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Physiological regulation of β-catenin stability by Tcf3 and CK1ε

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The wnt pathway regulates the steady state level of β-catenin, a transcriptional coactivator for the Tcf3/Lef1 family of DNA binding proteins. We demonstrate that Tcf3 can inhibit β-catenin turnover via its competition with axin and adenomatous polyposis for β-catenin binding. A mutant of β-catenin that cannot bind Tcf3 is degraded faster than the wild-type protein in *Xenopus* embryos and extracts. A fragment of β-catenin and a peptide encoding the NH_2 terminus of Tcf4 that block the interaction between β-catenin and Tcf3 stimulate β-catenin degradation, indicating this interaction normally plays an important role in regulating β-catenin turnover. Tcf3 is a substrate for both glycogen synthase kinase (GSK) 3 and casein kinase (CK) 1ε, and phosphorylation of Tcf3 by CKIε stimulates its binding to β-catenin, an effect reversed by GSK3. Tcf3 synergizes with CK1ε to inhibit β-catenin degradation, whereas CKI-7, an inhibitor of CK1ε, reduces the inhibitory effect of Tcf3. Finally, we provide evidence that CK1ε stimulates the binding of dishevelled (dsh) to GSk3 binding protein (GBP) in extracts. Along with evidence that a significant amount of Tcf protein is nonnuclear, these findings suggest that CK1ε can modulate wnt signaling in vivo by regulating both the β-catenin-Tcf3 and the GBP-dsh interfaces.

Introduction

Wnt signaling plays an important role in the development of all metazoan embryos and in the genesis of several human malignancies (for review see Morin, 1999). In the presence of a wnt signal, the transcriptional activation of target genes is initiated as a result of increased steady state levels of the transcriptional coactivator β-catenin. In the absence of wnt activity, soluble β-catenin is an unstable protein with an estimated half-life of 1–1.5 h in *Xenopus* embryos. Degradation of β-catenin occurs through the coordinated assembly of a complex that contains β-catenin, axin, glycogen synthase kinase (GSK)* 3, and the adenomatous polyposis coli (APC) protein. Within this complex, β-catenin is brought in the proximity of and is phosphorylated by GSK3 (Kishida et al., 1998). Phosphorylated β-catenin is subsequently recognized by the F-box protein β-TRCP (Maniatis, 1999), the specificity factor of an Skp1/cullin/F-box protein ubiquitin ligase complex (SCF) that catalyzes the covalent attachment of polyubiquitin chains to phosphorylated β-catenin. Polyubiquitin conjugates of β-catenin are rapidly degraded by the proteasome (Aberle et al., 1997).

Signaling through the wnt pathway is initiated upon binding of wnts to members of a family of seven transmembrane receptors (frizzled receptors). Through an as yet unknown mechanism, frizzled receptors activate a cytoplasmic protein, dishevelled (dsh), which interacts directly with axin via their DIX domains (Fukui et al., 2000; Julius et al., 2000; Salic et al., 2000). The GSK3 binding protein (GBP) (Yost et al., 1998) is recruited through its interaction with the PDZ domain of dsh and thus brought in the proximity of axin-bound GSK3. Binding of GBP to GSK3 inhibits its kinase activity against β-catenin, reducing its degradation by the SCF ubiquitin ligase complex and resulting in a increased steady state level of free β-catenin.

Free β-catenin interacts with the DNA binding proteins Tcf3 and Lef1 (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996) to form a bipartite transactivator that stimulates the transcription (van de Wetering et al., 1997) of immediate gene targets (for example, siamois [Brannon et al., 1997] and Xnr3 [McKendry et al., 1997] in *Xenopus*). In the absence of β-catenin, Tcf3/Lef1 proteins mediate repression when bound to the Groucho family of transcription factor, CREB binding protein (CBP), and CtBP. Interaction between Tcf3 and β-catenin occurs at the NH_2 terminus of Tcf3 and is separate from the Groucho and CtBP binding regions (for review see Barker et al., 2000).

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*Abbreviations used in this paper: APC, adenomatous polyposis coli; CBP, CREB binding protein; CK, casein kinase; dsh, dishevelled; GBP, GSK3 binding protein; GSK, glycogen synthase kinase; IGF, insulin growth factor; MBP, maltose-binding protein; SCF, Skp1/cullin/F-box protein complex.

Key words: β-catenin; Tcf; wnt; casein kinase; *Xenopus*

Models of the wnt pathway suggest a role for Tcf3/Lef1 as an unregulated scavenger of free β-catenin. However, CBP binds and acetylates a lysine in the β-catenin interaction domain of Tcf3, thereby lowering its affinity for β-catenin (Waltzer and Bienz, 1998). Some members of the Sox family of HMG box proteins also bind β-catenin and block its binding to Tcf (Zorn et al., 1999). In *Caenorhabditis elegans*, components of the mitogen-activated kinase pathway phosphorylate Tcf-bound β-catenin so as to block nuclear localization (Rocheleau et al., 1999). These studies indicate that the interaction between β-catenin and Tcf3 is dynamic and that regulating it may play an important role in modulating wnt signaling. We developed recently an in vitro system to examine the cytoplasmic components of the wnt signaling pathway using *Xenopus* egg extracts (Salic et al., 2000). We have used this system to study the effects of Tcf3 on β-catenin stability and the interaction between Tcf3 and β-catenin.

Components of the wnt pathway upstream of Tcf3 regulate β-catenin stability. However, a clear role for Tcf3 itself in the stabilization of β-catenin has not been demonstrated. In the present study, we show that Tcf3 inhibits the interaction between β-catenin and axin/APC and that Tcf3 and β-catenin interact significantly even in the absence of wnt signaling to modulate β-catenin turnover. We show that GSK3 and casein kinase (CK) 1ϵ both have direct but opposite effects in regulating the β-catenin–Tcf3 interaction. We also find that a significant fraction of Tcf3 is cytoplasmic in both *Xenopus* embryos and cultured cells, indicating that Tcf3 can act outside the nucleus to regulate β-catenin degradation. Additionally, we provide evidence that CKIϵ stimulates the interaction between dsh and GBP. These results suggest two possible mechanisms for the role of CK1ϵ in wnt signaling and provide further evidence that regulated formation of the β-catenin–Tcf3 complex and the dsh–GBP complex play a crucial role in wnt signaling.

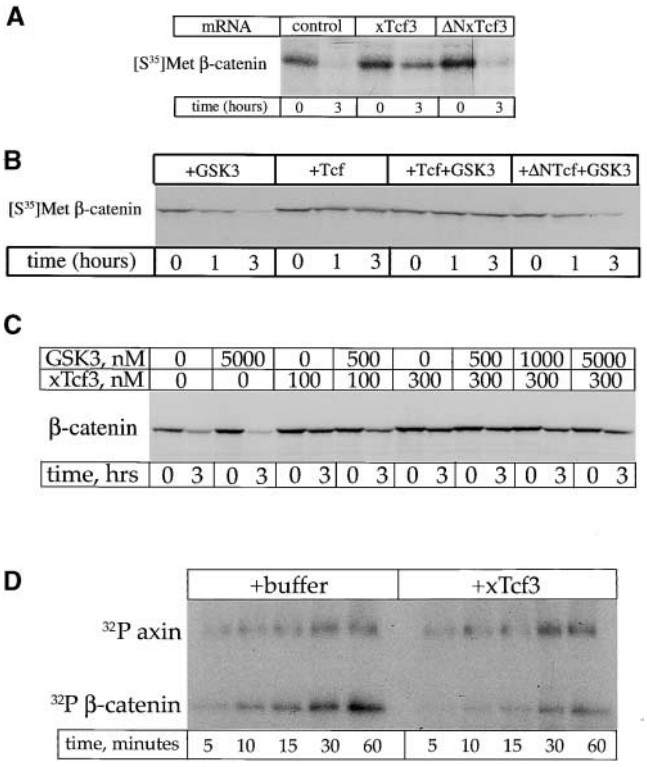

Figure 1. **Tcf3 blocks β-catenin degradation in extracts and phosphorylation in vitro.** (A) Translated Tcf3 but not ΔNTcf3 mRNA inhibits β-catenin degradation in extracts. (B) Purified Tcf3 protein (1 μM) blocks β-catenin degradation. This effect was not reversed by 1 μM GSK3. Purified ΔNTcf3 (1 μM) does not block β-catenin degradation. (C) Inhibition of β-catenin degradation by lower (100 nM) Tcf3 levels can be partially rescued by GSK3; higher Tcf3 (300 nM) levels cannot be rescued by even a large GSK3 excess. (D) Tcf3 inhibits the phosphorylation of β-catenin by GSK3 and axin in a purified system. In the same reaction, axin phosphorylation by GSK3 is not affected by Tcf3.

