-function of another worm CKI isoform, kin-20, which is most similar to mammalian ε, generates a distinct phenotype that is also produced by loss-of Wnt signals (J. Kimble, personal communication). Of note, when KIN-20 is expressed in frogs, it activates the Wnt pathway (data not shown). Epistasis tests placed CKI downstream of Dsh, and phosphorylation of Dsh may only be maximal with upstream activation. Formation of second axes, UV rescue, and transcriptional activation, however, may only require coupling to the downstream components. Another possible explanation is that the dose of γ required to activate Wnt in embryos is four times greater than any other isoform, yet this increased dose only produces a weak effect. For technical reasons, we only injected equal amounts of mRNA encoding all the isoforms for the oocyte phosphorylations. This dose of γ may therefore have been too low to observe Dsh phosphorylation in this assay.

It was previously reported that CKIα and a form of CKIε lacking the tail did not generate second axes (Sakanaaka et al., 1999). In addition, forms of CKI that lacked the C-terminal tail did not interact with Axin in coimmunoprecipitations. Based on these data, we concluded that the C-terminal tail was critical for activation of the Wnt pathway and that interaction with Axin was key to that signaling process (Sakanaaka et al., 1999). However, biochemical studies demonstrated that forms of CKI without the tail are activated (Cegielska et al., 1998; Graves and Roach, 1995). Consistent with that, our data demonstrate a strong activation of the Wnt pathway with forms of CKI—α, β, and CKIεCD—that lack the C-terminal tail. These data are reproducible in several distinct assays, including second axis formation, UV rescue, and induction of Wnt-specific targets. Furthermore, forms of CKIε and CKIγ3 that lack the tail (CKIεCD, CKIγ3CD) are slightly more active in the Wnt pathway than the wild-type enzymes. We also found that the tail of CKIε is neither required nor sufficient for binding to Axin in yeast and that the isolated CKIε kinase domain interacted strongly with Axin. We cannot reconcile our data with the previous report. However, in support of our data, Xenopus CKIα was recently isolated in an expression-cloning screen based on its ability to induce dorsal axes (Grammer et al., 2000). In addition, necessity tests suggest that CKIα is required in worms to transduce Wnt signals at least for an early cell polarity decision (Peters et al., 1999). Therefore, we conclude that the C-terminal tail of CKI is neither required for binding to Axin nor for activation of the Wnt pathway.
shown). This is consistent with the notion that distinct CKI isoforms function in the Wnt pathway, but may do so in a temporally and spatially regulated manner. Consistent with that notion, frog embryos contain at least four CKI isoforms (α, δ, ε, γ), and, although not yet studied in depth, they appear to have distinct expression patterns.

When Wnt binds to its receptor, Dsh becomes phosphorylated (Lee et al., 1999; Yanagawa et al., 1995). CKI binds to the region of Dsh that is required for this phosphorylation and for the subsequent activation of downstream components of the Wnt cascade (Peters et al., 1999; Sokol, 1996; Yanagawa et al., 1995). This suggests that CKI may be involved in both of these processes. In support of that, we previously demonstrated that CKI was required for Wnt signaling and that CKI increased Dsh phosphorylation in Xenopus oocytes (Peters et al., 1999). Here, we show that CKI directly phosphorylates Dsh in vitro and is required for the Wnt-dependent phosphorylation of Dsh in intact cells. Taken together, these data are consistent with a model that Wnt leads to the activation of CKI, which, in turn, phosphorylates Dsh. One observation that does not seem to fit with this model is that epistasis studies place CKI downstream of Dsh (Peters et al., 1999). These epistasis studies could be misleading as they were not done with true loss-of-function mutants. In addition, CKI, Dsh, and several other molecules in the Wnt pathway function in a large multiprotein complex, which could complicate epistatic analyses (Li et al., 1999; Sakanaka et al., 1999). However, biochemical studies support the placement of CKI downstream of Dsh; CKI directly binds to Axin, which functions downstream of Dsh in the Wnt cascade (Sakanaka et al., 1999). CKI could function downstream of Dsh in turn phosphorylate Dsh. For example, CKI and Dsh might associate in the absence of Wnt signaling. Upon ligand binding, Dsh could activate CKI, and the activated form of CKI could in turn phosphorylate Dsh. This phosphorylation might then disrupt the interaction of Dsh and CKI, releasing CKI to activate downstream components of the pathway. Alternatively, the phosphorylation of Dsh might simply be a marker of activation of CKI and not a requirement for Wnt pathway function. Indeed, while Rothbacher et al. have shown that Dsh phosphorylation is significantly higher on the dorsal side of the embryo during the onset of dorsalizing events, they also conclude that phosphorylation of Dsh is not required for second-axis formation (Rothbacher et al., 2000). This conclusion is based on deletion analysis involving large deletions of Dsh. Mapping and mutagenesis of specific Dsh phosphorylation sites coupled with functional analysis may be required to clarify the role of the Wnt/CKI-dependent phosphorylation of Dsh.

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