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Control of β-Catenin Phosphorylation/Degradation by a Dual-Kinase Mechanism

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Summary

Wnt regulation of β-catenin degradation is essential for development and carcinogenesis. β-catenin degradation is initiated upon amino-terminal serine/threonine phosphorylation, which is believed to be performed by glycogen synthase kinase-3 (GSK-3) in complex with tumor suppressor proteins Axin and adnomatous polyposis coli (APC). Here we describe another Axin-associated kinase, whose phosphorylation of β-catenin precedes and is required for subsequent GSK-3 phosphorylation of β-catenin. This "priming" kinase is casein kinase Iα (CKIα). Depletion of CKIα inhibits β-catenin phosphorylation and degradation and causes abnormal embryogenesis associated with excessive Wnt/β-catenin signaling. Our study uncovers distinct roles and steps of β-catenin phosphorylation, identifies CKIα as a component in Wnt/β-catenin signaling, and has implications to pathogenesis/therapeutics of human cancers and diabetes.

Introduction

Wnt signaling via β-catenin plays a central role in development and homeostasis (Wodarz and Nusse, 1998; Moon et al., 1997; Polakis, 2000). A key output of this pathway is the level of cytosolic β-catenin, which determines the activation of Wnt responsive genes. Without Wnt stimulation, β-catenin is constantly degraded by the proteosome (Orford et al., 1997; Aberle et al., 1997). This degradation strictly depends upon β-catenin phosphorylation, which occurs in a multiprotein complex composed of tumor suppressor proteins adnomatous polyposis coli (APC), Axin, and glycogen synthase kinase-3 (GSK-3; Zeng et al., 1997; Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Sakanaka et al., 1998; Itoh et al., 1998; Salic et al., 2000; reviewed in Kikuchi, 1999). It is believed that in this complex assembled by Axin, GSK-3 phosphorylates the β-catenin amino-terminal region (Peifer et al., 1994; Yost et al., 1996), thereby earmarking β-catenin for ubiquitination-dependent proteolysis (Winston et al., 1999; Kitagawa et al., 1999; Hart et al., 1999; Latres et al., 1999; Liu et al., 1999). Wnt signaling is suggested to inhibit β-catenin phosphorylation, thus inducing the accumulation of cytosolic β-catenin, which associates with the TCF/LEF (T cell factor/lymphocyte enhancer factor) family of transcription factors to activate Wnt/β-catenin-responsive genes (Molenaar et al., 1996; Behrens et al., 1996; Huber et al., 1996). Thus, β-catenin phosphorylation controls β-catenin protein level and Wnt signaling.

Four serine (S)/threonine (T) residues (S33, S37, T41, and S45) at the amino-terminal region of β-catenin (Figure 1A) are conserved from *Drosophila* to human and conform to the consensus GSK-3 phosphorylation site (Peifer et al., 1994). Indeed, β-catenin can be phosphorylated by GSK-3 in vitro (Yost et al., 1996), and these phospho-S/T residues are critical for β-catenin recognition by the F box protein β-Trcp, which is the specificity component of an ubiquitination apparatus (Winston et al., 1999; Kitagawa et al., 1999; Hart et al., 1999; Latres et al., 1999; Liu et al., 1999). The significance of S33, S37, T41, and S45 phosphorylation in β-catenin degradation is underscored by the observation that mutations at these S/T residues frequently occur in human colorectal cancer and several other malignancies (Figure 1A), which are associated with and most likely caused by deregulated accumulation of β-catenin (Morin et al., 1997; Korinek et al., 1997; Rubinfeld et al., 1997; Polakis, 2000). However, despite the critical importance of these S/T phosphorylation events in regulating β-catenin stability, surprisingly little is known about how β-catenin is phosphorylated in vivo.

Using a panel of antibodies that specifically recognize β-catenin phosphorylated at different S/T residues, we found, unexpectedly, that β-catenin phosphorylation in vivo requires CKIα, whose phosphorylation of β-catenin precedes and is obligatory for subsequent GSK-3 phosphorylation of β-catenin. Depletion of CKIα prevents β-catenin phosphorylation and degradation. These findings have important implications to the understanding of Wnt/β-catenin signaling, GSK-3 and CKIα function, and the pathogenesis and therapeutics of diseases.

Results

Distinct Roles and Steps of β-Catenin Phosphorylation

β-catenin is recognized by β-Trcp in a phosphorylation-dependent manner (Winston et al., 1999; Kitagawa et al., 1999; Hart et al., 1999; Latres et al., 1999; Liu et al., 1999). We showed that the β-catenin (S>A) mutant, which harbors alanine substitutions at S33, S37, T41, and S45, is not recognized by β-Trcp (Liu et al., 1999).

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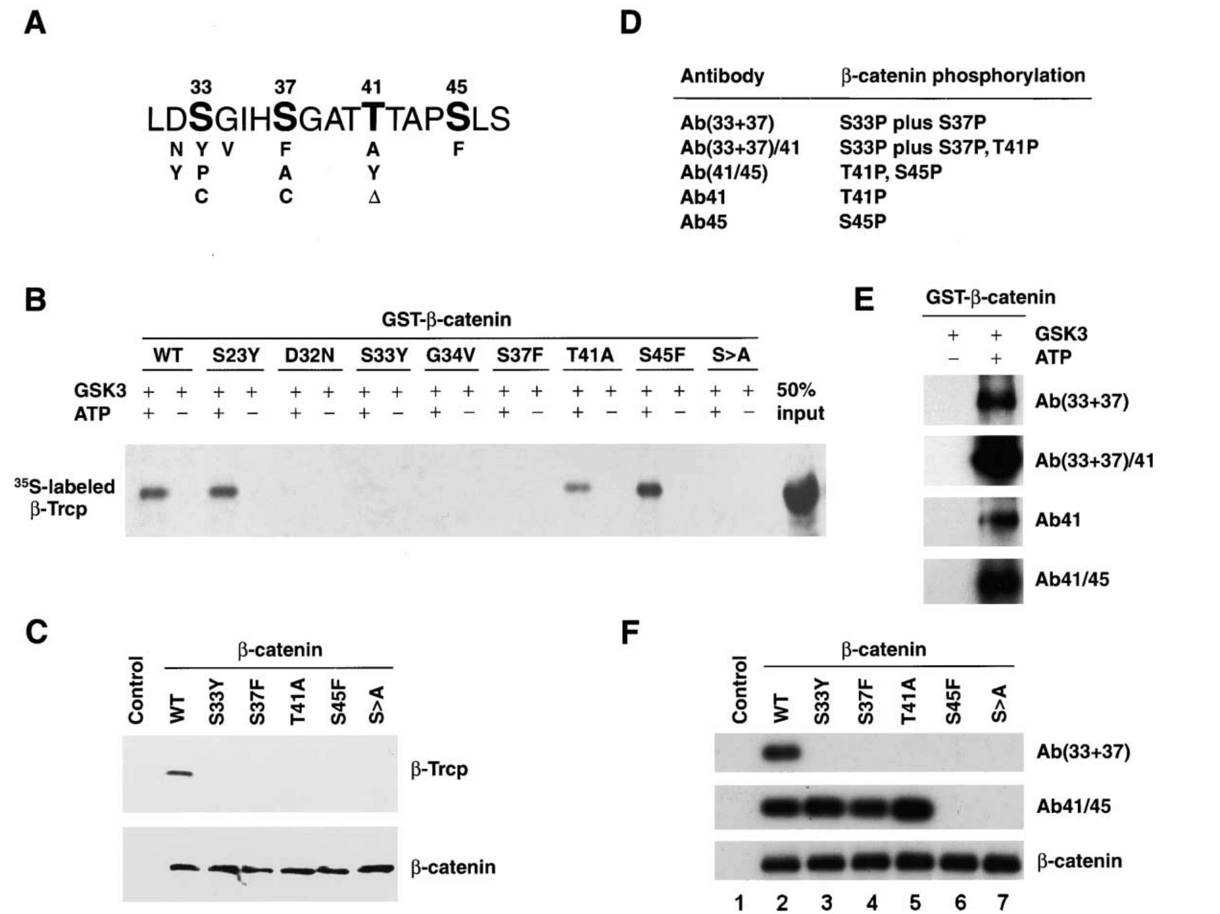