Results

Tcf3 inhibits both β-catenin degradation and phosphorylation by GSK3

To determine if Tcf3 can influence the rate of β-catenin degradation, we either translated Tcf3 mRNA in *Xenopus* extracts (Fig. 1 A) or added purified recombinant Tcf3 to the extracts (Fig. 1 B). In both cases, Tcf3 inhibited β-catenin degradation. An IC_{50} of 30 nM was determined by careful titration of Tcf3 (1 nM to 1 μM) into *Xenopus* extracts (unpublished data). As a test for the specificity of this interaction, we used an NH_2-terminal deletion mutant of Tcf3, ΔNTcf3, which cannot bind β-catenin in vitro (Molenaar et al., 1996). When ΔNTcf3 mRNA was translated in extracts, it had no effect on β-catenin degradation (Fig. 1 A); similarly, ΔNTcf3 purified protein (Fig. 1 B) had no effect. Thus, the inhibitory effect of Tcf3 on β-catenin degradation is dependent on its ability to interact with β-catenin through the NH_2-terminal domain. 1 μM of purified GSK3 (Fig. 1 B) did not reverse the inhibitory effect of Tcf3 (1 μM) on β-catenin degradation, suggesting that the effect of Tcf3 was not due to titration of GSK3. A more careful examination showed that excess GSK3 could partially rescue the inhibition of β-catenin degradation by lower levels of Tcf3 (100 nM); however, even a large excess of GSK3 did not reverse the effect of 300 nM added Tcf3 (Fig. 1C).

Phosphorylation of β-catenin by GSK3 is required for β-catenin degradation (Yost et al., 1996). β-catenin and axin are phosphorylated by GSK3 in vitro, and β-catenin phosphorylation is stimulated greatly by axin (Hart et al., 1998; Ikeda et al., 1998). We reconstituted this reaction in vitro using purified proteins and examined the effects of recombinant Tcf3 on the kinase reaction. 5 μM Tcf3 strongly inhibited β-catenin phosphorylation by GSK3 but had no effect on the phosphorylation of axin (Fig. 1 D). This result demonstrates that the effect of Tcf3 on β-catenin phosphorylation is not due to titration or inhibition of GSK3 but rather to the sequestration of β-catenin, preventing its interaction with axin/GSK3.

Tcf3 and axin/APC compete for β-catenin binding

Phosphorylation and subsequent degradation of β-catenin requires its association with both APC and axin. Since binding of Tcf3 to β-catenin blocks its degradation, we tested if this is due to blocking binding of β-catenin to axin and/or APC. As shown in Fig. 2 A, purified his-tagged Tcf3 but not his-tagged ΔNTcf3 blocked binding of β-catenin to axin beads. However, Tcf3 had no effect on GSK3 binding to

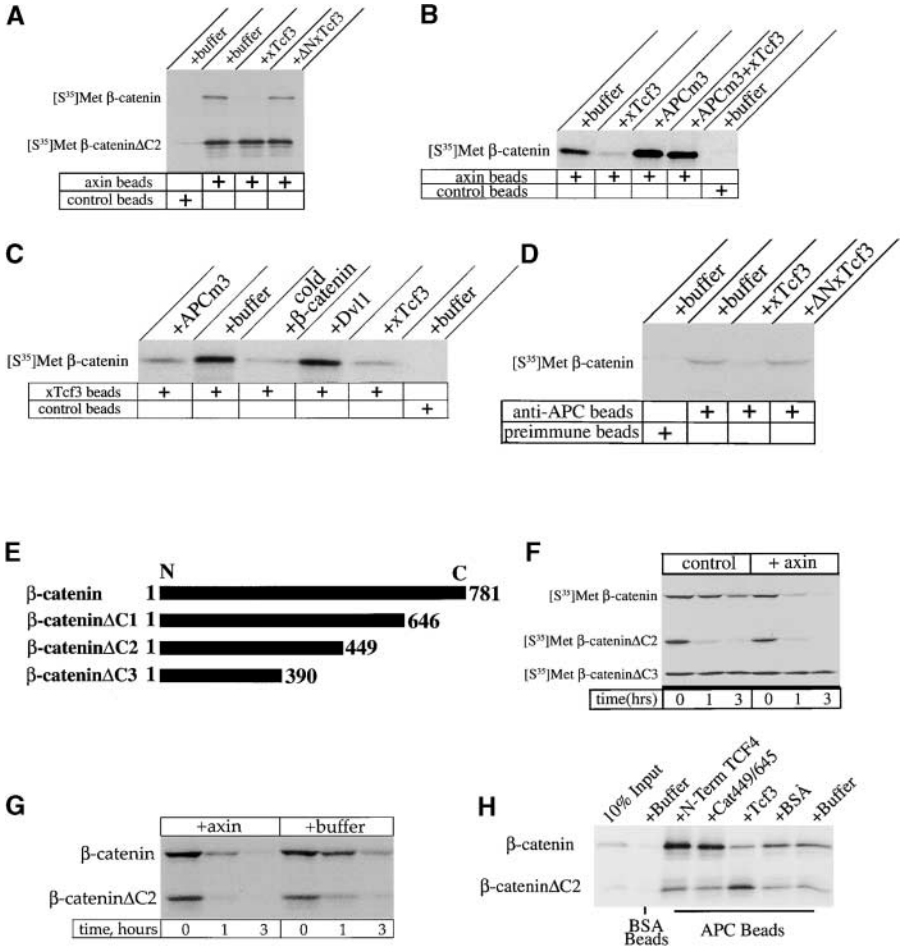

Figure 2. **Tcf3 competes with axin/APC for β-catenin (cold competitors were present at 1 μM in all experiments).** (A) Binding of β-catenin to axin beads is inhibited by Tcf3 but not by ΔNTcf3. In contrast, binding of the β-catenin ΔC2 mutant to axin is unaffected by Tcf3. (B) Tcf3 blocks the binding of β-catenin to axin beads, whereas APCm3 reverses this effect of Tcf3. (C) APCm3 inhibits the binding of β-catenin to Tcf3 beads. (D) 1 μM his_6-TCF3 blocks the interaction of β-catenin with endogenous APC in extracts, whereas his_6-ΔNTcf3 has no effect. (E) Scheme of the COOH-terminal β-catenin deletion constructs used to map the fragment of β-catenin responsible for stabilization by Tcf3. (F) Normal and axin-induced degradation of β-catenin, β-cateninΔC2, and β-cateninΔC3 in *Xenopus* extracts. β-cateninΔC3 is completely stable and does not respond to axin. (G) Axin stimulates the turnover of both β-catenin and β-cateninΔC2 in the same degradation reaction. (H) β-catenin and β-cateninΔC2 both bind to APC and binding is not disrupted by the Tcf4 NH_2-terminal peptide or by the cat449/645 fragment (at <2 μM).

axin (unpublished data). A purified 100-kD fragment of APC (APCm3) that spans the axin and β-catenin binding sites stimulates binding of β-catenin to axin beads (Fig. 2 B). In the presence of 100 nM APCm3, addition of up to 1 μM Tcf3 protein had no detectable effect on the binding of β-catenin to axin beads. Furthermore, binding of β-catenin to Tcf3 beads (Fig. 2 C) is effectively inhibited in the presence of 1 μM APCm3. Consistent with a dominant effect of APC in *Xenopus* extracts, a majority of the soluble β-catenin is in anti-APC immunoprecipitates (Salic et al., 2000). In extracts, 1 μM of added Tcf3 released radiolabeled β-catenin from APC immunoprecipitates (Fig. 2 D). As a control, 1 μM ΔNTcf3 had no detectable effect on β-catenin binding to endogenous APC. These results indicate that Tcf3 competes with both axin and APC for β-catenin binding. The failure of Tcf3 to block β-catenin binding to axin in the presence of APCm3 highlights the strong effect of APC in driving the degradation of free β-catenin by promoting its binding to axin and in inhibiting competing reactions driven by Tcf3. In contrast, the ability of Tcf3 to compete effectively with endogenous APC for β-catenin probably reflects the fact that most of β-catenin is not in a complex with axin/APC, since cellular axin levels are very low (10–20 picomolar; unpublished data).

