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The Casein Kinase I Family in Wnt Signaling

Renée M. McKay,[1] John M. Peters,[1] and Jonathan M. Graff[2]

Center for Developmental Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9133

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*. © 2001 Academic Press

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; Sakanaka *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; Sakanaka *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; Sakanaka *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; Sakanaka *et al.*, 1999). By epistasis analysis, CKIε was

[1] R.M.M. and J.M.P. contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: (214) 648-1196. E-mail: graff02@swvx12.swmed.edu.

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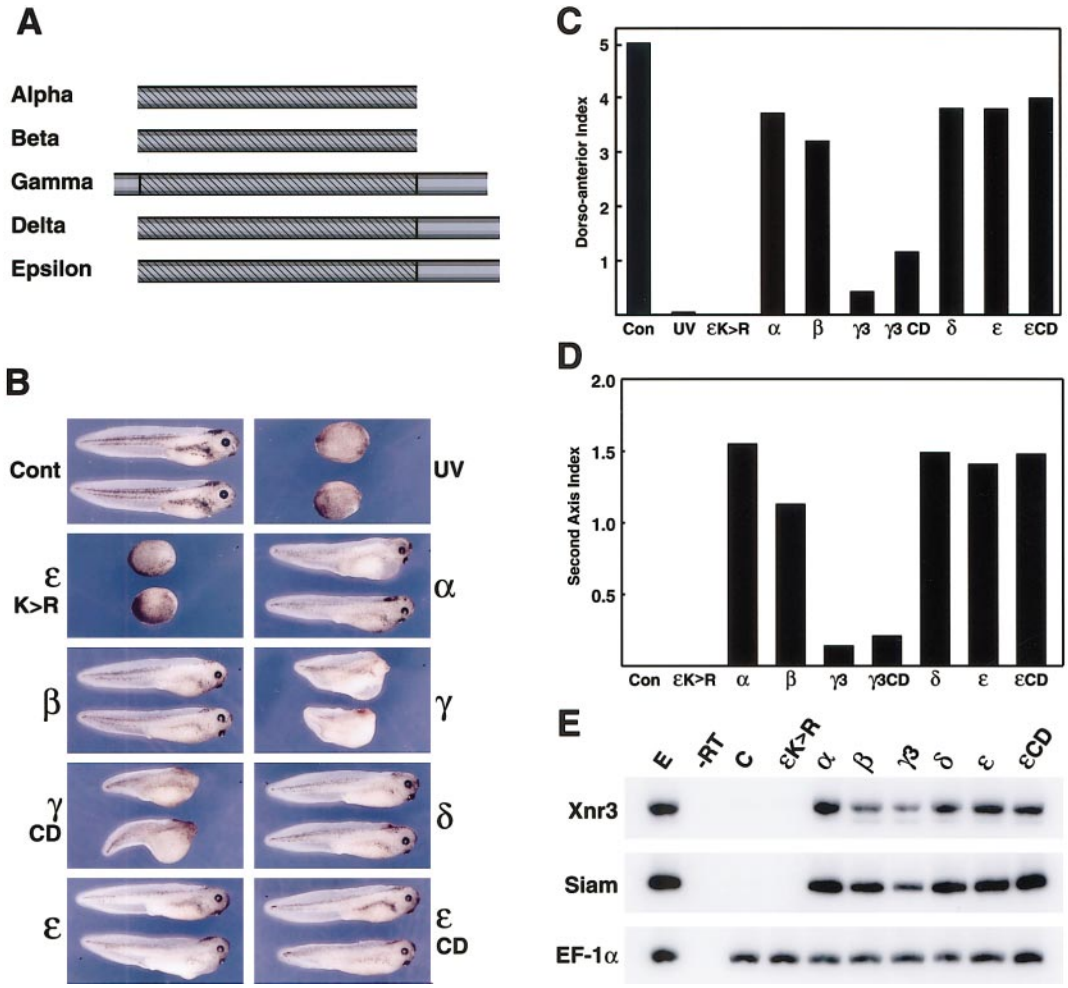

FIG. 1. The CKI isoforms activate the Wnt cascade. (A) Schematic of CKI isoforms; the catalytic kinase domains are hatched. (B, C) One-cell embryos were UV-irradiated, injected at the eight-cell stage, and tadpoles were photographed or scored with the dorso-anterior index (Kao and Elinson, 1988). Doses for α (175 pg), δ (350 pg), ϵ (350 pg), and ϵCD (catalytic domain, 175 pg) were chosen for optimal rescue; for β (25 pg), γ3 (1.4 ng), and γ3 CD (1.4 ng), 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 CKIϵ(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).