Figure 1. Distinct Roles and Steps of β-Catenin Phosphorylation

(A) Human β-catenin amino-terminal region containing S33, S37, T41, and S45. Examples of single amino acid substitutions or deletions (Δ) found in cancers are below the wild-type sequence.

(B) S33 and S37 phosphorylation constitutes β-Trcp recognition motif. Purified GST-β-catenin and mutants in equal amounts were each incubated with recombinant human GSK-3β in vitro with (+) or without (−) ATP and were then used to precipitate S^{35}-labeled β-Trcp. β-catenin was recognized by β-Trcp in a phosphorylation-dependent fashion. Mutations at D32, S33, G34, or S37 abolished this recognition, whereas mutations at T41, S45, or S23 (a control) did not. β-catenin (S>A) mutant, in which S33, S37, T41, and S45 were replaced by alanine, was not bound by β-Trcp.

(C) S33, S37, T41, and S45 are required for β-catenin recognition by β-Trcp in vivo. The wild-type or mutant β-catenin (Flag-tagged) was coexpressed in *Xenopus* embryos with β-Trcp (Myc-tagged). The wild-type β-catenin, but none of the mutants, coprecipitated β-Trcp (detected by a Myc antibody [Ab], top gel). Precipitated β-catenin and mutants were detected by a Flag Ab (bottom gel). Control indicates no injection. In the reverse precipitation, only the wild-type β-catenin was coimmunoprecipitated with β-Trcp (not shown).

(D) Abs for phosphorylated β-catenin.

(E) Purified GST-β-catenin was phosphorylated by GSK-3β with or without ATP as in (B), resolved by SDS-PAGE, and immunoblotted by indicated Abs, all of which only recognized phosphorylated β-catenin. Specific phosphoresidues recognized were determined using synthetic phosphopeptides (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/108/6/837/DC1).

(F) Differential steps and dependence of S45, T41, S37, and S33 phosphorylation in vivo. β-catenin and mutants (Flag) were individually expressed in *Xenopus* embryos and precipitated by a Flag Ab (bottom gel). The phosphorylation status was examined via blotting the precipitates with indicated Abs. Mutations at either T41 or S45 abolished phosphorylation at S33 and S37 (top gel, lanes 5 and 6), but neither mutations at S33 or S37 affected T41 or S45 phosphorylation (middle gel, lanes 3 and 4). The S45 mutation prevented T41 phosphorylation, but the T41 mutation did not prevent S45 phosphorylation (middle gel, lanes 5 and 6). Control indicates no injection.

We thus examined how β-catenin single-residue mutations found in cancer cells (Figure 1A) affect its recognition by β-Trcp. In a phosphorylation-coupled binding assay in vitro (Liu et al., 1999), single-residue substitutions at or surrounding S33 or S37 abolished phosphorylation-dependent recognition of β-catenin by β-Trcp, whereas mutations at T41 or S45 did not (Figure 1B). This result is consistent with the observation that a synthetic peptide containing phospho-S33 and -S37 binds to β-Trcp (Winston et al., 1999). Thus, phosphorylated S33 and S37, together with neighboring residues, constitute the recognition motif for β-Trcp. Indeed, S33, S37, and surrounding residues, but not T41 and S45, resemble the β-Trcp recognition motif in the transcription inhibitor IκB (Orford et al., 1997), which is also recognized and degraded in a phosphorylation-dependent fashion by β-Trcp (Winston et al., 1999; Spencer et al., 1999).

In contrast to these in vitro binding data, we found

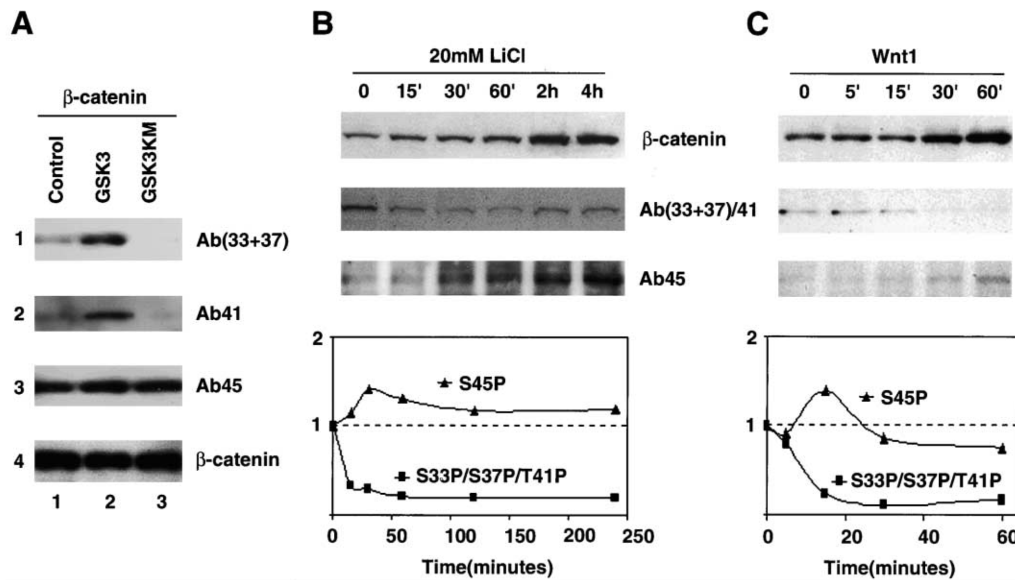

Figure 2. β-Catenin Phosphorylation at T41, S37, and S33 Depends on GSK-3 and Is Regulated by Wnt Signaling, whereas S45 Phosphorylation Requires a Distinct S45 Kinase

