Casein kinase Iε in the Wnt pathway: Regulation of β-catenin function

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Wnt and its intracellular effector β-catenin regulate developmental and oncogenic processes. Using expression cloning to identify novel components of the Wnt pathway, we isolated casein kinase Iε (CKIε). CKIε mimicked Wnt in inducing a secondary axis in *Xenopus*, stabilizing β-catenin, and stimulating gene transcription in cells. Inhibition of endogenous CKIε by kinase-defective CKIε or CKIε antisense-oligonucleotides attenuated Wnt signaling. CKIε was in a complex with axin and other downstream components of the Wnt pathway, including Dishevelled. CKIε appears to be a positive regulator of the pathway and a link between upstream signals and the complexes that regulate β-catenin.

Wnt regulates developmental and oncogenic processes through its downstream effector, β-catenin (1–3). Intracellular protein complexes, including Dishevelled (Dvl/Dsh), glycogen synthase kinase-3β (GSK-3β), axin and adenomatous polyposis coli (APC) protein, regulate cytosolic β-catenin protein levels. However, little is known about how Wnt or other upstream stimuli regulate these complexes. Most components of the Wnt pathway have been found by genetic approaches in *Drosophila*. Mutations in several molecules in the wingless (Wg, the *Drosophila* homologue of Wnt) pathway, such as Dvl/Dsh, β-catenin, and lymphoid enhancer factor-1 (Lef-1)/T cell factor (Tcf), caused segment polarity phenotypes in *Drosophila* similar to the Wg phenotype, suggesting that these molecules are positive regulators of the pathway (4, 5). Genetic studies in *Drosophila* also revealed that GSK-3β is a negative regulator of this pathway. GSK-3β is in a complex containing other negative regulators, axin and APC protein, and a positive regulator, β-catenin (6–10). β-catenin is an extensively studied effector in the pathway and has a pivotal role in both developmental processes and oncogenesis (2, 3). Upon Wnt stimulation, β-catenin protein is stabilized and moves to the nucleus where it forms a complex with and activates Lef-1/Tcf transcription factors (11, 12). Mutated forms of β-catenin appear to be involved in cancer and induce Lef-1/Tcf-dependent transcription even in the absence of Wnt stimulation (13, 14). The molecular mechanism by which Wnt regulates β-catenin is not yet fully understood. Here we show that casein kinase I (CKI)ε is an important regulator of β-catenin in the Wnt pathway and is a component of these complexes. CKIε mimicked Wnt in inducing a secondary axis in *Xenopus*, stabilizing β-catenin, and stimulating β-catenin-dependent gene transcription. Inhibition of endogenous CKIε by the kinase-defective form of CKIε (KN-CKIε) or antisense-oligonucleotide attenuated gene transcription stimulated by Wnt. CKIε was found in a complex with axin and other downstream components of the Wnt pathway, including Dvl. We propose that CKIε is a positive regulator of the Wnt pathway and is a possible functional link between upstream signals and the intracellular axin signaling complex that regulates β-catenin.

Materials and Methods

Plasmids. Human CKIδ cDNA was a gift from J. Kusuda (National Institute of Infectious Diseases, Tokyo). Human CKIα cDNA was isolated by PCR. Lysine-38 in mouse CKIε was mutated to phenylalanine to make KN-CKIε as described (15). CKIε, KN-CKIε, and ΔC-CKIε were constructed in pCS2+ vector (16). CKIδ and CKIα were constructed in pcDNA3.1 vector (Invitrogen). Myc-tagged Axin construct has been described (10). Myc-taggd Dv13 was from D. Yan (Chiron).

Library Screening. E14 mouse embryonic cDNA library [oligo(dT)-primed] was constructed in pCS2+ vector (16). Pools of RNA derived from the library were injected into the four-cell stage of the *Xenopus* embryos at the ventral side. A total of 6×10^5 independent clones were screened. Each pool for injection contains 25–50 clones.

***Xenopus* Experiments.** mRNAs were synthesized by using a mMESSAGE mMACHINE kit (Ambion, Austin, TX). The RNA samples (1–5 ng) were injected into the ventral side of the four-cell stage blastomeres. Embryos with secondary axis structure were counted 48–72 h after injection. β-catenin or XWnt-8 RNA was injected as a positive control (17, 18).

Reverse Transcription–PCR (RT-PCR). mRNA for RT-PCR was prepared from ventral halves of the *Xenopus* embryos at stage 10–10 1/2. RT-PCRs and primers were as described (19).