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 CKIϵ interacts with Dsh as well as Axin (Peters *et al.*, 1999; Sakanaka *et al.*, 1999). Wnt signaling, in cell lines, and CKIϵ, 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 CKIϵ binding (Peters *et al.*, 1999), suggesting that CKIϵ may be involved in the Wnt-dependent phosphorylation of Dsh.

In addition to CKIϵ, 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

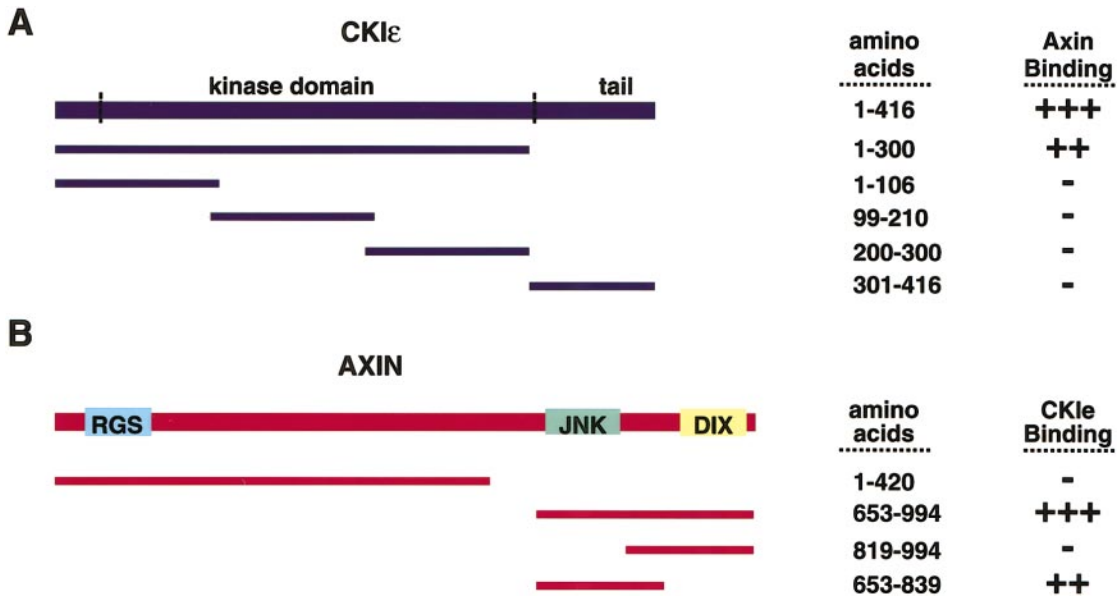

FIG. 2. CKI and Axin directly interact in yeast. (A) The C-terminal tail of CKI is neither necessary nor sufficient for binding to Axin. Various portions of CKIε were analyzed for the ability to associate with Axin in yeast. Relative strength of binding was assessed by a quantitative liquid culture β-galactosidase assay (Gietz *et al.*, 1997). (B) Full-length CKIε was tested for binding to various fragments of Axin.

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 CKIε 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 rescues, 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 εCD 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).