(A) Overexpression of GSK-3 increased phosphorylation at S33, S37 (gel 1), and T41 (gel 2) but not at S45 (gel 3) (compare lanes 1 and 2). GSK-3KM inhibited phosphorylation at S33, S37, and T41 (gels 1 and 2) but not at S45 (gel 3) (compare lanes 1 and 3). β-catenin (Flag) was expressed alone (control, lane 1) or coexpressed with GSK-3β (lane 2) or GSK-3βKM (lane 3) in *Xenopus* embryos and precipitated by a Flag Ab (panel 4). β-catenin phosphorylation was examined via blotting the precipitates with indicated Abs.

(B and C) LiCl- and Wnt-1-inhibited phosphorylation at S33/S37/T41, but not at S45 of the endogenous β-catenin in Rat2 cells. Cells were treated with (B) LiCl- (final concentration 20 mM) or with (C) Wnt-1-conditioned medium. Cytosolic extracts were prepared at indicated time points and analyzed for β-catenin protein level (top), phosphorylation at S33/S37/T41 (middle), or at S45 (bottom) via immunoblotting. Either LiCl or Wnt-1 increased β-catenin level. In the two graphs, β-catenin S33/S37/T41 or S45 phosphorylation is presented as a ratio between phosphorylation versus β-catenin protein level. The starting ratio at the control point (0 min) was set at 1 for both curves, which represented averages of at least three independent experiments.

that, in vivo, mutations at T41 and S45 also prevented β-Trcp binding to β-catenin in a manner indistinguishable from mutations at S33 and S37 (Figure 1C). These results explain why each of these mutations leads to deregulated β-catenin stabilization in cancer cells and further suggest that T41 and S45 may play a critical regulatory role in β-catenin phosphorylation and/or β-Trcp/β-catenin interaction. One possibility is that β-catenin phosphorylation at S33 and S37 depends on T41 and S45 phosphorylation, and this dependency is lost in vitro when excess GSK-3 is provided. Indeed, some in vitro studies suggest that GSK-3 is a processive kinase that sequentially phosphorylates S/T pentad-repeats from the carboxy- to amino-terminal direction (Harwood, 2001; Woodgett, 2001; Cohen and Frame, 2001).

To examine how β-catenin is phosphorylated in vivo, we generated a panel of antibodies (Abs) that specifically recognize β-catenin phosphorylated at different S/T residues (Figures 1D and 1E). Ab(33+37) detects phosphorylation at both S33 and S37; so does Ab (33+37)/41, which, in addition, also detects T41 phosphorylation to some extent. Ab(41/45) recognizes phosphorylation at either T41 or S45; Ab41 recognizes phosphorylated T41, and Ab45 detects phosphorylated S45 (Figures 1D and 1E; see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/108/6/837/DC1). We found that, in vivo, while the wild-type β-catenin was phosphorylated at both S33 and S37, either T41 or S45 mutation abolished S33 and S37 phosphorylation (Figure 1F). By contrast, neither S33 nor S37 mutation affected T41 or S45 phosphorylation (Figure 1F). Furthermore, T41 mutation did not effect S45 phosphorylation, but the S45 mutation prevented T41 phosphorylation (Figure 1F). These results suggest that β-catenin phosphorylation in vivo occurs in the carboxy- to amino-terminal direction, starting from S45 to T41, and then to S37 and S33, and the prior phosphorylation event is necessary for subsequent phosphorylation events to occur.

β-Catenin Phosphorylation by GSK-3 and an "S45" Kinase In Vivo

We then examined whether GSK-3 is responsible for S45, T41, S37, and S33 phosphorylation. Others and we have shown that the dominant-negative GSK-3 mutant, GSK-3KM, mimics, whereas the wild-type GSK-3 antagonizes, Wnt/β-catenin signaling (Pierce and Kimelman, 1995; Dominguez et al., 1995; He et al., 1995). Indeed, we found that GSK-3 enhanced, whereas GSK-3KM inhibited, T41, S37, and S33 phosphorylation (Figure 2A). Surprisingly, neither GSK-3 nor GSK-3KM had any effect on S45 phosphorylation (Figure 2A), implying that S45 is phosphorylated by a distinct kinase, which likely represents a so-called priming kinase (Harwood, 2001; Woodgett, 2001; Cohen and Frame, 2001). Although GSK-3 phosphorylation of some substrates, such as glycogen synthase, requires priming phosphorylation, it

is widely believed that the proximity of β-catenin and GSK-3 in the Axin complex forfeits priming phosphorylation (reviewed in Weston and Davis, 2001; Cohen and Frame, 2001).

We further examined whether phosphorylation of S45 and T41/S37/S33 of the endogenous β-catenin is differentially regulated. LiCl is a potent GSK-3 inhibitor (Stambolic et al., 1996; Hedgepeth et al., 1997) and indeed suppressed T41/S37/S33 but not S45 phosphorylation (Figure 2B). Wnt signaling is suggested to inhibit GSK-3 phosphorylation of β-catenin, and indeed, Wnt-1-conditioned medium blocked T41/S37/S33, but not S45, phosphorylation (Figure 2C). These results provided in vivo evidence and kinetics for Wnt inhibition of GSK-3 phosphorylation of β-catenin and further supported the existence of a "S45" kinase, whose regulation is distinct from that of GSK-3.