The degradation and binding data suggest that the interaction between β-catenin and Tcf3 could play an important role in vivo in regulating the degradation kinetics of β-catenin. To test if this is the case in vivo, we set out to generate a mutant of β-catenin that is unable to interact with Tcf3. β-catenin COOH-terminal truncation mutants are shown schematically in Fig. 2 E. One would predict that a mutant of β-catenin that cannot interact with Tcf3 would be unresponsive to added Tcf3 and would be degraded at a faster rate than wild-type β-catenin due to its inability to interact with endogenous Tcfs. Constructs were first tested in our degradation assay in *Xenopus* extracts. Full-length β-catenin and β-cateninΔC2 were degraded similarly (Fig. 2 F); β-cateninΔC1 also behaved identically to the full-length protein (unpublished data). Degradation of full-length β-catenin and β-cateninΔC2 was stimulated by axin (Fig. 2, F and G) and inhibited by lithium (unpublished data), dishevelled (Fig. 3 A), and APCm3 (unpublished data). Mutating the GSK3 phosphorylation sites (serine to alanine) in the NH_2-terminal region of β-cateninΔC1 and β-cateninΔC2 stabilized these proteins against degradation in extracts (unpublished data), further demonstrating that the stability of β-cateninΔC1 and β-cateninΔC2 is regulated similarly to wild-type β-catenin. In contrast to β-cateninΔC1 and β-cateninΔC2, β-cateninΔC3 was completely stable (Fig. 2 F), even when 20 nM axin was added to extracts (which normally accelerates the rate of wild-type β-catenin degradation greater than fourfold) (Salic et al., 2000). The stability of β-cateninΔC3 is likely due to its inability to bind axin (unpublished data), an interaction absolutely required for β-catenin degradation

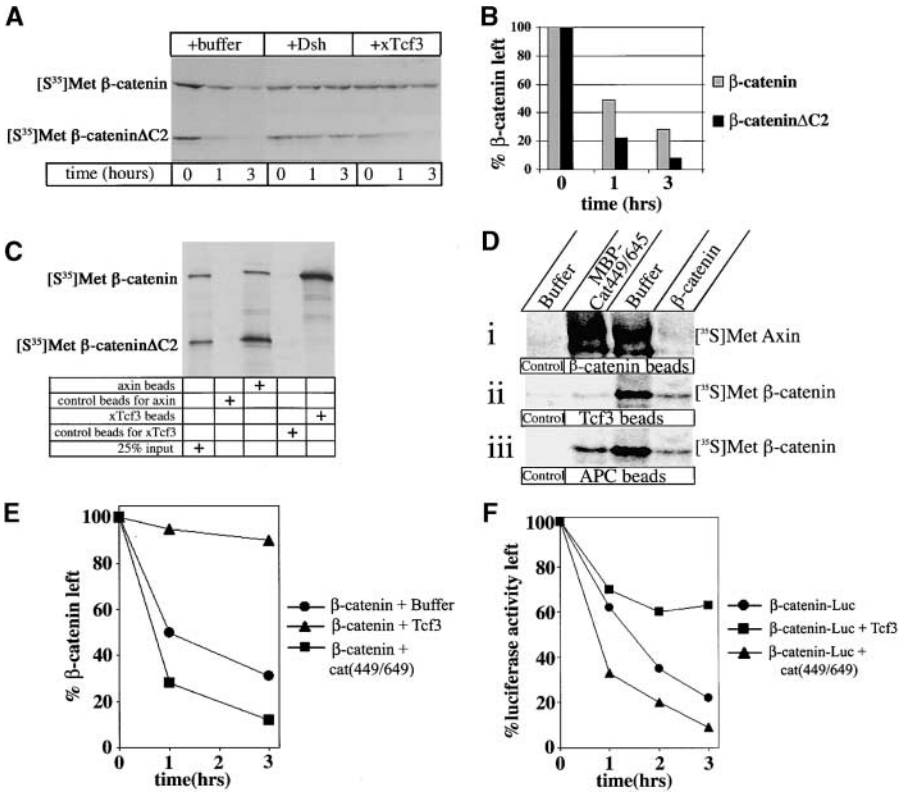

Figure 3. **Effects of Tcf3 on β-catenin mutants.** (A) Degradation of both β-catenin and β-cateninΔC2 is inhibited by 1 μM dsh, but only β-catenin is inhibited by 1 μM Tcf3. (B) Graphical representation of densitometry measurements of the autoradiogram in A shows the faster degradation rate of β-cateninΔC2 compared with β-catenin. (C) Both β-catenin and β-cateninΔC2 bind to axin, but only β-catenin binds to xTcf3 in vitro. (D) MBP-cat449/645 inhibits by >95% the binding of [35S]methionine-labeled β-catenin to Tcf3 in vitro (ii). MBP-cat449/645 (2 μM) had no effect on the binding of axin to β-catenin beads (i) and a moderate effect on the binding of β-catenin to APC beads (iii). Binding of radiolabeled β-catenin and axin to control beads was negligible. (E) Degradation of radiolabeled β-catenin in *Xenopus* extracts is stimulated by MBP-cat449/645 (200 nM). (F) β-catenin–luciferase is degraded more rapidly in embryos when coinjected with MBP-cat449/645. β-catenin–luciferase (4 ng) protein was injected into 2-cell stage *Xenopus* embryos with or without MBP-cat449/645 (4 ng). At the indicated times, embryos were processed for luciferase assays.

(Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998). Interestingly, degradation of β-cateninΔC2 is not inhibited by up to 1 μM Tcf3 (Fig. 3 A), consistent with its inability to bind Tcf3 (Fig. 3 C). β-cateninΔC2 still binds axin (Fig. 3 C). As with β-catenin, radiolabeled β-cateninΔC2 binds APC in vitro (Fig. 2 H) and can be immunoprecipitated from *Xenopus* extracts using anti-APC antibody beads (unpublished data). However, unlike full-length β-catenin, this interaction is not blocked by 1 μM Tcf3 added to the extracts (unpublished data). When compared with wild-type β-catenin, β-cateninΔC2 has a significantly faster degradation rate (Fig. 3, A and B), suggesting that endogenous Tcf3 regulates the rate of β-catenin turnover.

The interaction of Tcf3 with β-catenin controls the rate of β-catenin turnover in vitro and in vivo

To explore further the possibility that β-catenin degradation is normally inhibited by its interaction with Tcf3, we asked if perturbing this interaction would affect β-catenin degradation in vivo and in extracts. We first developed an inhibitor of this interaction. Based on our deletion analysis, a fragment of β-catenin (amino acids 449–645, cat449/645) between the COOH termini of β-cateninΔC1 and β-cateninΔC2 was expressed as a maltose-binding protein (MBP) fusion and tested for its ability to block the binding of β-catenin to Tcf3 beads. Excess cold β-catenin and cat449/645 (2 μM each) compete equally well with radiolabeled β-catenin for binding to Tcf3-beads (Fig. 3 D). In contrast to full-length β-catenin, cat449/645 does not compete for binding of axin to β-catenin beads, indicating that the fragment selectively interferes with the β-catenin–Tcf3 interaction. Lower than 2-μM levels of cat449/645 do not affect binding of β-catenin to APC (Fig. 2 H); however, higher cat449/645 concentrations can significantly inhibit binding to APC, suggesting some overlap between the Tcf3 and the APC binding sites on β-catenin.

Since cat449/645 below 2 μM blocks the interaction between β-catenin and Tcf3 without affecting the interaction between β-catenin and axin and APC, we tested its effect on the rate of β-catenin degradation. Addition of 200 nM MBP-cat449/645 to *Xenopus* extracts accelerated the rate of β-catenin degradation by ~50% (Fig. 3 E). Similarly, when injected into *Xenopus* embryos cat449/645 stimulated the degradation of coinjected β-catenin–luciferase by ~70% (Fig. 3 F). Paradoxically, high concentrations (>2 μM) of cat449/645 inhibited β-catenin degradation (unpublished data), probably reflecting the ability of high concentrations of this mutant to compete with wild-type β-catenin for binding to APC (Fig. 3 D). However, in the concentration range of cat449/645 where the β-catenin–APC interaction is not affected, the mutant blocks the Tcf–β-catenin binding and stimulates β-catenin turnover.