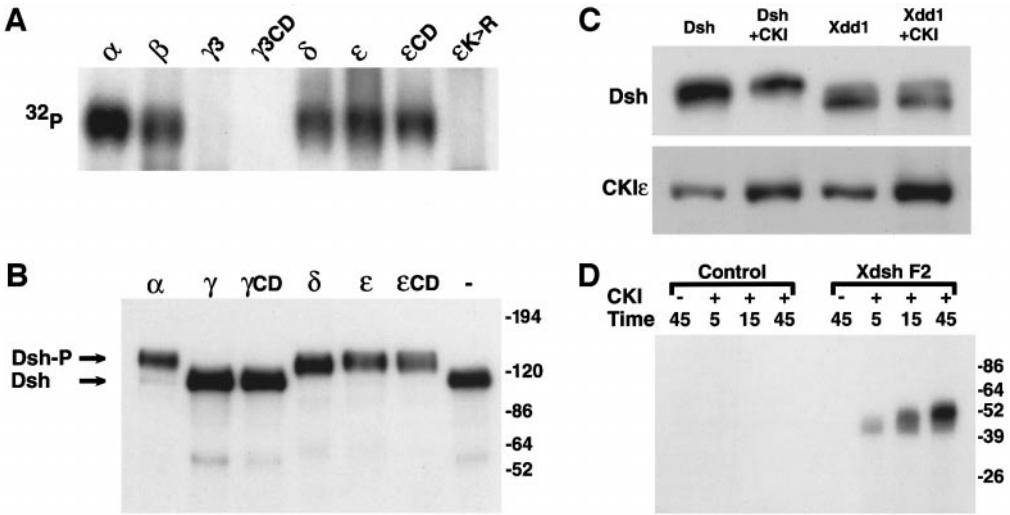

FIG. 3. CKI directly phosphorylates Dsh. (A) CKI isoforms α, β, δ, ϵ, or ϵCD increase Dsh phosphorylation. *Xenopus* oocytes were injected with the indicated CKI isoforms and myc-tagged Dsh, incubated in medium containing $^{32}P_i$, lysed, and immunoprecipitated with an antibody directed against the myc epitope. The immunoprecipitates were subjected to SDS–PAGE and autoradiography. (B) Expression of the CKI isoforms α, δ, ϵ, or ϵCD in oocytes decreased the electrophoretic mobility of Dsh. (C) CKIϵ decreases the mobility of Dsh but not Xdd1. NIH-3T3 cells were transfected with the indicated transcripts, and the mobility of Dsh or Xdd1 was assessed with Western blots. (D) CKI directly phosphorylates Dsh fragment 2 *in vitro*. Purified CKI and Dsh fragment 2 were incubated in the presence of [^{32}P]ATP and phosphorylation of Dsh fragment 2 assessed with autoradiography.

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, CKIγ3, CKIγ3CD, CKIδ, CKIϵ, or CKIϵCD and transferred to medium containing 0.3 mCi/ml $^{32}P_i$ 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 $MgCl_2$, 5mM DTT, 200 μM ATP, 2 μCi [γ-^{32}P]ATP at 18°C for the indicated periods of time, and phosphorylation evaluated by SDS–PAGE and autoradiography.

Cell culture. For the CKIϵ upshift of Dsh, 2×10^5 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 Xdd1-myc) + 0.6 μg CKIϵ (or pCS2+)]. For the blockade of endogenous Dsh phosphorylation, 2×10^5 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).

Yeast Two-Hybrid and Liquid β-Galactosidase Assays

The yeast two-hybrid assays were performed with standard methods (Chien *et al.*, 1991). CKIϵ fragments (1–300, 1–106, 99–210, 200–300, and 301–416) were cloned into pBTM116, and axin fragments (1–420, 653–994, 653–839, and 819–994) were cloned into pACT2. To measure binding, a semiquantitative liquid β-galactosidase assay was performed (Gietz *et al.*, 1997).

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 isoform, 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 isoform (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 isoform by generating a form 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; Deardorff *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

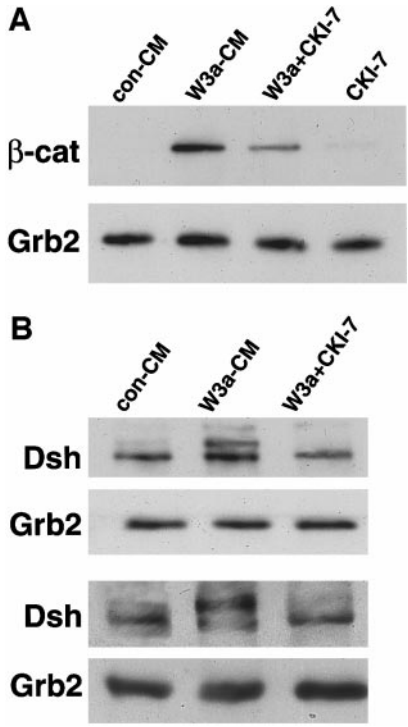

FIG. 4. The Wnt-dependent phosphorylation of endogenous Dsh requires CKI. (A) Western blot of total cell lysates from NIH-3T3 cells probed with an anti-β-catenin monoclonal antibody. Wnt3a-conditioned media induced β-catenin stabilization in NIH-3T3 cells. This stabilization was decreased by CKI-7, a 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.

(Brannon *et al.*, 1997; McKendry *et al.*, 1997). If the CKI isoforms activate the Wnt pathway, then they should induce the expression of these Wnt-specific markers. To evaluate this, we microinjected mRNA encoding the various isoforms and the inactive CKIε(K>R) into embryos, explanted animal caps, and analyzed Siamois and Xnr-3 expression. Control animal caps and caps expressing the CKIε(K>R) mutant did not induce Xnr-3 or Siamois (Fig. 1E). In contrast, all isoforms tested induced the expression of both Wnt-specific markers (Fig. 1E). This figure represents the highest level of activation of Xnr-3 and Siamois by the γ3 isoform, which, in general, produces the lowest level of gene induction in this assay, similar to what we observed in the two whole-embryo assays. Taken together, these data demonstrate that all the CKI isoforms can activate the Wnt pathway in *Xenopus* embryos. Although the γ isoforms were injected at a relatively high mRNA concentration and produced the least robust phenotype, it is plausible that this is due to low levels of protein expression.