Stimulation of S45 Kinase Activity by Axin

We next examined how S45 phosphorylation can be modulated. Axin is a scaffolding protein that assembles a multiprotein complex devoted to β-catenin phosphorylation/degradation (Figure 3A; Kikuchi, 1999). Interestingly, we found that expression of Axin stimulated phosphorylation of S45 and T41/S37/S33 (Figure 3B), implying that the S45 kinase, like GSK-3, may be a component of the Axin complex. We mapped the Axin domain that harbors the stimulatory activity for S45 phosphorylation. Neither the RGS domain, which mediates Axin-APC association, nor the DIX domain, which is involved in Axin interaction with Dishevelled (Li et al., 1999; Itoh et al., 2000; Salic et al., 2000) and with the Wnt coreceptor LRP5/6 (Mao et al. 2001), was required for S45 phosphorylation (Figures 3A–3C). Importantly, the GSK3 binding domain of Axin was also dispensable for S45 phosphorylation (Figures 3A and 3C). By contrast, deletion of Axin's β-catenin binding domain (AxinΔβcat) or the adjacent carboxyl region (AxinΔS45K) severely diminished the ability of Axin to promote β-catenin S45 phosphorylation (Figures 3A and 3C). In fact, the Axin fragment that contains the β-catenin binding domain plus the adjacent carboxyl domain, Axin(βcat+S45K), was sufficient to stimulate S45 phosphorylation, whereas the Axin fragment containing solely the β-catenin binding domain, Axin(βcat), was not (Figures 3A and 3D). These results suggest that, in addition to recruiting GSK-3 and β-catenin together, Axin may also bring the S45 kinase into the proximity of β-catenin, thereby promoting sequential phosphorylation of β-catenin by the S45 kinase and GSK-3.

Identification of the S45 Kinase as Casein Kinase Iα (CKIα)

We set out to purify the S45 kinase from rat brain extracts, which exhibited strong S45 kinase activity (Figure 4A). This activity was not depleted by an affinity column composed of β-catenin (thus, does not bind to β-catenin), or of the Axin fragment containing the GSK-3 binding domain, Axin(G3+βcat) (Figure 3A), which depleted both isoforms of GSK-3, α and β, from the brain extract (Figures 4A–4C). Thus, the S45 kinase is again distinct from GSK-3. By contrast, the S45 kinase activity was significantly diminished (Figure 4D) by depletion with Axin(βcat+S45K) (Figure 3A), which contains the S45 phosphorylation stimulatory activity, suggesting that S45 kinase indeed binds to Axin. We fractionated the S45 kinase activity by sequential chromatography via SP- and Blue-Sepharose columns (Figures 4E and 4F). Silver staining of the eluted fractions from the Blue-Sepharose column identified a protein band of about 37 kDa that correlated with the S45 kinase activity (Figure 4F). Further, this 37 kDa protein band could be depleted by Axin(βcat+S45K) that depleted the S45 kinase activity (Figure 4G). Mass spectrum analysis of the excised 37 kDa band revealed six peptide fragments that show perfect matches to the serine/threonine kinase CKIα (Figure 4H). Indeed, immunoblotting demonstrated that CKIα, which is 37 kDa, was copurified with the S45 kinase activity (Figure 4F) and was depleted by Axin-(βcat+S45K) (not shown).

CKIα Is Essential for β-Catenin Phosphorylation Degradation

We first confirmed that CKIα is a component of the Axin complex. We immunoprecipitated either overexpressed or the endogenous Axin from mammalian cells and detected the endogenous CKIα (and GSK-3) associated with Axin (Figures 5A and 5B). Further, Axin(βcat+S45K) but not Axin(βcat) precipitated the endogenous CKIα (Figure 5C). We next examined whether CKIα is able to phosphorylate β-catenin at S45 in vivo. Expression of CKIα increased S45 phosphorylation, whereas expression of a related but distinct member of the CKI family, CKIε, did not (Figure 5D). We then asked whether CKIα is required for S45 phosphorylation in vivo. We depleted the endogenous CKIα protein in 293T cells via double-strand (ds) RNA-mediated interference (RNAi; Elbashir et al., 2001). A ds RNAi oligo for CKIα significantly reduced the amount of CKIα protein (Figure 6A) and inhibited S45 phosphorylation (Figure 6B). CKIα RNAi also inhibited β-catenin S33/S37/T41 phosphorylation (Figure 6C), which depends on S45 phosphorylation. Most significantly, CKIα RNAi resulted in significant β-catenin protein accumulation (Figure 6D). CKIα RNAi did not alter the protein level of CKIε, GSK-3α and GSK-3β (Figures 6E and 6F), or tubulin (not shown), demonstrating the specificity of RNAi and further supporting the notion that reduction of S33/S37/T41 phosphorylation by CKIα RNAi was due to inhibition of S45 phosphorylation. As a negative control, the single-strand sense RNAi oligo had little effect on CKIα protein amount, β-catenin S45 phosphorylation, and the protein level (Figures 6A–6F).

CKIε was recently shown to stimulate β-catenin signaling (Peters et al., 1999; Sakanaka et al., 1999), thus functioning in a manner that is opposite to that of CKIα. We also depleted CKIε via a ds RNAi oligo, which significantly reduced the protein level of CKIε, but not of CKIα or tubulin (Figures 6G–6I). Contrary to depletion of CKIα, depletion of CKIε exhibited no effect on β-catenin phosphorylation or protein level (Figures 6J–6L). Thus, CKIα and CKIε have distinct functions in β-catenin signaling.

CKIα Is a Segment Polarity Gene in *Drosophila*

Wnt/β-catenin signaling is conserved from *Drosophila* to human (Wodarz and Nusse, 1998; Moon et al., 1997). We thus examined whether CKIα regulates degradation