The NH_2-terminal regions of Tcf3 and Tcf4 contain a conserved β-catenin binding domain (Molenaar et al., 1996). We wanted to determine if a peptide corresponding to the NH_2-terminal region of Tcf4 could similarly disrupt the interaction between β-catenin and Tcf3. A human Tcf4 NH_2-terminal peptide blocked the binding of β-catenin to Tcf3 beads (Fig. 4 A). As for cat449/645, the Tcf4 NH_2-terminal peptide had no effect on the interaction between β-catenin and axin (Fig. 4 B) or on the interaction between β-catenin and APC (Fig. 2 H). Addition of the Tcf4 NH_2-terminal peptide to *Xenopus* extracts stimulated the degradation of β-catenin by ~65% (Fig. 4 C), further demonstrat-

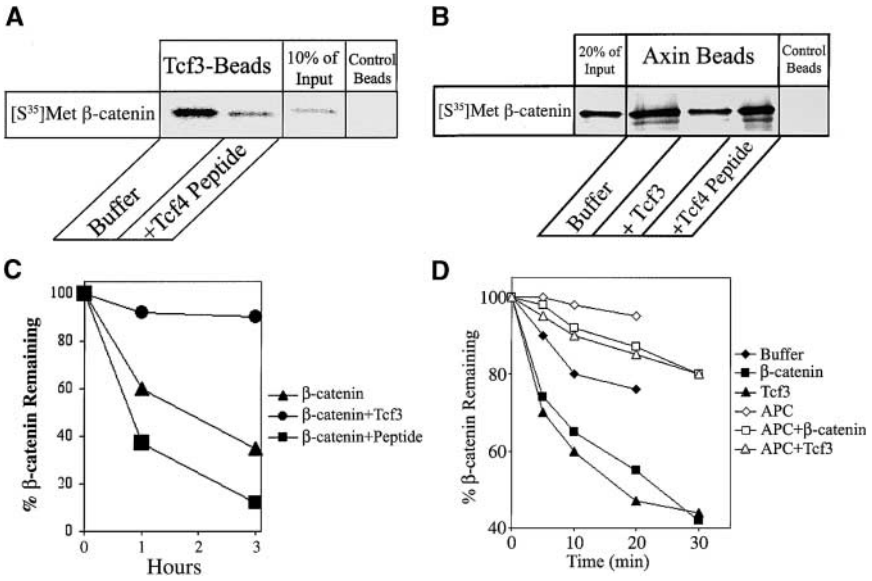

Figure 4. **Effects of an NH_2-terminal Tcf4 peptide on β-catenin stability and rate of axin–β-catenin dissociation.** (A) β-catenin binding to xTcf3 beads is inhibited by an NH_2-terminal Tcf4 peptide (10 μM). (B) In contrast, β-catenin binding to axin is unaffected by the presence of the NH_2-terminal Tcf4 peptide but is blocked by cold Tcf3. (C) Addition of the NH_2-terminal Tcf4 peptide (2 μM) to *Xenopus* extracts stimulates β-catenin degradation, which is in contrast to the inhibitory effect of Tcf3 (500 nM). (D) Half-life of the axin–β-catenin complex. Radiolabeled β-catenin bound to axin beads (in the presence or absence of 100 nM APC) was incubated with buffer, cold Tcf3 (2 μM) or cold β-catenin (2 μM) at room temperature. The β-catenin remaining on beads was measured as a function of time.

ing that Tcf proteins are normally involved in modulating β-catenin degradation. A control peptide had no effect on the degradation of β-catenin (unpublished data).

Although we have demonstrated that Tcf proteins can regulate the rate of β-catenin degradation, if Tcf and the proteins involved in β-catenin turnover do not normally colocalize in the cell such a mechanism might not be physiological. Specifically, Tcf is a transcription factor that may reside exclusively in the nucleus, whereas β-catenin degradation may occur solely in the cytoplasm. Therefore, we measured the amount of cytoplasmic and nuclear Tcf in *Xenopus* eggs, stage 7.5 *Xenopus* embryos, and cultured human 293 cells. As shown in Fig. 5 B, the vast majority of Tcf is cytoplasmic in eggs. Even in stage 7.5 embryos where the number of nuclei is ~2,000 per embryo (versus one per egg), a large fraction of Tcf is cytoplasmic (Fig. 5 A; 70% cytoplasmic fraction versus 30% in the nuclear fraction). We found a similar distribution of myc_6-tagged xTcf3 protein in stage 7.5 embryos that were injected with myc_6-tagged xTcf3 mRNA at the 2-cell stage. These findings are perhaps not surprising and may reflect two conditions found in a developing embryo: (a) the low nucleocytoplasmic ratio of the embryonic cells and (2) the rapid division cycles of the early embryo (30 min per cycle), which results in disassembly of the nucleus for half of the time. These two situations allow ample opportunity for Tcf to interact with cytoplasmic β-catenin to regulate its degradation. We next wanted to determine whether or not the high level of cytoplasmic Tcf protein was unique to *Xenopus* embryos and examined the cytoplasmic pool of Tcf in cultured cells. As shown in Fig. 5, C and D, fractionation of 293 cells indicates that ~40% of Tcf was cytoplasmic (compared with topoisomerase II, which is quantitatively pelleted with the nuclei). Therefore, in cultured 293 cells a significant amount of Tcf is present in the cytoplasm and thus capable of competing with APC and axin for β-catenin binding.

Tcf3 phosphorylation by GSK3 and CK1ε

We noted during the characterization of recombinant histidine-tagged Tcf3 (his_6-Tcf3) purified from baculovirus-infected *Sf9* cells that certain preparations exhibited kinase activity, resulting in a phosphorylated form of Tcf3 that comigrated with the unphosphorylated form by SDS-PAGE. Furthermore, addition of recombinant his_6-GSK3 to the reaction enhanced the phosphorylation of Tcf3 (unpublished data). Phosphorylation of Tcf3 was suppressed by 100 mM LiCl (a direct inhibitor of GSK3) (Klein and Melton, 1996) to the reaction (Fig. 6 A), which suggested that endogenous GSK3 copurified with recombinant his_6-Tcf3 from *Sf9* cells. This was confirmed by Western analy-

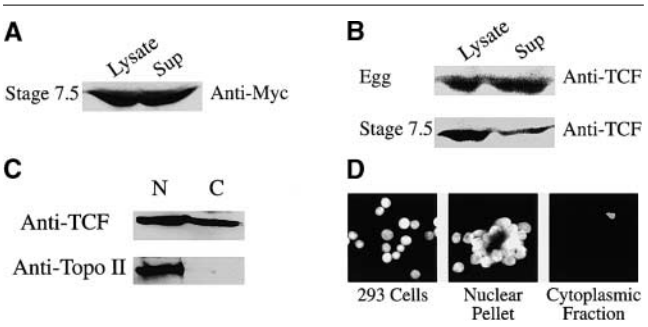

Figure 5. **A significant fraction of total cellular TCF protein in *Xenopus* embryos and in cultured cells is nonnuclear.** (A) Both cells of 2-cell embryos were injected with myc_6-Tcf3 RNA (250 pg/blastomere), gently homogenized at stage 7.5 (lysate), and centrifuged to pellet the nuclei (Sup). Equivalent volumes of lysate and supernatant were subsequently processed for Western analysis using an anti-myc antibody. Most of the detected myc-tagged Tcf3 is present in the supernatant fraction. (B) *Xenopus* eggs and stage 7.5 embryos were processed as described for the myc_6-Tcf3 RNA injected embryos except that an anti-Tcf antibody was used to detect endogenous Tcf. Nearly all of the Tcf detected is present in the supernatant fraction of eggs in contrast to stage 7.5 embryos. Lysates and supernatants were stained with Hoechst to confirm the presence (lysates) or absence (supernatants) of intact nuclei (unpublished data). Nuclear and cytoplasmic preparations from cultured 293 cells were blotted for topoisomerase II (C) and Tcf and stained with Hoechst (D). The nuclear pellet was brought to the same volume as the cytoplasmic fraction, and equivalent volumes were used for Western analysis.

