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The Casein Kinase I Family: Roles in Morphogenesis

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Wnt signals play important roles in development and oncogenesis and are transduced through at least two pathways: a canonical β-catenin-dependent and a β-catenin-independent cascade. Casein kinase I (CKI) is required in both invertebrates and vertebrates to transduce canonical Wnt signals. However, its role in the β-catenin-independent pathway was unknown. During vertebrate embryogenesis, the β-catenin-independent cascade is thought to control cell movements and has been postulated to be analogous to the *Drosophila* planar cell polarity pathway, which signals through the JNK cascade. Here, we report that blocking CKI function inhibits embryonic morphogenesis and activates JNK in cell lines. These studies suggest that CKI might also act in the β-catenin-independent pathway and indicate a role for CKI during convergence extension in early vertebrate development. © 2001 Academic Press

Key Words: **Casein Kinase I; morphogenesis; planar cell polarity; JNK; Dishevelled; Wnt.**

INTRODUCTION

The Wnt signaling pathway is critical for diverse developmental decisions made by both invertebrates and vertebrates (Cadigan and Nusse, 1997; McMahon and Moon, 1989; Moon *et al.*, 1997; Rocheleau *et al.*, 1997). Wnt signaling also plays a key role in control of cell proliferation; mutations in components of the Wnt pathway are found in many human cancers, including skin, liver, brain, and colon cancers (Chan *et al.*, 1999; Kinzler and Vogelstein, 1996; Koch *et al.*, 1999; Morin *et al.*, 1997; Polakis, 2000; White, 1998; Zurawel *et al.*, 1998). Components of the Wnt cascade are conserved, and, through a wide variety of approaches undertaken in several organisms, a molecular framework has been established (Cadigan and Nusse, 1997; Peifer and Polakis, 2000; Sokol, 1999; Wodarz and Nusse, 1998). In the canonical Wnt pathway, signaling through the Frizzled family of seven-transmembrane receptors (Bhanot *et al.*, 1999; Xu *et al.*, 1998) and dishevelled (Dsh) (Moon *et al.*, 1997; Yanagawa *et al.*, 1995) stabilizes β-catenin. β-Catenin then binds to and activates the transcription factor LEF-1/Tcf-3, inducing the expression of downstream target genes (Behrens *et al.*, 1996; Brannon *et al.*, 1997; McKendry *et al.*, 1997; Molenaar *et al.*, 1996). Glycogen synthase kinase 3 (GSK-3), APC, and Axin are negative regulators of Wnt signaling; Apc and Axin are mutated in human cancers (Ahmed *et al.*, 1998; Groden *et al.*, 1991; Kinzler *et al.*, 1991; Rubinfeld *et al.*, 1993; Satoh *et al.*, 2000; Su *et al.*, 1993; Zeng *et al.*, 1997).

In addition to the canonical Wnt β-catenin pathway, Dsh functions in another Wnt pathway that does not involve β-catenin stabilization (Boutros and Mlodzik, 1999). In *Drosophila*, this β-catenin-independent cascade is termed the planar cell polarity pathway and it transduces its signal via some of the same molecules that function in the classical Wnt pathway, including Frizzled, Dsh, and potentially others (Boutros and Mlodzik, 1999). Downstream of Dsh, the planar cell polarity pathway diverges from the canonical Wnt/β-catenin pathway and acts through an unknown mechanism thought to involve the JNK cascade and rho/rac (Boutros *et al.*, 1998; Eaton *et al.*, 1996; Strutt *et al.*, 1997). In cell culture, Dsh can activate both the β-catenin and the JNK pathways (Boutros *et al.*, 1998; Li *et al.*, 1999). However, inhibitors of the canonical Wnt pathway, including Axin, also activate the JNK cascade (Boutros *et al.*, 1998; Zhang *et al.*, 1999).

In *Xenopus* embryos, the β-catenin-independent pathway appears to control a set of cell movements termed convergence extension that occur during gastrulation (Tada and Smith, 2000). Recent work in *Xenopus* demonstrated that

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cells blocked from undergoing convergence extension have a defect in cell polarity (Wallingford *et al.*, 2000), which suggests that planar cell polarity in flies and convergence-extension movements in frogs may be analogous processes. A role for Wnt signaling in convergence extension is indicated by embryonic expression of inhibitors or dominant-negative forms of Wnts (1, 8, 11), Frizzleds (7, 8), or Dsh, which dramatically shorten the anterior–posterior axis, producing the "bent-back" phenotype (Deardorff *et al.*, 1998; Djiane *et al.*, 2000; Hoppler *et al.*, 1996; Moon *et al.*, 1997; Sokol, 1996; Tada and Smith, 2000). As these embryos contain all tissue types, the bent-back phenotype is thought to result from an inhibition of cell movements rather than a change in cell fate. These results contrast with those obtained upon injection of the negative Wnt regulators Axin and GSK-3 or a dominant-negative form of Tcf-3; these embryos are ventralized and contain no dorsal tissues (He *et al.*, 1995; Molenaar *et al.*, 1996; Zeng *et al.*, 1997). Ventralized embryos are also observed when β-catenin is depleted from oocytes or embryos by anti-sense or morpholino technology (Heasman *et al.*, 1994, 2000). The molecular mechanism that underlies the distinct phenotypes observed when different components of the Wnt pathway are blocked has yet to be resolved.