Cell Culture, Immunoprecipitation, and Western Blotting. S2 stable cell lines were generated by transfecting CKIε and sgg under the control of methallothionein promoter with pMK33 vector that contains hygromycin-resistant gene for selection marker. S2 cells were lysed 24 h after induction by $CuSO_4$. Transfection of 293 cells and immunoprecipitation was performed as described (10). Cytosolic fraction of 293 cells were prepared from supernatant by ultracentrifugation (100,000 $g \times 30$ min) after lysis in hypotonic buffer.

Antisense Oligonucleotide Transfection. Antisense oligonucleotides against human CKIε (CK-ASa; 5′-gcggcagaagttgaggtatgttgag-3′, CK-ASb; 5′-cgtaggtaagagtagtcgggcttgt-3′) or control oligonucleotide (5′-cgccgtcttcaactccatacaactc-3′) (final concentration 100 nM) were transfected into 293 cells by using cationic peptoid reagents (20) followed by transfection with Lef-1, Lef-1 reporter, and Wnt-1 plasmids using Lipofectamine (Life Technologies, Grand Island, NY).

Results and Discussion

To find additional regulators in the Wnt pathway, we used a screen for molecules that could mimic the developmental effects of Wnt. In *Xenopus* embryos, ectopic expression of Wnt elicits formation of a secondary axis (21). We injected pools of RNA

Abbreviations: CKI, casein kinase I; GSK-3β, glycogen synthase kinase-3β; Lef-1, lymphoid enhancer factor-1; Tcf, T cell factor; RT-PCR, reverse transcription–PCR; KN-CKIε, kinase-defective form of CKIε.

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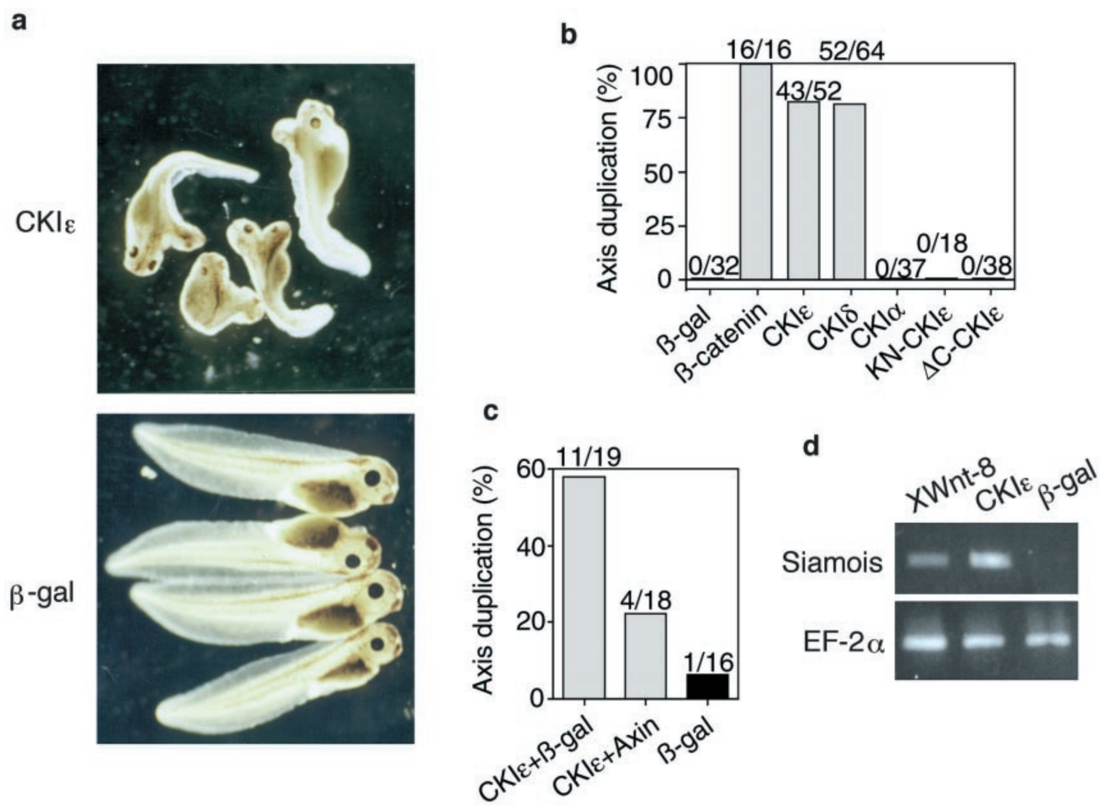