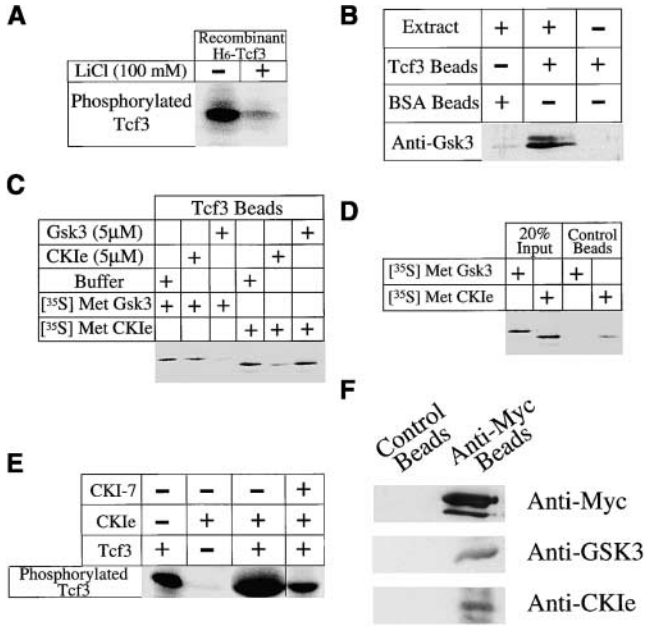

Figure 6. **GSK3 and CK1ϵ bind and phosphorylate Tcf3.** (A) Tcf3 purified from *Sf9* cells contains lithium-sensitive kinase activity. Recombinant his_6-Tcf3 (1μg) was incubated in 10 μl kinase buffer (described in Materials and methods) for 30 min at room temperature either in the presence or absence of 100 mM LiCl, which normally inhibits GSK3 activity. Phosphorylation of Tcf3 is dramatically decreased in the presence of lithium. (B) Tcf3 beads pull down GSK3 from *Xenopus* extracts. Beads (control-BSA or Tcf3-coupled) were incubated with *Xenopus* egg extracts, washed, eluted, and analyzed by Western blotting with a monoclonal anti-GSK3 antibody. (C) Both GSK3 and CK1ϵ bind Tcf3. Radiolabeled in vitro–translated GSK3 and CK1ϵ bind Tcf3 beads. Binding of radiolabeled GSK3 and CK1ϵ to Tcf3 beads was abolished by addition of excess cold protein (5 μM of his_6-GSK3 and MBP-CK1ϵ, respectively), demonstrating specificity of the binding reaction. However, excess cold his_6-GSK3 does not block CK1ϵ binding to Tcf3, whereas excess cold MBP-CKIϵ fails to block GSK3 binding to Tcf3, which suggests the existence of independent nonoverlapping sites for GSK3 and CK1ϵ binding on Tcf3. (D) CK1ϵ can phosphorylate Tcf3. Incubating Tcf3 with MBP-CK1ϵ enhances its phosphorylation. Endogenous kinase activity seen for Tcf3 alone reflects copurification of GSK3. (E) The enhancement of Tcf3 phosphorylation by CK1ϵ can be readily reversed by addition of CKI-7 (100 μM), a specific CK1 inhibitor, which indicates that Tcf3 is a substrate for both GSK3 and CK1ϵ. (F) Both GSK3 and CK1ϵ coimmunoprecipitates with myc-tagged Tcf3. Both cells of 2-cell embryos were injected with myc_6-Tcf3 RNA (500 pg/blastomere), homogenized at stage 7.5, and precipitated with either anti-myc antibodies coupled to beads or to control beads. Western blotting with antibodies against GSK3 and CK1e indicates that both proteins coimmunoprecipitates with myc-tagged Tcf3 protein.

sis using an anti-GSK3 antibody (unpublished data). To rule out the possibility that GSK3 gratuitously copurified with Tcf3 on the Ni^{2+} beads (which have ion exchange properties), we tested whether Tcf3 beads could be used to purify GSK3 from *Xenopus* extracts. We found that Tcf3 beads but not control BSA beads were capable of purifying GSK3 from *Xenopus* extracts (Fig. 6 B). No GSK3 was eluted from Tcf3 beads that were not preincubated in *Xenopus* extracts, which ruled out the possibility that the eluted GSK3 originated from *Sf9* cells during the purification of Tcf3.

CK1ϵ was demonstrated recently to be a positive regulator of wnt signaling (Peters et al., 1999; Sakanaka et al., 1999). Although these studies suggested physical associations between CK1ϵ and both axin (Sakanaka et al., 1999) and dsh (Peters et al., 1999; Sakanaka et al., 1999), the mechanism of action of CK1ϵ in wnt signaling has not been determined. A dominant negative dsh mutant (Xdd1) that lacks the putative CK1ϵ binding domain (PDZ domain) fails to block the ability of CK1ϵ to stimulate wnt signaling in vivo (Sokol, 1996), a result inconsistent with a model in which CK1ϵ mediates wnt signaling by binding to the PDZ domain of dsh. An analysis of the primary sequence of Tcf3 indicates that it contains multiple potential phosphorylation sites for both GSK3 and CK1ϵ. Furthermore, many of the phosphorylation sites for GSK3 and CK1ϵ are overlapping and are conserved within the Tcf family.

We examined the interaction of GSK3 and CK1ϵ with Tcf3. Radiolabeled GSK3 and CK1ϵ both significantly bound Tcf3 beads (Fig. 6, C and D). Furthermore, the binding sites of GSK3 and CK1ϵ on Tcf3 appear to be distinct from one another, since GSK3 binding cannot be competed with excess cold CK1ϵ. Conversely, the binding of CK1ϵ to Tcf3 beads is not abolished when incubated with excess cold GSK3. As with GSK3, CK1ϵ can phosphorylate recombinant Tcf3 in vitro. CK1ϵ can enhance the phosphorylation of Tcf3 in an in vitro kinase assay above the level due to that of contaminating GSK3 (Fig. 6 E). Furthermore, the enhanced Tcf3 phosphorylation by CK1ϵ is partially inhibited when CKI-7 (IC_{50}, 10–30 μM; purchased from Seikagaku Corporation), a selective inhibitor of CK1 activity (Chijiwa et al., 1989), is included in the kinase reaction. The association between Tcf3 and GKS3/CK1ϵ represents bona fide in vivo interactions, since both endogenous *Xenopus* GSK3 and CK1e immunoprecipitated with an anti-myc antibody from extracts made from embryos injected with myc_6-xTcf3 mRNA (Fig. 6 F). Although Tcf3 binds GSK3, it does not inhibit the activity of GSK3 against axin (Fig. 1 D).

Effects of Tcf3 phosphorylation on its activity

These experiments establish that both GSK3 and CK1ϵ can bind and phosphorylate Tcf3 and suggest a possible role for both GSK3 and CK1ϵ in modulating Tcf3 activity. We therefore tested if these kinases affect the interaction of Tcf3 with β-catenin. Tcf3 can inhibit the degradation of β-catenin (Fig. 1, A and B). CK1ϵ and Tcf3 act synergistically to inhibit β-catenin degradation: as shown in Fig. 7 A, concentrations of CK1ϵ and Tcf3 that by themselves are not inhibitory together cause significant inhibition of β-catenin degradation. In addition, CKI-7 blocks the effect of Tcf3 in a dose-dependent manner in *Xenopus* extracts (Fig. 7 B). As expected, 100 μM CKI-7 (even in the presence of 10 nM Tcf3) actually accelerates the rate of degradation of β-catenin in extracts compared with a buffer control. Thus, CK1ϵ activity is required for inhibition of β-catenin degradation by Tcf3. The simplest model consistent with these experimental results is that phosphorylation of Tcf3 by CK1ϵ promotes the interaction of Tcf3 with β-catenin. As shown in Fig. 7 C, preincubation of Tcf3 beads with CK1ϵ increases their affinity for β-catenin nearly fourfold compared with