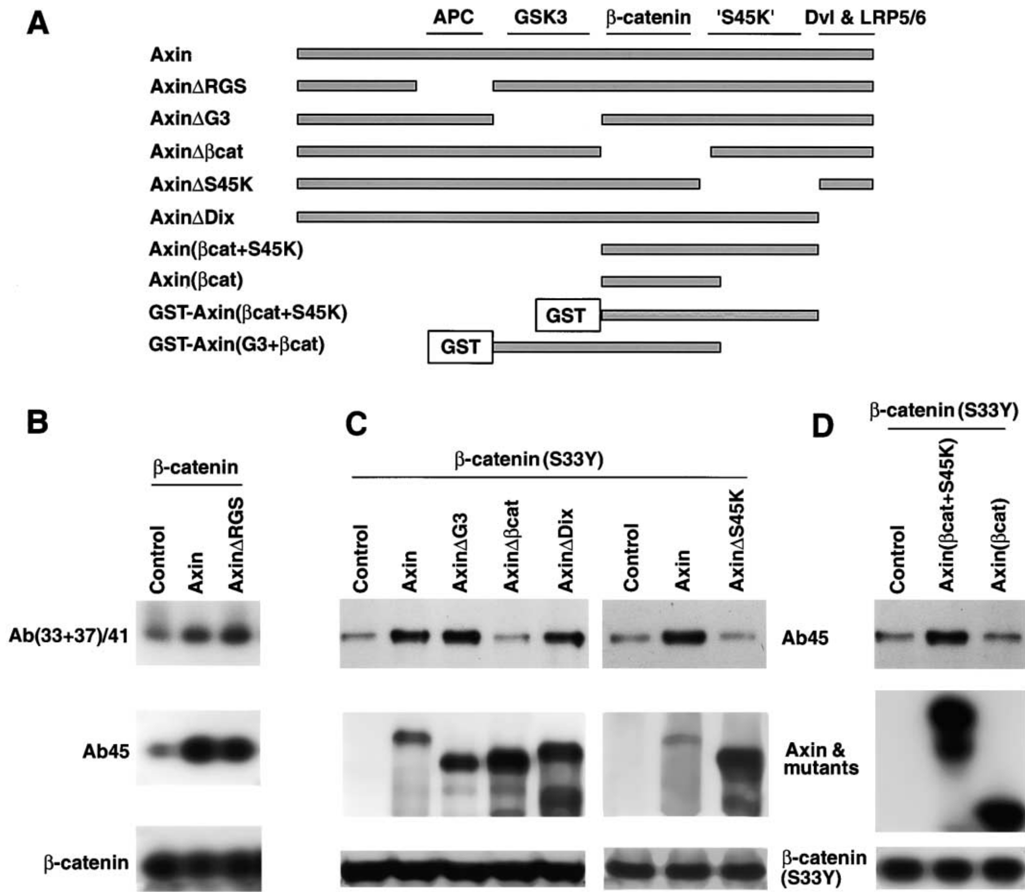

Figure 3. Stimulation of S45 Phosphorylation by Axin

(A) Axin and Axin mutants. Axin's binding regions for APC (the RGS domain), GSK-3, β-catenin, Dishevelled (Dvl), and LRP5/6 (the DIX domain) and the putative S45 kinase are indicated on top.

(B) Axin expression stimulated phosphorylation at S45 (middle gel) and S33/S37/T41 (top gel). β-catenin (Flag) was expressed alone (control) or together with Axin or AxinΔRGS (Myc) in *Xenopus* embryos and precipitated by a Flag Ab (bottom gel). β-catenin phosphorylation was examined by blotting the precipitates with indicated Abs.

(C) Axin's β-catenin binding region and the adjacent carboxyl region are required for stimulating S45 phosphorylation. β-catenin (S33Y) mutant (Flag) was used for analysis because the mutation does not affect S45 phosphorylation, and β-catenin (S33Y) protein level is constant regardless of coexpressed Axin or mutants. Deletion of Axin's binding regions for APC, GSK-3, Dishevelled, and LRP5/6 did not affect the ability of Axin to stimulate S45 phosphorylation, but deletion of either Axin's β-catenin binding region or the adjacent carboxyl region abolished Axin's ability to stimulate S45 phosphorylation (top gel). The protein expression level for the wild-type Axin is always lower than that for Axin mutants.

(D) Axin's β-catenin binding region plus the adjacent carboxyl region are sufficient for stimulating S45 phosphorylation. Deletion of the adjacent carboxyl region abolished the activity (top gel). In (C) and (D), β-catenin (S33Y) (Flag) was expressed alone (control) or coexpressed with Axin or Axin mutants (Myc) in *Xenopus* embryos and precipitated by a Flag Ab (bottom gel). Precipitates were examined for S45 phosphorylation (top gel). Axin and its mutants in embryo extracts were examined via a Myc Ab (middle gel).

of Armadillo (Arm), the *Drosophila* ortholog of β-catenin. Strikingly, RNAi depletion of *Drosophila* CKIα resulted in a dramatic increase of Arm protein in S2 cells (Figure 7A). Furthermore, RNAi depletion of CKIα in *Drosophila* embryos generated a naked cuticle phenotype (Figure 7B) and a strong expansion of the expression domain of wingless (wg, *Drosophila* wnt-1; Figure 7C), which itself is an Arm target gene. This is reminiscent of the phenotype caused by loss-of-function mutations in *Drosophila* Axin (Hamada et al., 1999; Willert et al., 1999) or GSK-3 (zeste-white 3/shaggy) gene (Siegfried et al., 1994) or by wg overexpression (Noordermeer et al., 1992). CKIα RNAi did not alter the level of other proteins such as tubulin, and RNAi for other unrelated genes did not affect Arm amount (Figure 7A) or the cuticle phenotype (not shown), demonstrating the specificity of CKIα depletion. Arm protein accumulation in S2 cells and the segment polarity phenotype in embryos resulted from CKIα RNAi, therefore, suggest together that CKIα function is conserved and essential for β-catenin degradation in both *Drosophila* and human.

Discussion

We provided compelling evidence that β-catenin phosphorylation in vivo is sequentially carried out by two distinct kinases, CKIα and GSK-3. CKIα phosphorylation of S45 proceeds and is required for subsequent GSK-3

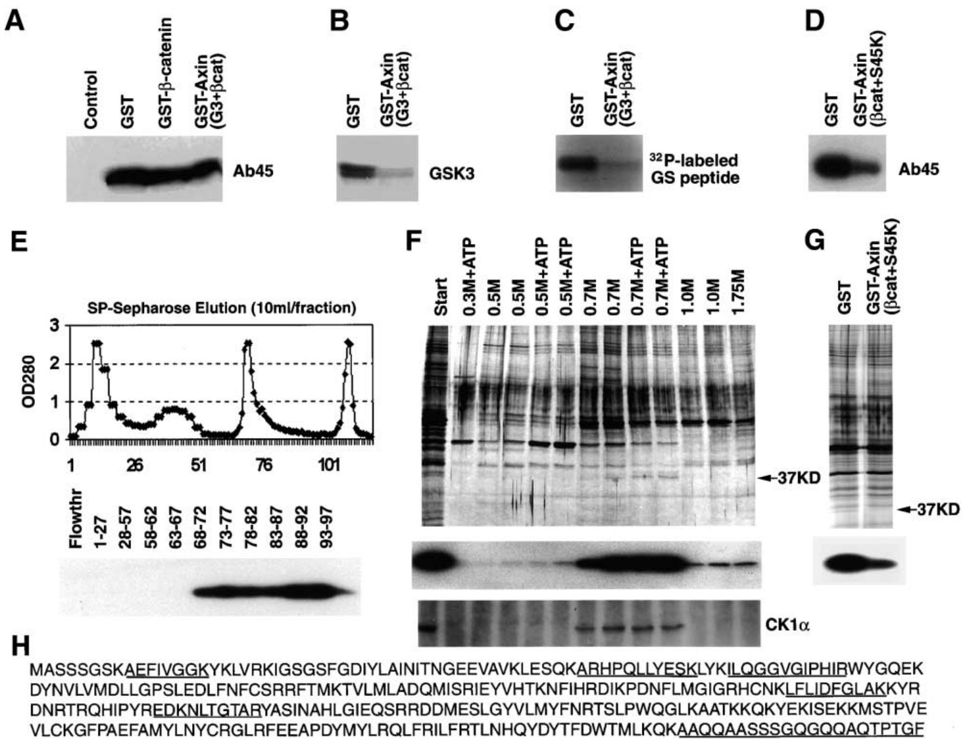

Figure 4. Identification of the S45 Kinase as CKIα

(A–C) Identification in rat brain extracts an S45 kinase activity that is independent of GSK-3.