Fig. 1. CKIε/δ induces a secondary axis in *Xenopus* embryos. (*a*) Examples of the embryos injected with CKIε or β-galactosidase (β-gal) RNA. (*b*) Percentage of embryos with duplicated axis injected with β-gal, β-catenin, CKIε, CKIδ, CKIα, KN-CKIε, or ΔC-CKIε RNA as indicated. The number of the embryos with duplicated axis relative to the total number of injected embryos is indicated above each bar. (*c*) Axin inhibits secondary axis formation induced by CKIε. CKIε RNA was coinjected with β-gal or axin RNA (1–2 ng). (*d*) Induction of *Siamois* by CKIε. *Siamois* expression was analyzed by RT-PCR from dorsal halves of the embryos injected with XWnt-8, CKIε, or β-gal. EF-1 expression was a loading control.

derived from a mouse embryonic cDNA library into the ventral side of *Xenopus* embryos and searched for a gene that caused secondary axis formation. In this screen, we isolated several clones including β-catenin and Wnt-1 that have been shown previously to induce a secondary axis, validating the efficacy of this approach in discovering genes in the Wnt pathway. We also isolated a full-length cDNA for mouse CKI, which is 98.8% identical to the human CKIε isoform. The CKI gene family consists of seven different genes in mammals, CKIα, β, γ1, γ2, γ3, δ, and ε (15, 22). CKIε and δ isoforms, the most closely related, share 98% identity in the kinase domain and are 53% identical in a C-terminal domain that is not present in other CKI isoforms. This C-terminal domain appears to negatively regulate kinase activity (15, 23). CKIα is 74% identical to CKIε in the kinase domain and has no C-terminal extension. We showed that the ventral injection of CKIε RNA induced a secondary axis in *Xenopus* embryos (Fig. 1 *a* and *b*). CKIδ, like CKIε but not CKIα, induced a secondary axis when injected at the ventral side of the embryos (Fig. 1*b*). A point mutant of CKIε that was defective in kinase activity (KN-CKIε) did not induce a secondary axis (Fig. 1*b*). These data suggested that axis-inducing activity is specific for the CKIε/δ isoform and depends on its kinase activity. Because the C-terminal domain is unique for CKIε/δ isoforms, we tested whether deletion of this domain altered activity. When the truncated CKIε (ΔC-CKIε) RNA was injected into *Xenopus* embryos, we did not see any effects on axis formation (Fig. 1*b*). Both CKIα and ΔC-CKIε had kinase activity *in vitro* comparable to or greater than that of wild-type CKIε when they were expressed in mammalian cells (on a per-cell basis assessed by kinase assays of CKIε immunoprecipitates) or *in vitro*-translated (data not shown) even though they were not effective in inducing a secondary axis. Therefore, CKI kinase activity was not sufficient for mimicking Wnt. The requirement for the C-terminal domain of CKIε suggested that this part of the molecule is involved in linking CKIε to the Wnt pathway. Indeed further studies (see below) confirmed that this domain is important for the interaction of CKIε with a signaling complex.

To demonstrate that CKIε activates Wnt signaling, we coinjected CKIε with axin, which is a known inhibitor of the Wnt pathway that acts by linking β-catenin to GSK-3β (6–10, 17). Axin inhibited the induction of a secondary axis by CKIε (Fig. 1*c*). This finding suggests that the CKIε effect is mediated through β-catenin in a manner analogous to the effects of Wnt. One of the downstream target genes of β-catenin in *Xenopus* is *Siamois* (18, 19). *Siamois* is a homeobox gene induced by Wnt and responsible for its dorsalizing activity. CKIε overexpression at the ventral side of *Xenopus* embryo also induced *Siamois* expression detected by RT-PCR (Fig. 1*d* and ref. 19). Our observations in the *Xenopus* experiments that CKIε mimicked Wnt, both in its gene regulation and developmental effects, suggested that CKIε might be a component of the Wnt pathway.