CKI Binds to the C Terminus of Axin

A previous report demonstrated that CKIε coimmunoprecipitated 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—CKIεCD, CKIα, CKIβ, and CKIγCD—that lack 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 coimmunoprecipitations might suggest that the interaction with Axin is not physiologically relevant. We therefore tested the requirement of the C-terminal tail of CKIε for binding to Axin. Using a directed yeast two-hybrid assay, we expressed CKIε, the C-terminal tail of CKIε, and the CKIε catalytic domain lacking the tail (CKIεCD) 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 CKIε is therefore neither sufficient nor necessary for binding to Axin in yeast.

To delineate the region of Axin that bound to CKI, we expressed several different fragments of Axin in yeast with CKIε as bait. CKIε did not bind fragments of Axin encoding amino acids 1–420 or 819 to the stop (Fig. 2B). However, CKIε 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 CKIε 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 coinjected *Xenopus* oocytes with mRNA encoding the various CKI isoforms, the isolated catalytic domains of γ3 and ε, or the catalytically inactive CKIε(K>R) as a negative control, along with myc-tagged Dsh. We then incubated the oocytes in $^{32}P_i$, lysed them, immunoprecipitated Dsh with a monoclonal antibody directed against the myc-tag, and subjected the precipitates to SDS–PAGE. Autoradiography demonstrated that α, β, δ, ε, and the εCD increased Dsh phosphorylation (Fig. 3A). The exception was the γ3 isoform, which, as in the functional assays, produced the lowest response.

Wnt induces the phosphorylation of Dsh *in vivo*, and the phosphorylated form of Dsh migrates with a lower mobility on SDS–PAGE (Lee *et al.*, 1999; Yanagawa *et al.*, 1995). If CKI is responsible for the Wnt-dependent phosphorylation of Dsh, then CKI should also upshift Dsh. To evaluate this, we coexpressed myc-Dsh with the CKI isoforms in *Xenopus* oocytes, lysed the oocytes, separated the proteins with SDS–PAGE, and evaluated the migration of Dsh with a Western blot. α, β (not shown), δ, ε, and the ε kinase domain decreased the mobility of Dsh; however, the γ3 isoform did not (Fig. 3B). This reproduces the results observed with incorporation of radioactive phosphate (Fig. 3A).

The Wnt-dependent phosphorylation and upshift of Dsh had previously been demonstrated in cell lines rather than *Xenopus* oocytes (Lee *et al.*, 1999; Yanagawa *et al.*, 1995). This phosphorylation and upshift requires the presence of a small stretch of about 80 amino acids that are present in the middle of Dsh and are specifically deleted in the construct Xdd1 (Sokol, 1996; Yanagawa *et al.*, 1995). As these 80 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 vivo*. 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, mimicking Wnt (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 CKI increases the phosphorylation of Dsh *in vivo*, directly phosphorylates Dsh *in vitro*, phosphorylates the appropriate central region of Dsh, and upshifts Dsh, we sought to determine whether CKI was required for the Wnt-dependent phosphorylation of Dsh observed in intact cells. Previous studies demonstrated that CKI-7, a CKI inhibitor, blocked Wnt-dependent responses in *Xenopus* embryos (Peters *et al.*, 1999). To test whether CKI-7 could also block Wnt function in intact cells, we assessed the ability of CKI-7 to decrease the Wnt-induced stabilization of β-catenin. To that end, NIH-3T3 cells were incubated with conditioned media from control cells, Wnt3a-conditioned media (Shibamoto *et al.*, 1998), or Wnt3a-conditioned media plus CKI-7. The cells were then lysed and

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 media 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-dependent Wnt cascade (Peters *et al.*, 1999; Sakanaka *et al.*, 1999). In *Xenopus*, CKIε induces second axes, rescues UV-treated embryos, and induces expression of Wnt-signaling markers (Peters *et al.*, 1999; Sakanaka *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 (Sakanaka *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 rats, and cell budding in yeast (Gross *et al.*, 1997; Kloss *et al.*, 1998; Santos *et al.*, 1996).

In this study, we have begun to characterize the role and the mechanism of action of CKI in Wnt signaling. We find that all of the CKI isoforms activated the canonical Wnt pathway in *Xenopus* embryos. In addition, expression of each of the isoforms, with the exception of γ, led to Dsh phosphorylation in *Xenopus* oocytes. The lack of Dsh phosphorylation by γ, coupled with its ability to activate the Wnt pathway, could be interpreted to suggest that Dsh phosphorylation is not required for Wnt signaling. However, several other explanations are also possible. For example, measuring Dsh phosphorylation in oocytes and Wnt signaling in embryos may not reflect the same events. Consistent with that, preliminary studies indicate that the Wnt pathway cannot be activated in oocytes as measured by the lack of β-catenin stabilization and activation of transcriptional reporters (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 (Sakanaka *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, it was concluded that the C-terminal tail was critical for activation of the Wnt pathway and that the interaction with Axin was key to that signaling process (Sakanaka *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.

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). 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 yet still 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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