Casein kinase I (CKI) was recently identified as a new component of the canonical Wnt pathway (Peters *et al.*, 1999; Sakanaka *et al.*, 1999). Gain-of-function studies in *Drosophila* cells, *Xenopus* embryos, and mammalian cell lines as well as loss-of-function studies in *Caenorhabditis elegans* embryos, *Xenopus* embryos, and mammalian cell lines demonstrated that CKI is a conserved component of the Wnt cascade (Peters *et al.*, 1999; Sakanaka *et al.*, 1999). Epistasis analysis placed CKI between Dsh and GSK-3 in the Wnt pathway, and, consistent with this placement, CKI was shown to interact with both Dsh and Axin in coimmunoprecipitations and yeast two-hybrid assays (Peters *et al.*, 1999; Sakanaka *et al.*, 1999). CKI therefore interacts with two proteins that have been shown to have roles in both the β-catenin and JNK pathways: Axin, a negative regulator of the canonical Wnt signaling pathway but an activator of the JNK cascade, and Dsh, a positive regulator of both pathways.

CKI is a family of closely related kinases that includes the α, β, γ, ϵ, and δ isoforms, all of which contain highly conserved kinase domains (Gross and Anderson, 1998). The isoforms are distinguished by amino and carboxyl extensions of varying lengths that flank the kinase domain. 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). Loss-of-function studies in *C. elegans* indicated a role for CKI in worm embryogenesis (Peters *et al.*, 1999); however, the endogenous role of CKI during vertebrate development has yet to be determined.

While CKI is a conserved component of the canonical Wnt pathway, several questions regarding CKI remain. What is the endogenous role of members of the CKI family in vertebrate development? Is CKIϵ required for dorsal axis formation, or is it involved in convergence-extension movements, or both? Does CKI play a role in the JNK pathway? In this report, we examine several of these questions. Our results indicate that the CKI family plays a role in convergence-extension movements during *Xenopus* embryogenesis; blocking CKI in embryos results in the bent-back phenotype. We also find that inhibiting CKI activates JNK in cell lines, suggesting a role for CKI in JNK signaling.

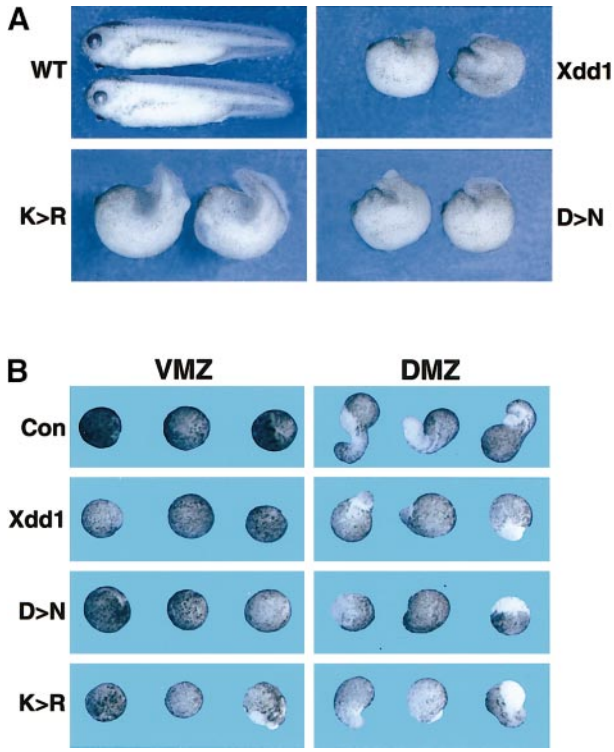

FIG. 1. Dominant-negative forms of CKIϵ block convergence extension. (A) CKIϵ(K>R) (250 pg), CKIϵ(D>N) (250 pg), and Xdd1 (1 ng) were injected into both dorsal blastomeres at the four-cell stage, and the embryos were photographed as tadpoles. (B) Dorsal marginal zones (DMZs) and ventral marginal zones (VMZs) were dissected from embryos injected with mRNAs encoding CKIϵ(K>R) (1 ng), CKIϵ(D>N) (1 ng), and Xdd1 (1 ng), incubated in 0.5× MMR, and photographed at stage 18. VMZs do not elongate. Control (Con) DMZs elongate and CKIϵ(K>R), CKIϵ(D>N), and Xdd1 inhibit those movements.