(A) In an in vitro kinase assay, the rat brain extract phosphorylated GST-N3 (the β-catenin amino-terminal region containing S33, S37, T41, and S45; Liu et al., 1999) at S45 as detected by blotting GST-N3 with Ab45. This S45 kinase activity was not depleted by GST, GST-β-catenin, or GST-Axin(G3+βcat). Control indicates no brain extracts added.

(B and C) GST-Axin(G3+βcat) depleted both GSK-3α and GSK-3β from the brain extract, as assayed by blotting with Abs that recognize both GSK-3α (top) and GSK-3β (bottom) (B), and by in vitro phosphorylation with [^{32}P]ATP of a GSK-3 substrate peptide (with the primed phosphate) derived from glycogen synthase (GS) (C).

(D) The brain S45 kinase activity was significantly depleted by GST-Axin(βcat+S45K). S45 phosphorylation on GST-N3 in the in vitro kinase assay was examined with Ab45.

(E) Fractionation of the S45 kinase in the brain extract via a SP-Sepharose column. All S45 kinase activity, but less than 10% of the total protein, bound to the column after initial loading. Elution was done with increasing NaCl concentrations (0.15 M for fractions 1–57, 0.3 M for fractions 58–97, and 2M for the remaining fractions). Fractions were pooled as shown and analyzed for S45 kinase activity (bottom gel) as done in (A). The highest S45 kinase activity was eluted into fractions 88–97, which had lowest protein levels as reflected by OD 280 nm (top graph).

(F) Fractions 88–97 from the SP-Sepharose column were pooled together and fractionated using a Blue-Sepharose column. All S45 kinase activity was retained by the column, which was sequentially washed with increasing concentrations of NaCl with or without 5 mM ATP as indicated. Different fractions were collected and subjected to silver staining (top gel) and in vitro S45 kinase assay (middle gel) as done in (A). The kinase activity was eluted at 0.7 M NaCl with or without ATP, and correlated fully with the appearance of a 37 kDa band. The bottom gel shows CKIα immunoblotting, performed after the identification of the 37 kDa band as CKIα.

(G) GST-Axin(βcat+S45K) but not GST depleted the 37 kDa band and the S45 kinase activity from fractions 88–97 of the SP-Sepharose column, as visualized by silver staining (top gel) and the in vitro S45 kinase assay (bottom gel) done as in (A).

(H) The 37 kDa band from the gel contained a single kinase, CKIα (and several other proteins) as revealed by mass spectrum analysis. Underlined peptide sequences were perfect matches between mass spectra of the band (trypsin-digested) and CKIα.

phosphorylation of T41, S37, and S33 (Figure 8). Our findings identify CKIα as an essential component that controls β-catenin phosphorylation degradation. This understanding of β-catenin phosphorylation at a single-residue resolution enables us to look into how β-catenin mutations found in human cancers disrupt distinct steps in β-catenin degradation. Thus, mutations surrounding S33 and S37 abolish β-catenin recognition by β-Trcp and the ubiquitination of β-catenin; mutations at T41 prevent GSK-3 phosphorylation of S37 and S33 and thus β-Trcp recognition; and mutations at S45 block the priming phosphorylation by CKIα and consequently all phosphorylation events by GSK-3. Each of these mutations causes β-catenin to escape recognition by β-Trcp and subsequent degradation (Figure 8).

CKIα was among the first protein kinase activities discovered, yet its function and regulation remains poorly understood. Like GSK-3, CKIα is expressed ubiquitously and appears to be constitutively active (Gross and Anderson, 1998), consistent with its role in β-catenin degradation. The finding that β-catenin is a CKIα substrate in vivo therefore identifies CKIα as a central player in cell fate determination and growth control. Indeed, CKIα controls segment polarity during *Drosophila* embryogenesis (Figure 7). Interestingly, β-catenin phosphorylation by CKIα and by GSK-3 are both stimulated

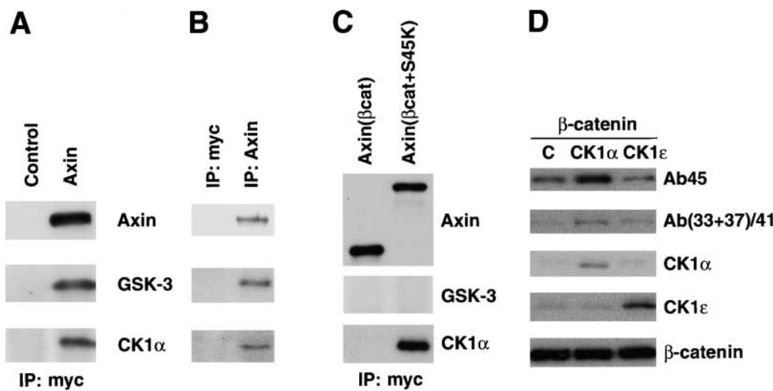

Figure 5. CKIα Is a Component of the Axin Complex and Promotes β-Catenin S45 Phosphorylation

(A) Overexpressed Axin (Myc) precipitated the endogenous CKIα and GSK-3 in 293T cells. Immunoprecipitation (IP) was done with a Myc Ab. Control indicates mock transfection.

(B) The endogenous Axin precipitated the endogenous CKIα and GSK-3 in mouse L cells. IP was done with Axin Abs (which only recognize mouse/rat Axin) or a Myc Ab (control).

(C) Transfected Axin(βcat+S45K) but not Axin(βcat) (both Myc-tagged) precipitated the endogenous CKIα in 293T cells. Neither precipitated GSK-3. IP was done with a Myc Ab. In (A)–(C), precipitates were blotted with Abs for GSK-3, CKIα, or Axin, or Myc (for tagged Axin). Only GSK-3β was shown, since GSK-3α and IgG (both 50 kDa) were not resolved on the gel.