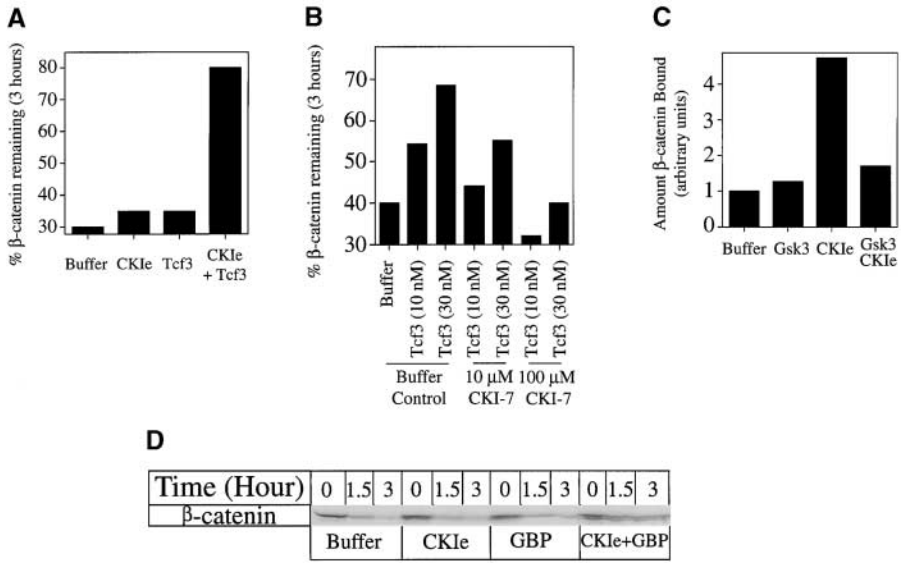

Figure 7. **Effect of CK1ε on Tcf3–β-catenin interaction.** (A) Tcf3 and CK1ε act synergistically to inhibit β-catenin degradation. His_6-Tcf3 (3 nM) and MBP-CK1ε (200 nM) were added to *Xenopus* extracts either alone or together. Inhibition of β-catenin degradation is dramatically enhanced by addition of both Tcf3 and CK1ε (nearly 80% remaining after 3 h) compared with addition of either Tcf3 or CK1ε alone (35% remaining after 3 h). (B) The CK1ε inhibitor CKI-7 inhibits the effect of Tcf3 or β-catenin stabilization. CKI-7 inhibits the effects of 10 and 30 nM Tcf3 in a dose-dependent manner. At high CKI-7 concentrations (100 μM), the effect of Tcf3 is abolished and β-catenin degradation is actually stimulated when compared with the buffer control. (C) CK1ε stimulates the binding of Tcf3 to β-catenin. Preincubation of Tcf3 beads with 1 μM CK1ε in kinase buffer stimulates its binding to β-catenin (compared with preincubation in buffer alone). This effect of CK1ε was decreased by addition of 1 μM GSK3 to the kinase reaction. GSK3 by itself had no effect on the binding of Tcf3 to β-catenin. A nearly fivefold increase in the binding of Tcf3 to β-catenin is seen when Tcf3 beads were preincubated with CK1ε. (D) CK1ε acts synergistically with GBP to inhibit β-catenin degradation. 50 nM GBP or 200 nM CK1ε has no effect on β-catenin degradation; however, together they dramatically inhibit β-catenin degradation in extracts.

untreated beads. Furthermore, although preincubating Tcf3 beads with GSK3 had very little effect on β-catenin binding, GSK3 abolished the effect of CK1ε. One prediction from these experiments is that blocking GSK3 activity would further potentiate the effect of CK1ε. As expected, GBP acts synergistically with CK1ε to inhibit β-catenin degradation (Fig. 7 D). Failure of GSK3 to inhibit the effects of Tcf3 in our earlier experiments (Fig. 1 B) was probably due to the high concentration of Tcf3 (1 μM) used. In fact, the effect of adding Tcf3 at concentrations closer to its IC_{50} for β-catenin degradation (100 nM) can be reversed by the addition of 1 μM GSK3 (Fig. 1 C).

In previous studies, injections of CKI-7 did not block the effects of overexpressed β-catenin in *Xenopus* embryos (Peters et al., 1999), which suggested that CK1ε acts upstream of β-catenin. However, it is unclear whether or not epistasis experiments performed by injecting components of the wnt pathway upstream of β-catenin (wnt and dsh) can boost the high level of β-catenin readily obtained by direct injection of β-catenin mRNA. It should be noted that the concentrations of CKI-7 used in our in vitro experiments is 10-fold greater than in these experiments and is still only sufficient to inhibit 50% of the CK1 activity. We found that injection of comparable concentrations of CKI-7 into *Xenopus* embryos were toxic and arrested cell division soon after injection.

Role of CK1ε in mediating dsh activity

Given the fact that CK1ε has been shown to bind axin (Sakanaka et al., 1999) and dsh (Peters et al., 1999), it appears that CK1ε like GSK3 simultaneously affects many components of the wnt pathway. Dsh acts synergistically with CK1ε in *Xenopus* embryos (Fig. 8 A). Embryos injected ventrally at the 4–8-cell stage with low doses of Dsh (100 pg) or CK1ε (50 pg) RNA develop normally (100%, $n = 30$), whereas injection of embryos with both Dsh and CK1ε resulted in axis duplication (68%, $n = 50$). Dsh acts synergistically with CK1ε to inhibit β-catenin degradation in *Xenopus* extracts (Fig. 8 B), consistent with the in vivo results. Preliminary experiments indicate that both GSK3 and CK1ε can bind the PDZ domain of dsh (unpublished data). Since GBP binds the PDZ domain of dsh, we tested whether CK1ε or GSK3 can alter the affinity of dsh for GBP. CK1ε stimulates the binding of radiolabeled dsh to GBP in *Xenopus* extracts four- to fivefold greater than in buffer- or GSK3-treated extracts (Fig. 8 C). This result indicates that in addition to promoting the association between Tcf3 and β-catenin, CK1ε stimulates the binding of GBP to dsh. Interestingly, in the absence of *Xenopus* extracts CK1ε has no effect on binding of dsh to GBP, suggesting that extracts contain an activity that mediates the effect of CK1ε on the dsh–GBP interaction.

Discussion

We have demonstrated both in vivo in *Xenopus* embryos and in vitro in *Xenopus* egg extracts that Tcf3 inhibits the rate of β-catenin degradation in a dose-dependent manner. We show that the inhibition of β-catenin degradation by Tcf3 protein has physiological relevance, since a significant fraction of the total Tcf protein resides in the cytoplasm in *Xenopus* eggs and early embryos and in cultured 293 cells. Thus, Tcf can effectively compete with the degradation machinery (axin/APC/GSK3 complex) for β-catenin binding.

The inhibitory effect of Tcf3 depends on its ability to bind β-catenin because an NH_2-terminal deletion mutant of Tcf3 that lacks the β-catenin binding site has no effect on β-catenin degradation. A β-catenin COOH-terminal deletion mutant (β-cateninΔC2) is regulated in a manner indistinguishable from the wild-type protein except it is no longer sensitive to Tcf3 and is degraded at a faster rate than

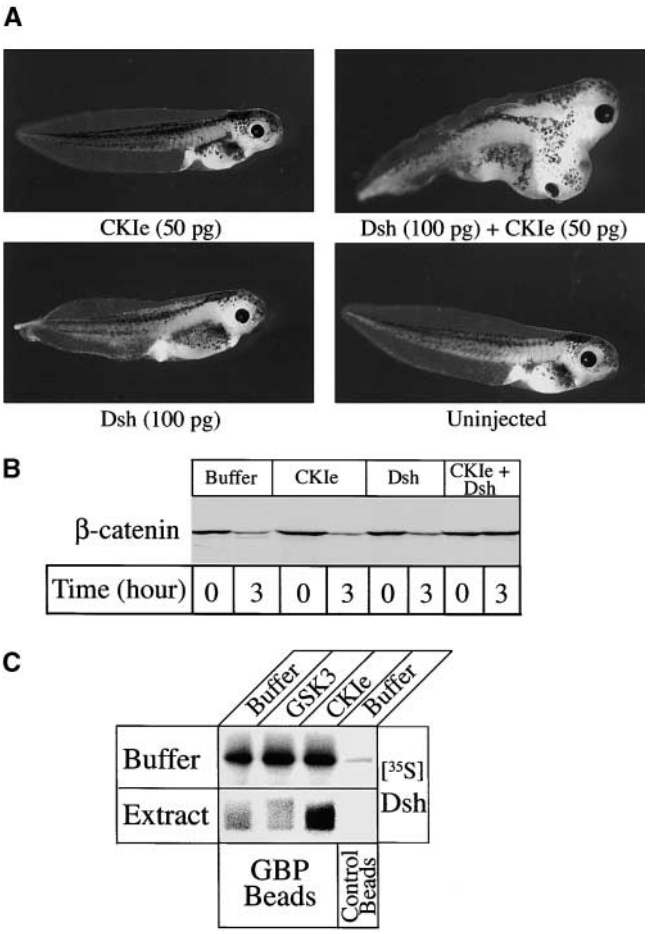

Figure 8. **Role of CK1ε in mediating dsh activity.** (A) dsh acts synergistically with CK1ε in *Xenopus* embryos to induce axis duplication. Embryos were injected with 50 pg CK1ε, 100 pg dsh, or 50 pg CK1ε plus 100 pg dsh RNA in one ventral blastomere of 4–8-cell stage embryos. (B) Dsh and CK1e act synergistically to inhibit β-catenin degradation. Dsh protein (100 nM) and CK1e protein (500 nM) were added to extracts alone or in combination. (C) GBP cross-linked beads were incubated with radiolabeled dsh and either buffer, 1 μM CK1ε, or 1 μM GSK3 in the presence or absence of *Xenopus* extracts. CK1ε stimulates the binding of GBP to radiolabeled dsh in *Xenopus* extracts. No differences were detected when binding was performed in the absence of extracts.