MATERIALS AND METHODS

Constructs and RNA Synthesis

The CKI isoforms [α and β: bovine; δ, γ2, γ3, γ3 KD (amino acids 36–354): human] were cloned into the plasmid pCS2+. The D>N dominant-negative forms were generated with SOEing PCR (Ho *et al.*, 1989) by converting an aspartic acid in the conserved protein kinase domain VIb to asparagine (amino acid 128 of XCKIϵ). All

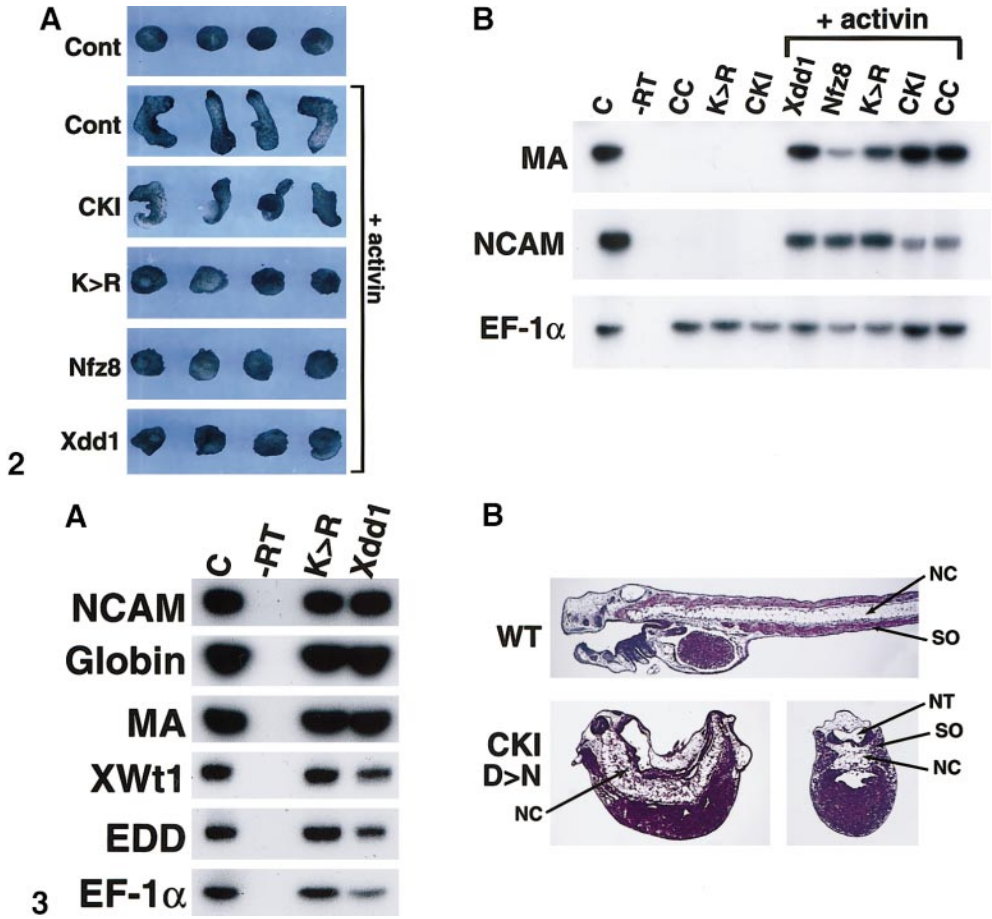

FIG. 2. Dominant-negative CKIε blocks activin-dependent cell movements, but does not alter cell fate in animal caps. (A) One-cell embryos were injected with mRNAs encoding CKIε (250 pg), CKIε(K>R) (250 pg), Nfz8 (1 ng), and Xdd1 (1 ng). Animal caps were explanted, incubated in buffer or activin, and photographed. (B) The animal caps from (A) were analyzed by RT-PCR. (C, control whole embryos; CC, control caps.) Activin induces expression of muscle actin (MA) and NCAM, markers of dorsal cell fates (Kintner and Melton, 1987; Mohun *et al.*, 1984). EF-1α, a ubiquitously expressed message, serves as a loading control (Krieg *et al.*, 1989).

FIG. 3. Dominant-negative CKIε does not alter differentiation in whole embryos. (A) Embryos were injected into both dorsal blastomeres at the four-cell stage with mRNA encoding CKIε(K>R) (250 pg) or Xdd1 (1 ng), and whole embryos were evaluated for expression of the indicated markers. Neural (NCAM), mesodermal [globin, muscle actin (MA), Wilms tumor (XWt1)], and endodermal [endodermin (EDD)] markers are equally expressed in all samples. C, control whole embryos. (B) Sagittal and transverse sections of CKIε(D>N)-injected embryo (stage 35) shows the presence of somites (SO), notochord (NC), and neural tissue (NT).

constructs and oligonucleotide sequences are available upon request. 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 whole embryo loss-of-function phenotypes, the dominant-negative CKI isoforms were injected into both dorsal blastomeres at the four-cell stage or into the animal pole at the one-cell stage, and then photographed as tadpoles. For the elongation assays, PIF was diluted 1:3 and added to the animal caps at stage 8 (Deardorff *et al.*, 1998; Sokol, 1996).

Morpholino

An anti-CKIε morpholino (5′-TCCCCACTCTCAGCTCCATGTTTAC-3′) was purchased from Gene Tools, LLC. The control morpholino was the standard control morpholino designed by and purchased from Gene Tools, LLC.

Cell Culture

A total of 2×10^5 cells (293 or NIH-3T3) were seeded in six-well dishes, grown to near confluence, and transfected with 2 μg DNA. The transfection was done with Lipofectamine (Gibco/BRL) for 3 h following the manufacturer's protocol. Twenty-four hours later, the cells were rinsed with PBS and 600 μM CKI-7 (U.S. Biologics),

or ethanol carrier (as a control) was added for 1 h. For Western blot analysis, cells were lysed in 1% Triton X-100, 50 mM Tris–HCl, pH 8.0, and 150 mM NaCl with protease inhibitors. Then, the samples were subjected to SDS–PAGE, transferred to nitrocellulose using a semidry transfer apparatus, and probed with the appropriate antibodies: anti-CKIε mAb (Transduction Labs), anti-Grb2 mAb (Transduction Labs), or anti-phospho-c-Jun antibody (NEB).