(D) Overexpression of CKIα, but not CKIε, increased β-catenin S45 phosphorylation. Likely as a consequence, CKIα also increased S33/S37/T41 phosphorylation. β-catenin (Flag) cDNA was transfected alone (control, C) or together with CKIα or CKIε cDNA. Cell extracts were made 2 days after transfection and immunoblotted with indicated Abs.

by Axin (Figure 3). In fact, CKIα and GSK-3 bind to different regions of Axin such that they "sandwich" β-catenin in the Axin complex, thereby promoting effective β-catenin phosphorylation (Figures 3A and 8). As Wnt signaling inhibits only GSK-3 but not CKIα phosphorylation of β-catenin (Figure 2), CKIα may represent a node at which other signaling pathways regulate β-catenin protein level. As depletion of CKIα causes β-catenin accumulation in a manner similar to a lack of function of GSK-3, APC, or Axin (Figures 6 and 7), CKIα is a candidate tumor suppressor.

A priming kinase for GSK-3 in β-catenin phosphorylation is not fully expected. Besides Wnt signaling, GSK-3 is also a key enzyme in insulin signal transduction, which inhibits GSK-3 phosphorylation of, and thereby activates, glycogen synthase (GS) (Harwood, 2001; Woodgett, 2001; Frame et al., 2001). GSK-3 is thus a major therapeutic target for treating type II or non-insulin-dependent diabetes mellitus (NIDDM), which accounts for 90% of all diabetes cases. Insulin and Wnt inhibit GSK-3 phosphorylation of GS and β-catenin, respectively. This specificity appears to depend, at least in part, on whether GSK-3 is inhibited via phosphorylation by Akt upon insulin signaling (Cross et al., 1995; Dajani et al., 2001; Frame et al., 2001) or via Dishevelled and GBP (GSK-3 binding protein) binding to GSK-3 and Axin upon Wnt signaling (Li et al., 1999; Ding et al., 2000; Farr et al., 2000; Salic et al., 2000). While GSK-3 phosphoryla-

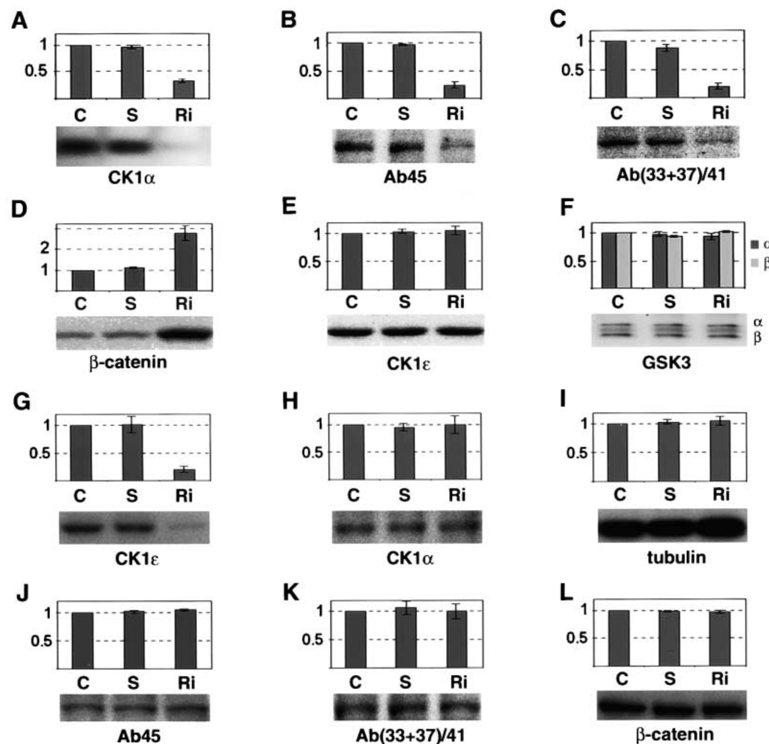

Figure 6. CKIα Is Essential for β-Catenin S45 Phosphorylation and Degradation in 293T Cells

(A–F) CKIα RNAi (Ri) decreased the CKIα protein level (A), β-catenin S45 phosphorylation (B), and S33/S37/T41 phosphorylation (C), but increased β-catenin protein level (D). Note that graphs in (B) and (C) reflect the ratio between β-catenin phosphorylation versus β-catenin protein level. CKIα RNAi did not affect the protein level for CKIε (E), GSK-3α and GSK-3β (F), or tubulin (not shown).

(G–L) CKIε RNAi (Ri) decreased the protein level of CKIε (G) but not of CKIα (H) or tubulin (I), but had no effect on β-catenin S45 phosphorylation (J), S33/S37/T41 phosphorylation (K), or β-catenin protein level (L). Cell extracts were made 3 days after transfection with the double-strand (ds) RNAi oligo (Ri) or the corresponding single-strand sense oligo (S, as a negative control). C (control) indicates without RNAi transfection or with mock transfection. Graphs represent averages from at least four independent experiments, with the value for the control set as 1 arbitrarily.

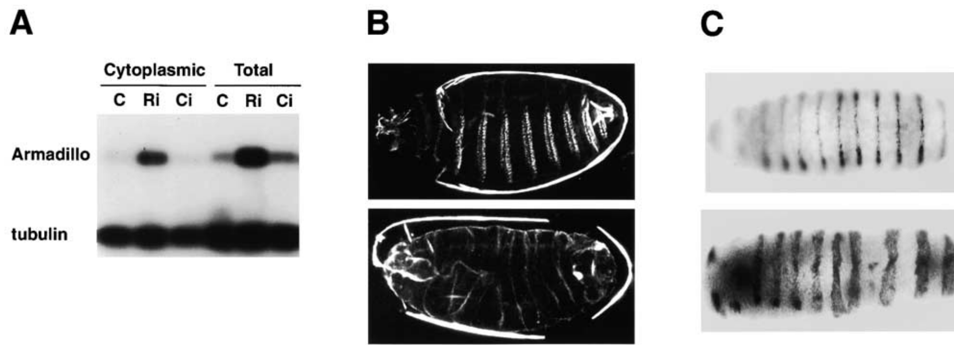

Figure 7. *Drosophila* CKIα Is Required for Armadillo (Arm) Degradation and Segment Polarity

(A) CKIα RNAi induced Arm accumulation. S2 cells were either untreated (control, C), or treated with ds RNA for *Drosophila* CKIα (Ri) or for *Xenopus* β-Trcp (Ci, a negative control). The cytoplasmic or total cell extract was made 3 days after RNAi treatment and immunoblotted with an Ab for Arm or tubulin.

(B) *Drosophila* embryo injected with CKIα ds RNA (bottom) exhibited a naked cuticle phenotype. A total of 109 of 460 CKIα-RNAi injected embryos (24%), but none of the 64 embryos injected with buffer alone (top), exhibited the phenotype shown.