the full-length protein in *Xenopus* extracts. Based on experiments with this mutant, we defined a fragment of β-catenin (cat449/645) that can disrupt the interaction between β-catenin and Tcf but not the interaction of β-catenin with axin/APC. Addition of this purified fragment accelerates the rate of β-catenin degradation in both *Xenopus* extracts and embryos. Similarly, a peptide encoding the NH_2-terminal β-catenin binding region of Tcf4 (which also blocks the binding of β-catenin to Tcf3) stimulates β-catenin degradation. Our attempts to determine whether this peptide has any effect on axis formation have been unsuccessful because injections of this peptide into *Xenopus* embryos at concentrations sufficient to block Tcf3–β-catenin interaction severely perturbed gastrulation. Although the effects of the Tcf4 peptide and the cat449/645 fragment might seem modest, these results should be viewed in the context of the effects of known regulators of the wnt pathway (for example, axin and dsh), which alter the rate of β-catenin degradation only three- to fourfold.

Structure-function analysis of β-catenin (von Kries et al., 2000) and the x-ray crystal structure of its complex with the β-catenin binding domain of Tcf3 (Graham et al., 2000) indicate that the Tcf/Lef1 binding site of β-catenin forms a positively charged groove. This interacts with the β-catenin binding domain of Tcf3 consisting of an extended structure that can be divided into three sites. A comparison of this structure to β-cateninΔC2 indicates that this mutant is missing one of the flanking regions and part of the central region involved in Tcf–β-catenin interaction.

Our results suggest that cytoplasmic Tcf3 competes for β-catenin binding with both APC and axin (Fig. 8 A); however, once a trimeric complex consisting of β-catenin, axin, and APC is formed Tcf3 is unable to compete effectively for β-catenin binding (Fig. 2 B). By sequestering β-catenin in the cytoplasm or enhancing its export from the nucleus (Henderson, 2000), APC inhibits signaling through the wnt pathway by further blocking assembly of the β-catenin–Tcf3 bipartite complex. Therefore, in the absence of APC the effect of Tcf proteins on wnt signaling would predominate. Loss of APC function would not only result in an increase in the amount of free β-catenin available to bind Tcf3 but would also allow Tcf3 to compete effectively with axin for β-catenin binding.

Factors that change the concentration of Tcf3 protein or its affinity for β-catenin can alter the amount of the β-catenin–Tcf complex. Decreases or increases in the ratio of soluble β-catenin to Tcf3 can modulate the transcription level of target genes, since Tcf3 acts as a transcriptional repressor in the absence of β-catenin and as a transcriptional activator when bound to β-catenin (Cavallo et al., 1998; Roose et al., 1998). A model of the wnt pathway in which the level of signaling is continually being modulated (rather than simply being in the "on" or "off" position) is an attractive one. In fact, experiments in which cells have been transfected with axam (Kadoya et al., 2000), an axin binding protein that blocks its interaction with dsh, show a reduced level of β-catenin. This result indicates that even in the absence of ligand a significant amount of signal modulation occurs in the wnt pathway.

A major mechanism by which the rate of β-catenin degradation is regulated by a wnt ligand is through its control of GSK3 kinase activity (Fig. 8 B). Previous studies of GSK3 have indicated that it controls nearly every level of β-catenin degradation: (a) phosphorylation of APC to facilitate its interaction with β-catenin, (b) phosphorylation of axin to promote its stability and possibly to increase its affinity for β-catenin, and (c) phosphorylation of β-catenin so as to allow recognition by the SCF ubiquitin ligase complex. Studies using promoter-based assays to assess the activation state of the wnt pathway have found disparities between the steady-state levels of β-catenin and the transcriptional activation of reporter constructs. Using a biochemical approach, we have revealed an additional role for GSK3 in the wnt pathway that may account for these discrepancies: GSK3 phosphorylates Tcf3 thereby decreasing its affinity for β-catenin and antagonizing the activity of CK1ε. This model helps explain why insulin growth factor (IGF) 1 can enhance the stability

of β-catenin but not transcriptional activation of downstream targets (Playford et al., 2000), whereas addition of the GSK3 inhibitor lithium resulted in a dramatic increase in β-catenin–Tcf–mediated transcriptional activation. IGF-1 promotes the stability of β-catenin by stimulating its tyrosine phosphorylation without affecting the activity of GSK3. Increases in β-catenin levels mediated by IGF-1 may not be sufficient to overcome the inhibitory effect of GSK3 on β-catenin–Tcf interaction. It will be interesting to explore the biochemical state of Tcf in early *Xenopus* development and its contribution to wnt signaling, particularly the role of its interactions with GSK3 and CK1ε.

Our initial experiments indicated that GSK3 does not readily reverse the inhibitory effect of Tcf3 at high concentration (1 μM), but subsequent experiments revealed that GSK3 reverses the effect of Tcf3 at lower concentrations that are close to the IC_{50} of Tcf3 for β-catenin degradation. These conflicting results demonstrate the subtleties of these experiments and the hazards inherent in overexpression studies and highlight the advantages of using a biochemical system to study the wnt pathway. The concentrations of individual components can be manipulated so as to reveal regulations that might otherwise be overlooked in experiments involving overexpression of components by transfection of DNA into cultured cells or injection of RNAs into embryos. CK1ε is both necessary and sufficient for wnt signaling (Peters et al., 1999; Sakanaka et al., 1999). Our results suggest that one of the roles of CK1ε in wnt signaling is to phosphorylate Tcf3 thereby increasing its affinity for β-catenin. In *Xenopus* extracts, CK1ε and Tcf3 synergize to inhibit β-catenin degradation. Studies with the CK1ε inhibitor CKI-7 (Fig. 6 C) indicate that CK1ε regulates the formation of the β-catenin–Tcf3 complex: CKI-7 blocks the inhibitory effect of Tcf3 on β-catenin degradation and therefore increases the rate of β-catenin degradation above basal levels. The mechanism by which GSK3 and CK1ε oppose each other's actions is not known. Intriguingly, our analysis of the crystal structure of β-catenin complexed with the β-catenin binding domain of Tcf3 (Graham et al., 2000) indicates that one of the three β-catenin binding regions of Tcf3 (amino acids 49–64 of *Xenopus* Tcf3) contains a conserved and overlapping phosphorylation site for both GSK3 and CK1ε (which is also present in other members of the Tcf/LEF-1 family that bind β-catenin). This may indicate that binding and/or phosphorylation of Tcf3 by one kinase may preclude the binding and/or phosphorylation of Tcf3 by the other kinase.

The interaction between β-catenin and Tcf appears to be regulated in multiple ways. In *Drosophila*, the CBP acetylates a lysine in the β-catenin binding domain of dTcf, thereby lowering its affinity for β-catenin (Waltzer and Bienz, 1998). However, in vertebrates CBP has been shown to bind to the COOH-terminal region of β-catenin to activate Tcf-mediated gene transcription (Takemaru and Moon, 2000). Certain Sox proteins have been shown to interact with β-catenin and block its interaction with Tcf proteins (Zorn et al., 1999). ICAT, a β-catenin interacting protein inhibits the interaction of β-catenin with Tcf4 and represses β-catenin–Tcf4–mediated gene transcription (Tago et al., 2000). In *C. elegans*, the LIT-1 protein phosphorylates POP-1, a Tcf/LEF-related protein, to downregulate its activity (Rocheleau et al., 1999). Interestingly, phosphorylation of POP-1 requires an active LIT-1 kinase complex containing WRM-1, a β-catenin–like homologue belonging to a divergent wnt pathway. Finally, the bipartite β-catenin–Tcf complex can directly bind Smad4 to stimulate expression of a target gene, *twin* (Nishita et al., 2000). Each of these interactions could also modulate β-catenin stability, leading to pleiotropic and often synergistic effects in wnt signaling. Similarly to GSK3, CK1ε appears to regulate the wnt pathway at more than one level. CK1ε stimulates the binding of dsh to GBP in *Xenopus* extracts. Although CK1e can directly bind dsh, the mechanism by which CK1ε promotes dsh–GBP interaction is not clear, since this effect of CK1ε was only detected in the presence of *Xenopus* extracts. Interestingly, the amount of dsh bound to GBP in the presence of *Xenopus* extracts was much less than the amount of dsh bound to GBP in the absence of extracts. Dsh is heavily phosphorylated in *Xenopus* extracts (unpublished data), which possibly inhibits its binding to GBP. Addition of CK1ε to *Xenopus* extracts increases the binding of dsh to GBP to levels comparable to that seen in the absence of extract. These results suggest the existence of an as yet unidentified factor(s) that functions to inhibit the binding of GBP to dsh; one role of CK1ε may be to counteract the activity of this factor.