RESULTS

Dominant-Negative Forms of CKIε Block Convergence-Extension Movements in Xenopus Embryos

The first CKI family member that we studied through sufficiency tests was the ε isoform (Peters *et al.*, 1999). To analyze the endogenous role of CKIε during vertebrate embryogenesis, we attempted to inhibit its function by expressing two dominant-negative forms of CKIε, CKIε(K>R) and CKIε(D>N) (Peters *et al.*, 1999). These dominant negatives block Wnt- and CKIε-dependent processes in *Xenopus* (Peters *et al.*, 1999). We microinjected these mutant constructs into both dorsal blastomeres of four-cell-stage *Xenopus* embryos and allowed them to develop. Both dominant negatives generated embryos with shortened axes and with the tail bending back over the head (Fig. 1A). This "bent-back" phenotype is similar to that described for dominant-negative forms of Dsh (Xdd1) (Fig. 1A), Frizzleds, and Wnts (Deardorff *et al.*, 1998; Hoppler *et al.*, 1996; Sokol, 1996). Thus, blocking CKIε function in early embryos alters morphogenesis.

A possible explanation for the bent-back phenotype was that convergence-extension movements require CKIε and, thus, were blocked by the dominant negatives. Convergence extension is a morphogenetic movement that is the main driving force for dorsal axis elongation (Keller, 1991). To test whether the dominant-negative forms of CKIε blocked convergence-extension movements, we turned to the dorsal marginal zone (DMZ) assay (Graff *et al.*, 1994). Explanted DMZ spontaneously undergo cell movements and elongate. This contrasts with ventral marginal zones (VMZs), which do not elongate, but rather form a ball of tissue. To test whether CKIε was necessary for convergence extension in DMZs, two-cell-stage embryos were microinjected with the dominant-negative forms of CKI, and, as a positive control, Xdd1, a dominant-negative form of Dsh that blocks convergence-extension movements (Sokol, 1996). DMZs isolated from uninjected embryos and dominant-negative-injected embryos were cultured until stage 17 and then scored for elongation. While DMZs from control embryos elongated extensively, DMZs from embryos injected with dominant-negative forms of CKIε(D>N and K>R) or Dsh (Xdd1) did not elongate to the same extent (Fig. 1B).

Dominant-Negative Forms of CKIε Block Morphogenesis in Animal Cap Explants

To further evaluate the role of CKIε in cell movements, we employed the animal cap assay. Isolated animal caps do not undergo convergence extension and form round balls of epidermis. However, when incubated with activin, the caps express dorsal markers and undergo extensive cell movements (Fig. 2A) that are thought to mimic endogenous convergence extension (Deardorff *et al.*, 1998; Smith, 1987; Sokol, 1996). To determine whether inhibiting CKIε function could block these movements, one-cell-stage embryos were injected with dominant-negative forms of CKIε(K>R or D>N), Dsh (Xdd1), or Frizzled-8 (Nfz8), and animal caps were explanted at the blastula stage. The caps were then incubated with activin to induce convergence-extension movements. While untreated caps were round, caps from uninjected embryos or from embryos injected with wild-type CKIε underwent extensive elongation in the presence of activin (Fig. 2A). The dominant-negative forms of CKIε, Dsh, and Frizzled blocked this activin-induced elongation (Fig. 2A).

Blocking CKI Does Not Inhibit Tissue Differentiation

Activin is thought to generate cell movements by forming dorsal tissues (Smith *et al.*, 1989). So, it was plausible that inhibiting CKIε blocked the activin-dependent induction of dorsal fates and the loss of cell movements was simply a secondary effect. To address this possibility, we evaluated the animal caps for tissue differentiation by scoring for expression of the dorsal markers muscle actin and NCAM (Kintner and Melton, 1987; Mohun *et al.*, 1984). Control, untreated animal caps did not express these dorsal markers, while activin induced the expression of both markers (Fig. 2B). CKIε(K>R), CKIε(D>N) (not shown), Xdd1, and Nfz8 did not eliminate the activin-induced expression of muscle actin or NCAM (Fig. 2B).

To extend the molecular analysis from animal caps to the whole-embryo phenotype, we analyzed wild-type tadpoles, Xdd1-, and CKIε(K>R)-injected tadpoles for the presence of various tissue-specific markers. Xdd1 and CKIε(K>R) were injected into both dorsal blastomeres at the four-cell stage and the embryos allowed to develop to stage 32. Wild-type and bent-back tadpoles were harvested and scored for the expression of markers by RT-PCR. CKIε(K>R), like Xdd1, did not affect expression of the neural marker NCAM, the mesodermal markers globin, muscle actin, and XWt1, or the endodermal marker EDD (Fig. 3A). Histological analysis of the bent-back, CKIε(D>N)-tadpoles confirmed the presence of these tissues as well (Fig. 3B). Therefore, the dominant-negative forms of CKIε do not alter cell fate, but rather inhibit morphogenesis.