To understand the mechanism by which CKIε activates the Wnt pathway, we examined the effect of CKIε on β-catenin protein level. Wnt-1 stabilizes cytosolic β-catenin protein by suppressing GSK-3β (24, 25). We made *Drosophila* Schneider S2 cell lines that stably expressed CKIε controlled by a metallothionein promoter. Overexpression of CKIε caused accumulation of endogenous armadillo (arm) protein, the *Drosophila* homologue of β-catenin (Fig. 2*a*). We also showed that β-cate-

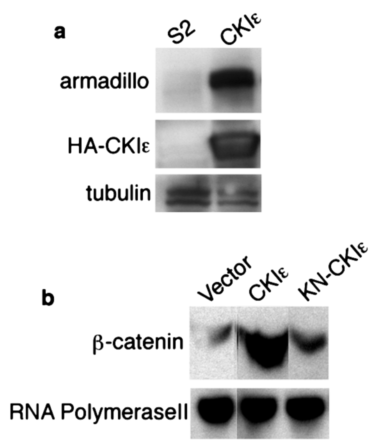

Fig. 2. β-catenin stabilization induced by CKIε. (*a*) *Drosophila* S2 Schneider cell lysates blotted with armadillo antibody and hemagglutinin (HA) antibody recognized transfected CKIε. Tubulin was a loading control. (*b*) Cytosolic fraction from 293 cells transfected with vector, Wnt-1, CKIε, and KN-CKIε blotted with β-catenin antibody. RNA polymerase II was a loading control.

nin protein level was increased by transiently overexpressing CKIε in 293 cells (Fig. 2*b*). These results suggest that CKIε activates the Wnt pathway by stabilizing β-catenin.

To further study the role of CKIε in the Wnt pathway, we measured Lef-1 reporter gene activity in mammalian cells. The transcription factor Lef-1/Tcf forms a complex with β-catenin in response to Wnt stimulation (11, 12). When expressed in 293 cells, Wnt-1 stimulated the expression of a Lef-1 reporter gene transcription 4- to 6-fold over vector transfected cells (10). CKIε and CKIδ activated the Lef-1 reporter gene about 10-fold (Fig. 3*a*). However CKIα and ΔC-CKIε did not activate the Lef-1 reporter gene (Fig. 3*a*), consistent with the *Xenopus* injection experiments (Fig. 1). Coexpressing axin inhibited the Lef-1 reporter activation induced by CKIε (Fig. 3*b*). These data confirm the results from *Xenopus* experiments, suggesting that CKIε activates the Wnt pathway through an effect on the β-catenin-Lef-1/Tcf complex.

KN-CKIε inhibited the activation of Lef-1 reporter by Wnt-1 (Fig. 3*c*), suggesting the involvement of endogenous CKIε during the Wnt signal. These data suggest that KN-CKIε acts as a dominant negative kinase and blocks the upstream signal coming from Wnt-1. To further assess the physiological importance of CKIε in the Wnt pathway, we used antisense-oligonucleotides to reduce the endogenous CKIε protein level, which resulted in the inhibition of Lef-1 reporter activity induced by Wnt-1 (Fig. 3*d*). Taken together the *Xenopus, Drosophila*, and mammalian cell experiments showed that CKIε activates the Wnt pathway and appears to be a significant positive regulatory component that is required for a full Wnt effect.

Some of the downstream molecules in the Wnt pathway have been shown to form complexes containing negative regulators (GSK-3β, axin, and adenomatous polyposis coli tumor suppressor protein) and a positive regulator (β-catenin) *in vivo* (6–10). We examined the possibility that CKIε is also in a complex with these molecules. We found that axin, a negative regulator of the