Both Tcf and β-catenin are required for axis formation in *Xenopus* development. β-catenin is stabilized on the dorsal side of the embryo during early development, and when zygotic transcription starts at the midblastula transition, β-catenin translocates into the nuclei of dorsal cells. How β-catenin stabilization and nuclear translocation on the dorsal side takes place is still unclear, although both processes are perhaps essential for axis formation. Regulation of β-catenin stability by Tcf might play an important role in these processes.

Materials and methods

Egg extracts

Xenopus egg extracts were prepared as described previously using a modified version of a method used for making cell cycle extracts (Salic et al., 2000). Centrifugation was performed at 4°C in a microfuge. After a packing spin (30 s at 30 *g*), the eggs were crushed at 21,000 *g* for 5 min. The cytoplasmic layer was removed and spun two more times at 21,000 *g* for 5 min. After the addition of protease inhibitors and an energy regeneration cocktail (energy mix) (Murray and Kirschner, 1989), extracts were either used immediately or snap-frozen in liquid nitrogen and stored at −80°C.

Immunoprecipitation of myc_6-Tcf3

Both cells of 2-cell embryos were injected with myc_6-Tcf3 RNA (500 pg/blastomere) and homogenized at stage 7.5 by resuspending the embryos three times with a clipped P-200 pipet tip (cut so that the opening was slightly smaller than the diameter of an embryo). The lysate (100 μl) was incubated with 25 μl of anti-myc antibody-coupled beads (Santa Cruz Biotechnology, Inc.) or control beads for 2 h at 4°C. Beads were washed and the samples eluted (see Binding assay). The eluted samples were subjected to SDS-PAGE followed by Western blotting using an anti-myc antibody (9E10; Santa Cruz Biotechnology, Inc.), an anti-GSK3β antibody (Transduction Laboratory), and an anti-CK1ε antibody (Transduction Laboratory).

Tcf fractionation

Egg and embryonic lysates were prepared as described above. Supernatants were obtained by centrifuging the lysates for 2 h at 100,000 *g* at 4°C. Equivalent volumes of lysate and supernatant were subjected to SDS-PAGE followed by Western blotting using either an anti-myc antibody (9E10; Santa Cruz Biotechnology, Inc.) or an anti-Tcf monoclonal antibody (Exalpha Biological). Nuclear and cytoplasmic extracts were prepared from cultured 293 cells as described previously (Heintz and Stillman, 1988) with modifica-

tions. Cells were washed in a cold hypotonic buffer (lysis buffer; 25 mM Hepes, pH 7.7, 5 mM CH_3COOK, 0.5 mM $MgCl_2$, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 100 μM PMSF) and disrupted with 25 strokes in a dounce homogenizer. Nuclei were pelleted at 4,000 rpm for 5 min in an Eppendorf centrifuge. The supernatant was removed, recentrifuged at 4,000 rpm for 5 min, and transferred to a new tube. The nuclear pellet was washed twice with PBS and resuspended in lysis buffer with a volume equivalent to the supernatant fraction. SDS-PAGE and Western blotting was performed on the nuclear and cytoplasmic fractions using an anti–topoisomerase II monoclonal antibody (StressGen Biotechnologies) and an anti-Tcf monoclonal antibody (Exalpha Biological).

mRNA synthesis, translation in extracts, and degradation assays

Capped mRNAs were synthesized in vitro from linearized plasmid DNA templates, purified using RNAeasy spin columns (QIAGEN), and resuspended in water to 0.2–0.5 mg/ml. For experiments requiring translations, fresh extracts prepared as described above were supplemented with placental ribonuclease inhibitor (Promega) (1:100 ratio), mixed with mRNA to a final concentration of 50–100 ng/μL, and incubated for 1–2 h at room temperature. Translations were terminated by the addition of cycloheximide (100 μg/mL) and were either used immediately in degradation assays or snap-frozen in liquid nitrogen and stored at −80°C.

Degradation assays

For degradation assays, 6–8 μL of *Xenopus* egg extracts were supplemented with 0.1 μL cycloheximide (10 mg/mL), 0.2 μL energy mix, 0.2 μL purified bovine ubiquitin (14 mg/mL), and 0.1–0.3 μL [^{35}S]methionine-labeled β-catenin. The reactions were incubated at room temperature for 3 h, and 1-μL aliquots were removed at 0, 1, and 3 h for analysis by SDS-PAGE and autoradiography.

Plasmids and recombinant proteins

Xenopus Tcf3, ΔNTcf3 (Molenaar et al., 1996), dsh, and β-catenin were subcloned by PCR in the pCS2+ vector. β-catenin deletion mutants were constructed by PCR in pCS2+ and verified by in vitro translation of a protein of the appropriate size. *Xenopus* GBP and CK1ε were cloned from a stage 14 *Xenopus* plasmid library. MBP-GBP, MBP-cat449/645 (amino acids 449–645 of *Xenopus* β-catenin), and MBP-CKIε were expressed in bacteria. His-tagged *Xenopus* Tcf3 (his_6-Tcf3), his_6-ΔN Tcf3 (lacking amino acids 1–31 of *Xenopus* Tcf3), his_6-APCm3 (amino acids 1342–2075 of *Xenopus* APC), and his_6-GSK3 were produced in baculovirus-infected *Sf9* cells.

Kinase reactions

Kinase reactions were performed for 30 min at room temperature in kinase buffer (20 mM Hepes, pH 7.5, 300 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 0.2% Tween-20, 50 μM ATP, and 0.25 μCi/μL [γ-^{32}P]ATP). For the phosphorylation of Tcf3 beads by CK1ε and GSK3, Tcf3 beads (5 μl) were incubated with 1 μM GSK3 or MBP-CK1ε in a 50-μl reaction containing 20 mM Hepes, pH 7.5, 300 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 0.2% Tween-20, and 2 mM ATP. Reactions were performed for 1 h at room temperature with rotation. Beads were subsequently washed with 3× 1 ml of binding buffer (see below) before addition of β-catenin.

Binding assays

Binding reactions using MBP fusion proteins were performed with the fusion proteins bound to amylose beads (New England Biolabs, Inc.). Other proteins were immobilized by cross-linking to Ultralink beads (Pierce Chemical Co.). For binding experiments performed in extracts, *Xenopus* egg extracts were diluted 1:5 in buffer containing 20 mM Hepes, pH 7.5, 300 mM NaCl, 1% Tween-20, 1 mg/mL BSA, and protease inhibitors. Binding to purified or in vitro–translated proteins ([^{35}S]methionine-labeled using the Promega TNT kit) was performed in the same buffer (2 h at 4°C). After incubation, beads were washed with 3 mL of buffer A (20 mM Hepes, pH 7.5, 300 mM NaCl, 1% Tween-20), 3 mL of buffer B (20 mM Hepes, pH 7.5, 400 mM NaCl, 1% Tween-20), and 3 mL of buffer C (20 mM Hepes, pH 7.5, 50 mM NaCl, 1% Tween-20). Bound proteins were eluted with hot sample buffer and analyzed by SDS-PAGE followed by either autoradiography or Western blotting. Western analysis of GSK3 was performed using a monoclonal antibody obtained from Transductions Laboratories. NH_2-terminal Tcf4 peptide was obtained from Zymed Laboratories.

β-catenin–luciferase assays

10 ng of recombinant β-catenin–luciferase protein (1 mg/ml in 20 mM Hepes, pH 7.4, and 50 mM NaCl) purified from baculovirus-infected *Sf9* cells was injected into 2-cell stage *Xenopus* embryos and processed as described previously (Salic et al., 2000).

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