Blockade of Morphogenesis by Dominant-Negative CKI Occurs Zygotically

Endogenous cell movements occur after the midblastula transition (MBT), which marks the onset of zygotic transcription (Keller, 1991). If the blockade of convergence extension by CKI inhibition reflects an endogenous role for CKI, then the effect should also occur post-MBT. To address this, we microinjected the animal pole of one-cell-stage embryos with plasmid DNA encoding CKIε(D>N), and, as a positive control, the dominant-negative form of Frizzled-8, Nfz8 (Deardorff *et al.*, 1998). As zygotic transcription begins post-MBT, plasmid-driven expression ensures a late effect (Deardorff *et al.*, 1998; Newport and Kirschner, 1982). After injection, animal caps were explanted, incubated with and without activin, and evaluated for activin-dependent elongation. CKIε(D>N), expressed from either plasmid DNA or mRNA, blocked elongation as did the positive control plasmid, Nfz8 (Fig. 4A). In contrast, wild-type CKIε plasmid DNA or mRNA (not shown) did not (Fig. 4A). We conclude that the blockade of convergence-extension movements by CKIε inhibition occurs zygotically.

β-Catenin Does Not Rescue the Blockade of Cell Movements

As described above, Wnt signals through at least two pathways, a β-catenin-dependent and a β-catenin-independent cascade (Moon *et al.*, 1997; Sokol, 2000; Tada and Smith, 2000; Wallingford *et al.*, 2000). To determine whether β-catenin plays a role in convergence extension, we attempted to rescue the CKIε(K>R) blockade of activin-induced animal cap elongation with β-catenin. To that end, we microinjected one-cell-stage embryos with CKIε(K>R) mRNA alone or with mRNA encoding β-catenin and then incubated the caps with activin. CKIε(K>R) blocked elongation, and β-catenin did not reverse the effect (Fig. 4B). This is consistent with the idea that the endogenous role of CKIε in cell movements involves a β-catenin-independent pathway.

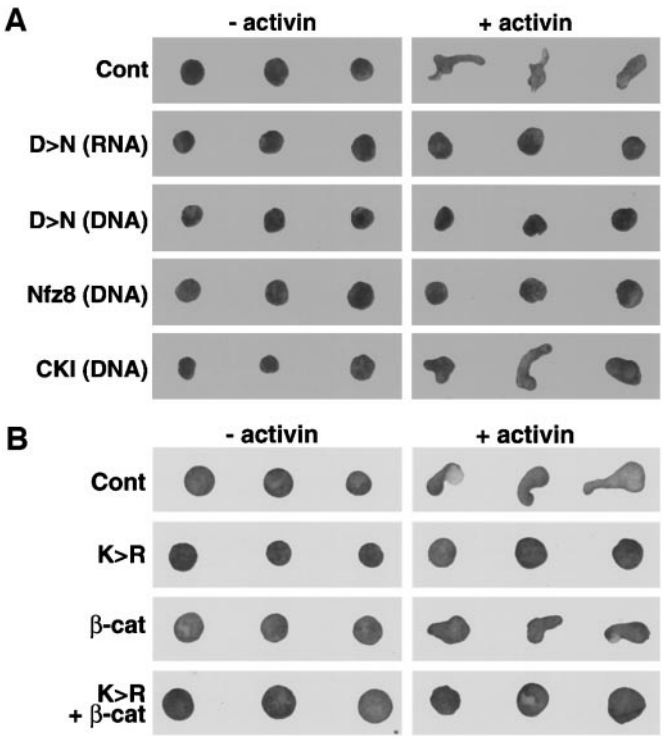

FIG. 4. The inhibition of morphogenesis occurs zygotically and is β-catenin independent. (A) Zygotic expression of CKIε(D>N) blocks cell movements. Embryos were injected at the one-cell stage with CKIε(D>N) (1 ng) mRNA or with plasmid DNA encoding CKIε(D>N) (1 ng), Nfz8 (1 ng), or wild-type CKIε (1 ng). Animal caps were explanted, incubated with or without activin as indicated, and then photographed. (B) β-catenin does not rescue elongation. Embryos were injected at the one-cell stage with mRNA encoding CKIε(K>R) (250 pg), β-catenin (1 ng), or CKIε(K>R) (250 pg) + β-catenin (1 ng), and caps were treated with activin and photographed.