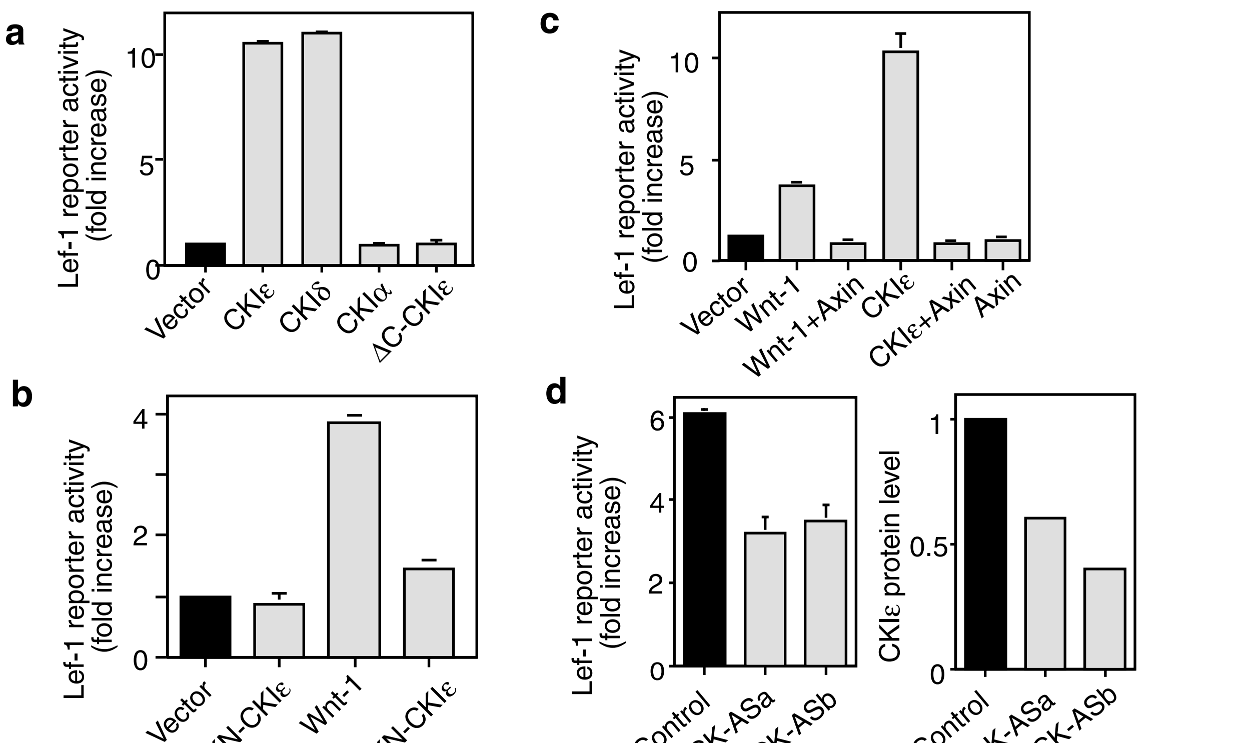

Fig. 3. Lef-1 reporter gene activity induced by CKIε. Lef-1 reporter gene assay was performed as described (10). Representative data from several independent experiments are shown. (*a*) The effects of CKI isoforms on Lef-1 activity. (*b*) Axin inhibits Lef-1 reporter gene activity induced by CKIε. (*c*) KN-CKIε blocks Lef-1 activity induced by Wnt-1. (*d*) CKIε antisense-oligonucleotides inhibit Lef-1 reporter gene activity induced by Wnt-1. (*Left*) Wnt-1 induced Lef-1 reporter activity. CKI-ASa and CKI-ASb are two different antisense-oligonucleotides from the human CKIε coding sequence. The control-oligonucleotide is the reverse sequence of ASa. (*Right*) Endogenous level of CKIε normalized to levels of 14-3-3 protein (loading control) after transfection of oligonucleotides.