Blocking CKI Activates the JNK Cascade

In *Drosophila*, a β-catenin-independent Wnt pathway controls cell polarity (Boutros and Mlodzik, 1999). Recent evidence suggests that vertebrate convergence-extension movements may be an equivalent of fly planar cell polarity (Wallingford *et al.*, 2000). For example, overexpression of wild-type Dsh as well as certain mutant forms of Dsh, disrupts cell polarity in flies and blocks cell movements in frogs (Boutros *et al.*, 1998; Sokol, 1996; Wallingford *et al.*, 2000). The β-catenin-independent pathway that controls cell polarity/movements is thought to involve JNK signaling (Djiane *et al.*, 2000; Dong *et al.*, 1996; Sokol, 2000). As dominant-negative forms of CKIε blocked convergence extension, we asked whether modulating CKI function would also affect JNK signaling. In tissue culture cells, c-Jun becomes phosphorylated upon activation of the JNK cascade (Boutros *et al.*, 1998; Minden and Karin, 1997). To evaluate JNK activation, we transfected 293 cells with wild-type or dominant-negative CKIε, lysed the cells, and measured c-Jun phosphorylation with a phospho-specific antibody. We also blocked endogenous CKI function using CKI-7, a specific and selective pharmacological inhibitor of CKI (Chijiwa *et al.*, 1989). Both CKIε(K>R) and CKI-7 increased c-Jun phosphorylation, while wild-type CKIε had no effect (Fig. 5A). Similar results were obtained in NIH-3T3 cells (Fig. 5B). Of note, CKI binds to two components of the canonical Wnt pathway, Dsh and Axin, that can activate JNK (Fig. 5). Therefore, blocking CKI activity, through two distinct approaches and in two different cell lines, activated the JNK cascade.

Other CKI Isoforms Block Convergence-Extension Movements

In *Xenopus*, inhibiting Wnt signals produces two distinguishable phenotypes: ventralized embryos or bent-back

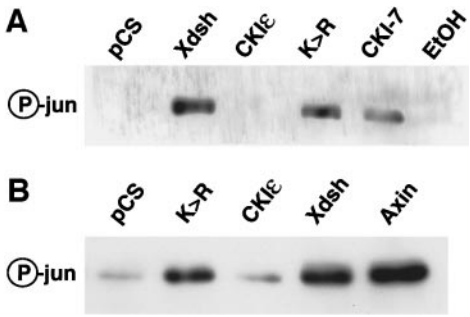

FIG. 5. Blocking CKI activates the JNK cascade. (A) 293 and (B) NIH-3T3 cells were transfected with c-Jun (0.8 μg) and 1.2 μg of pCS2+ (control), Xdsh, Axin, CKIε, or CKIε(K>R), or treated with 600 μM CKI-7 or ethanol carrier. The cell lysates were transferred and probed with an anti-phospho-c-Jun antibody to measure JNK activation (Boutros *et al.*, 1998). CKIε(K>R), CKI-7, Xdsh, and Axin activated the JNK cascade. A Grb2 mAb was used to ensure equal loading (not shown).

embryos (Deardorff *et al.*, 1998; Djiane *et al.*, 2000; He *et al.*, 1995; Heasman *et al.*, 1994; Hoppler *et al.*, 1996; Molenaar *et al.*, 1996; Sokol, 1996; Sumanas *et al.*, 2000; Yost *et al.*, 1998; Zeng *et al.*, 1997). The dominant-negative forms of CKIε produced the bent-back phenotype and did not block primary axis formation. However, CKI is a large family of enzymes and another CKI isoform might play a role in primary axis formation. We therefore extended the loss-of-function experiments and expressed dominant-negative forms (D>N) of the CKI α, β, γ2, γ3, and δ isoforms in embryos. All of the CKI(D>N) isoforms, with varying penetrance, produced the same bent-back phenotype, while β-galactosidase did not (Figs. 6A and 6B).

Although none of the dominant-negative isoforms of CKI altered primary axis formation, there were many possible explanations for the lack of a ventralized phenotype: the dominant negatives may not be sufficiently potent, the timing of the blockade may be too late, or redundancy among the isoforms could confound the dominant-negative studies. To attempt to address these issues, we decreased CKIε levels with morpholino oligonucleotides and we blocked CKI function with CKI-7. Morpholino oligonucleotides bind specifically to their complementary target RNA and block mRNA translation (Heasman *et al.*, 2000). We injected embryos with an anti-CKIε morpholino and found that CKIε protein expression was reduced but not eliminated (Fig. 6C). The decrease in CKIε protein levels appeared specific, as expression of the Grb2 protein was not affected (Fig. 6C). Notably, the anti-CKIε morpholino-injected embryos developed primary dorsal axes (Figs. 6D and 6E). However, these axes were shortened, consistent with a role for CKIε in morphogenesis (Figs. 6D and 6E). Embryos injected with a control morpholino developed normally (Fig. 6E). We also inhibited CKI activity with CKI-7, which potently inhibits several CKI isoforms (Chijiwa *et al.*, 1989), by microinjecting either one-cell or two-cell-stage embryos with a range of CKI-7 doses (6–60 ng). Although 3 ng of CKI-7 blocked Wnt and Dsh function in *Xenopus* embryos (Peters *et al.*, 1999), doses up to 60 ng did not alter primary axis formation (Fig. 6F). Taken together, these data suggest that, if CKI is required for axis formation, it is necessary prior to the time at which we inject embryos.

DISCUSSION

Wnt signaling controls critical developmental decisions in a wide range of organisms (Cadigan and Nusse, 1997; Moon *et al.*, 1997). Mutations in components of the Wnt pathway underlie mouse and human neoplasms (Nusse, 1992; Polakis, 2000). This highlights the key role that Wnt signaling plays in biology and disease and underscores the necessity to characterize its mechanisms of action. Through a diverse array of biochemical, molecular, and genetic experiments, insight has been shed on the Wnt pathway with several elements of the pathway already characterized (Moon *et al.*, 1997; Sokol, 1999; Wodarz and Nusse, 1998). In *Xenopus*, the canonical β-catenin-dependent Wnt signaling pathway controls primary axis formation (He *et al.*, 1995; Heasman *et al.*, 1994; Molenaar *et al.*, 1996; Zeng *et al.*, 1997). A β-catenin-independent Wnt pathway also exists, and, in *Xenopus*, this pathway is important for the convergence-extension movements that are the main driving force for axis elongation (Keller, 1991; Tada and Smith, 2000; Wallingford *et al.*, 2000). CKI is a recently identified component of the β-catenin-dependent Wnt cascade (Peters *et al.*, 1999; Sakanaka *et al.*, 1999). In this study, we have begun to characterize the role of the CKI family in early development and its role in the β-catenin-independent pathway. To examine the role that CKI plays during vertebrate development, we inhibited CKI function in *Xenopus* embryos with dominant-negative forms of CKI, with a morpholino that blocks CKIε mRNA translation, and with CKI-7, a pharmacological inhibitor of CKI. Our results suggest that CKI plays a role in convergence-extension movements: dominant-negative forms of CKI generated embryos with shortened axes and a bent-back phenotype (Figs. 1A and 6A). This phenotype is similar to that observed with dominant-negative forms of Wnt, Frizzled, and Dsh (Deardorff *et al.*, 1998; Hoppler *et al.*, 1996; Sokol, 1996). Reducing CKIε protein levels with the anti-CKIε morpholino also produced embryos with shortened axes (Figs. 6D and 6E). However, microinjection of CKI-7 had no effect on cell movements (Fig. 6F). Previous data suggested that CKI-7 is only active for a brief period in embryos (not shown). The lack of effect of CKI-7 on cell movements may therefore be due to inactivation or degradation of the inhibitor before gastrulation, when morphogenesis occurs. Our results with the dominant-negative forms of CKI and the anti-CKIε morpholino indicate a role for CKI in convergence-extension movements.