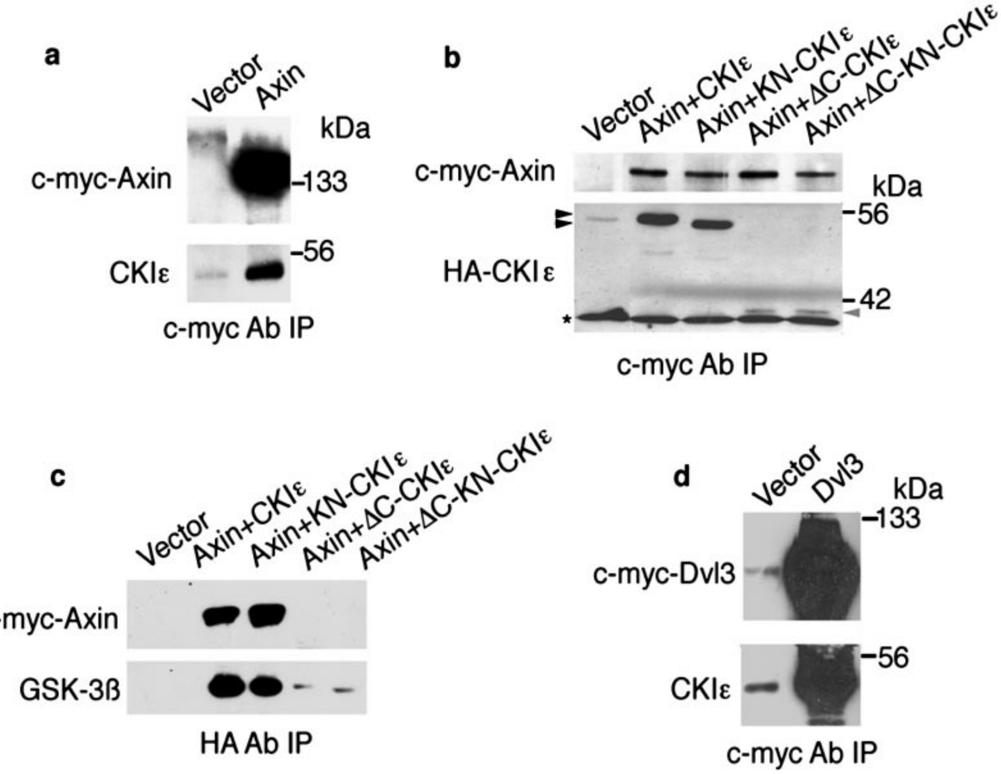

Fig. 4. CKIε forms a complex with the other molecules in the Wnt pathway. (*a*) Endogenous CKIε coimmunoprecipitated with transfected myc-tagged axin. (*b*) The C-terminus domain of CKIε is required for binding to axin. Myc-axin and hemagglutinin (HA)-CKIε constructs (indicated by arrows) were cotransfected in 293 cells. Myc-axin immune complexes were analyzed by immunoblotting with myc (detect axin) and HA (detect CKIε) antibodies. Dye front was marked as ⋆. (*c*) GSK-3β is in a complex with CKIε and axin. Myc-axin and HA-CKIε constructs (indicated) were cotransfected in 293 cells. HA-CKIε immune complexes were analyzed by immunoblotting with myc (detect axin) and GSK-3β antibodies. (*d*) Endogenous CKIε is coimmunoprecipitated with transfected myc-tagged Dvl3.

Wnt pathway, bound to CKIε. Endogenous CKIε was coimmunoprecipitated with overexpressed axin (Fig. 4*a*). Binding of axin to ΔC-CKIε and ΔC-KN-CKIε was much reduced compared to wild-type CKIε and KN-CKIε (Fig. 4*b*). These results suggest that the C-terminal domain of CKIε is important for its interaction with axin, which may be the reason that ΔC-CKIε and CKIα did not activate the Wnt pathway in *Xenopus* or mammalian cells (Figs. 1 and 3). To further study the complex of CKIε with axin and GSK-3β, we showed that GSK-3β coimmunoprecipitated with CKIε, but much less with ΔC-CKIε, and only in the presence of axin (Fig. 4*c*). This finding suggests that CKIε is a positive regulatory molecule in the Wnt pathway and its interaction through its C terminus with the axin-GSK-3β complex is likely to be important for its activity. Furthermore, we also detected endogenous CKIε in the complex with overexpressed Dvl3 (Fig. 4*d*), an upstream molecule of the axin-GSK-3β complex.

In this study, we cloned a CKIε gene by using the *Xenopus* system as an indicator of Wnt-inducing effects. Recently a CKIε gene of *Drosophila* was identified as the clock gene (26). Homozygous mutation in the *Drosophila* CKIε gene produces embryonic lethality, which also suggests the involvement of CKIε in an early process that is probably unrelated to its "clock" function. It will be interesting to examine in detail the embryonic phenotype of CKIε-defective flies related to pathway genes.

In summary we report a signaling molecule in the Wnt pathway, CKIε, that induces secondary axis formation in *Xenopus*, Lef-1-dependent transcription, and stabilization of β-catenin in both fly and mammalian cells. A critical role of CKIε in Wnt signaling is supported by several observations: inhibition of endogenous CKIε by KN-CKIε or by antisense-oligonucleotides blocked Wnt-1 effects; endogenous CKIε is present in a complex with axin, GSK-3β, and Dvl, known components of the Wnt pathway; and the C-terminal domain of CKIε is required for its interaction with the axin complex and for the ability of CKIε to mimic Wnt. It will be important to determine the substrates of CKIε in this pathway and the upstream signals between Wnt receptors and CKIε. It is also possible that stimuli other than Wnt regulate the ability of CKIε to impinge on this pathway.

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