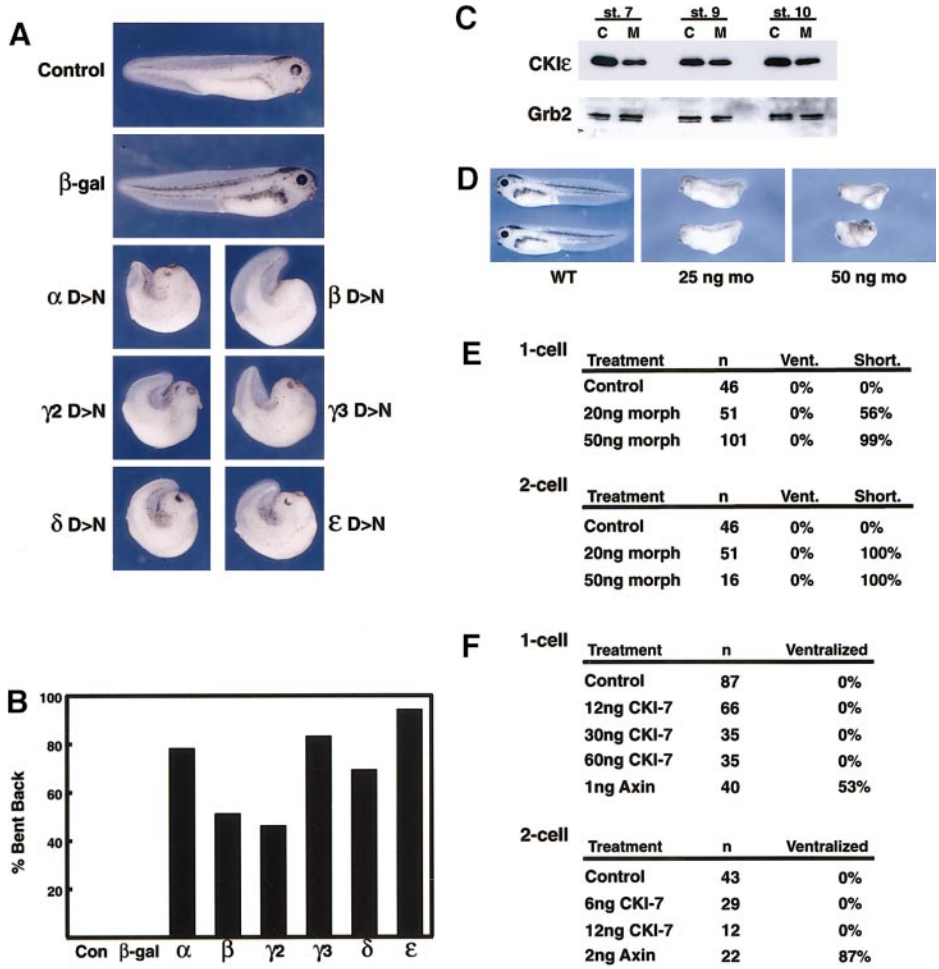

FIG. 6. Inhibiting CKI generates truncated axes. (A, B) mRNAs encoding β-galactosidase (2 ng) or D>N forms of CKIα (1 ng, $n = 50$), CKIβ (1.5 ng, $n = 43$), CKIγ2 (1 ng, $n = 48$), CKIγ3 (1 ng, $n = 58$), CKIδ (1 ng, $n = 58$), or CKIε (1 ng, $n = 47$) were injected into one-cell embryos. Tadpoles were then photographed (A) or scored for the percentage displaying the bent-back phenotype (B). (C) Fifty nanograms of anti-CKIε morpholino (M) was injected at the one-cell stage, and CKI levels were analyzed at stages 7, 9, and 10 with a CKIε monoclonal antibody. Grb-2 was used as a loading and specificity control. C, Control embryos. (D, E) An anti-CKIε morpholino shortens, but does not ventralize, embryos. An anti-CKIε morpholino was injected at the one-cell or two-cell stage, and embryos were photographed (D) and phenotypically scored (E) scored as tadpoles. A control morpholino had no effect (not shown). (F) CKI-7 did not alter endogenous axial patterning. One- and two-cell embryos were injected with the CKI inhibitor, CKI-7, or, as a positive control, with Axin.

It has been proposed that the convergence-extension pathway in *Xenopus* is analogous to the planar cell polarity cascade described in *Drosophila* (Djiane *et al.*, 2000; Sokol, 2000; Tada and Smith, 2000; Wallingford *et al.*, 2000). The planar cell polarity pathway is a β-catenin-independent Wnt pathway that is thought to signal through the JNK cascade (Boutros and Mlodzik, 1999; Eaton *et al.*, 1996; Weber *et al.*, 2000). We found that inhibiting CKI blocked convergence-extension movements and activated JNK. The results that wild-type Dsh, mutant Dsh, and dominant-negative CKI can both activate JNK and interfere with convergence-extension movements suggest a role for the JNK cascade in this process (Boutros *et al.*, 1998; Wallingford *et al.*, 2000). The current data regarding the role of the JNK cascade in vertebrate convergence extension do not allow a unifying paradigm. For example, some studies suggest that blocking JNK inhibits convergence extension and others imply that activating JNK inhibits convergence extension (Dong *et al.*, 1996; Djiane *et al.*, 2000). Taken together, the currently available studies support a role for the JNK cascade in vertebrate convergence extension and suggest a model in which altering the level of JNK activation, either positively or negatively, can affect proper convergence extension in the developing embryo.

In *Xenopus* embryos, CKIε generates complete dorsal axes in both the second axis assay and the UV-rescue assay

(Peters *et al.,* 1999), which suggests a possible role for CKI in dorsal axis specification. Yet, blocking CKI function with dominant negatives, CKI-7, or the CKIε morpholino did not alter dorsal axis formation. These data are consistent with other reports in which components of the Wnt pathway that act upstream of GSK-3 were blocked in embryos. For example, embryos microinjected with inhibitors of Wnts, Frizzleds, or Dsh all contained dorsal axes and had the bent-back phenotype (Deardorff *et al.,* 1998; Hoppler *et al.,* 1996; Hsieh *et al.,* 1999; Sokol, 1996). Of note, embryonic expression of dominant-negative forms of Frizzled-7 blocked morphogenetic movements but not axis formation (Djiane *et al.,* 2000). However, when Frizzled-7 was blocked by antisense-depletion in oocytes, primary axis formation was inhibited (Sumanas *et al.,* 2000). This suggests that many, if not all, components of the canonical β-catenin pathway might be important in axial determination. One possible explanation for the lack of effect on primary axis formation is that the dominant-negative forms of CKI, Wnt, Dsh, and Frizzled are not potent enough to completely inhibit the endogenous gene products. Another possibility is that dorsal axis specification occurs very early and the dominant negatives are not expressed soon enough to alter this process. When might the endogenous process occur? In the dominant-negative experiments, mRNA is microinjected and must then be translated. In an attempt to circumvent this delay, we microinjected the drug CKI-7, which should rapidly inhibit CKI function (Chijiwa *et al.,* 1989). However, no dose of CKI-7, nor any time of microinjection into embryos, affected axis formation. This suggests that the upstream components of the pathway are required prior to the time in which we are able to microinject the drug. So, the key event(s) for axis specification might occur soon after fertilization. To resolve this issue and to determine the role that the CKI family plays in dorsal axis formation may require depletion of maternal stores of CKI mRNA in oocytes (Heasman *et al.,* 1994). To date, we have tested 25 different CKIε anti-sense oligonucleotides and none have depleted the CKIε message to a substantial degree (not shown).

Dominant-negative forms (D>N) of the other CKI isoforms also generated the same bent-back phenotype as observed with the dominant-negative forms of CKIε (Figs. 6A and 6B). Although it is possible that all of the different CKI isoforms play a role in convergence-extension movements, it seems unlikely. An alternative explanation is that all of the dominant negatives interact or interfere with a common target. Preliminary data support this idea, as dominant negatives of one isoform can inhibit the activity of a different CKI isoform (not shown). Taken together, the dominant-negative studies and the morpholino data are consistent with the idea that CKI plays a role in embryonic morphogenesis. Several lines of evidence indicate that alterations in cell movements might contribute to carcinogenesis (Bilder *et al.,* 2000; Peifer and Polakis, 2000). So, the role that Wnt signaling plays in carcinogenesis and cell movements might be related.

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