(C) CKIα-RNAi-injected embryos (bottom) exhibited an expansion of the wg expression domain when compared with the wild-type embryo (top), as assayed by whole-mount in situ hybridization at stage 13.

tion of GS requires priming phosphorylation and occurs without the assistance of any scaffolding protein, GSK-3 and β-catenin are placed next to each other by Axin, which stimulates β-catenin phosphorylation by GSK-3 in vitro (Kikuchi, 2000). It is thus widely believed that the Axin-GSK-3-β-catenin complex bypasses the requirement for priming phosphorylation (reviewed in Weston and Davis, 2001; Cohen and Frame, 2001). Some studies using in vitro kinase assays suggest that the intrinsic property of GSK-3 to differentially phosphorylate primed (such as GS) versus "nonprimed" (such as β-catenin) substrates may underlie the distinct out-

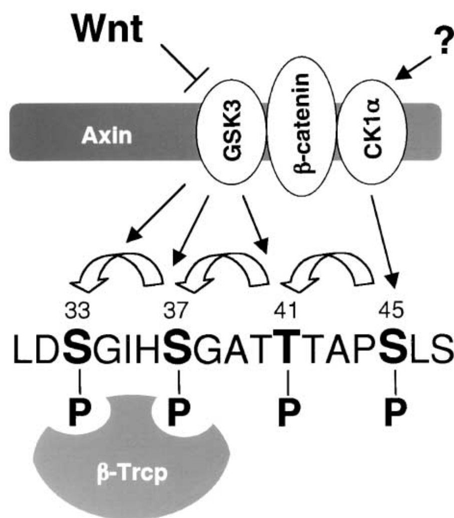

Figure 8. A Model for β-Catenin Phosphorylation and Recognition by β-Trcp

CKIα, GSK-3, and β-catenin each bind a different domain of Axin such that CKIα and GSK-3 sandwich β-catenin in the Axin complex. CKIα phosphorylation of S45 allows GSK-3 to phosphorylate T41, then S37 and S33. Phosphorylation of S37 and S33 creates the recognition site for β-Trcp. Wnt signaling inhibits GSK-3 phosphorylation of T41, S37, and S33. How CKIα phosphorylation of S45 is regulated remains unknown.

comes of GSK-3 inhibition by insulin versus by Wnt (Thomas et al., 1999; Frame et al., 2001). In fact, a strategy for therapeutic inhibition of GSK-3 in treating NIDDM is based on the assumption that inhibitors specific for GSK-3 phosphorylation of primed GS could be developed that would not inhibit GSK-3 phosphorylation of nonprimed β-catenin and thus would not induce β-catenin accumulation and cancer. Our data reveal that, like GS, β-catenin phosphorylation by GSK-3 also requires a critical priming step and thus suggests a need to reevaluate our thinking on the specificity of GSK-3 inhibition by insulin and Wnt and on therapeutic drug design for GSK-3 activity.

The observation that GSK-3 phosphorylation of its major physiological substrates, GS and β-catenin, depends on priming kinases is fully consistent with recent analyses of the GSK-3 atomic structure (Dajani et al., 2001; ter Haar et al., 2001) and further raises the question whether GSK-3 phosphorylation of most, if not all, substrates requires priming phosphorylation. In vitro, GSK-3 can phosphorylate other components in the Wnt pathway, including APC (Rubinfeld et al., 1996), Axin (Yamamoto et al., 1999; Jho et al., 1999; Willert et al., 1999), and TCF (Lee et al., 2001). GSK-3 phosphorylation of APC (Rubinfeld et al., 1996) and Axin enhances their ability to bind to β-catenin and stabilizes Axin protein (Yamamoto et al., 1999; Jho et al., 1999; Willert et al., 1999), all contributing to β-catenin degradation. It will be important, therefore, to examine whether GSK-3 phosphorylation of APC, Axin, or TCF in vivo requires priming phosphorylation, and whether CKIα and/or other kinases serve as the priming kinase(s) for these substrates. It is also of interest to note that GSK-3 phosphorylation of different substrates requires distinct priming kinases (casein kinase II for GS, CKIα for β-catenin, and protein kinase A for CREB). These dual-kinase systems may provide exquisite combinatory control for GSK-3 substrate selectivity.

Another member of the CKI family, CKIε, has been shown to stimulate β-catenin signaling (Peters et al., 1999; Sakanaka et al., 1999), and thus it functions in a

manner that is opposite to that of CKIα. CKIα and CKIε share related kinase but different flanking domains and have been known to exhibit distinct functions (Gross and Anderson, 1998). For example, CKIε, but not CKIα, rescues growth arrest in a yeast mutant deficient for Hrr25, a CKIε homolog (Fish et al., 1995). We found that only CKIα, but not CKIε, is capable of phosphorylating β-catenin at S45 (Figure 5D). Importantly, depletion of CKIα, but not CKIε, prevents β-catenin phosphorylation and degradation (Figure 6). In addition, CKIε fails to compensate for CKIα depletion and the consequent inhibition of β-catenin phosphorylation (Figure 6). Previous experiments also demonstrated that CKIε, but not CKIα, can activate β-catenin signaling in mammalian cells and *Xenopus* embryos (Sakanaka et al., 1999). However, one study suggests that CKIα and CKIε can each activate Wnt signaling upon overexpression in *Xenopus* embryos (McKay et al., 2001). We speculate that inherent shortcomings of certain overexpression experiments may account for this discrepancy. It remains unclear how CKIε functions positively in β-catenin signaling. CKIε has been shown to bind Dishevelled (Peters et al., 1999; Sakanaka et al., 1999), Axin (Sakanaka et al., 1999), and TCF (Lee et al., 2001) and can phosphorylate Dishevelled (Peters et al., 1999), APC (Rubinfeld et al., 2001), and TCF (Lee et al., 2001) but not β-catenin (Peters et al., 1999). As such, CKIε may modulate the binding of Dishevelled to GBP, of β-catenin to TCF (Lee et al., 2001), or of other components to the Axin complex (Gao et al., 2002). Identification of the in vivo substrate(s) of CKIε will elucidate further how CKI proteins play distinct roles in Wnt signal transduction.

Experimental Procedures

Plasmids

The following plasmids were described: CS2+β-catenin (Flag), pGEX-β-catenin, and CS2+β-Trcp (Myc) (Liu et al., 1999); SP64-GSK-3β and SP64-GSK-3βKM (He et al., 1995); CS2+Axin and CS2+AxinΔRGS (both Myc-tagged) (Zeng et al., 1997); and CS2+CKIε (Peters et al., 1999). Rat CKIα was subcloned into CS2+. Substitutions or deletions in β-catenin and Axin were generated by the QuickChange Kit (STRATAGENE). AxinΔG3, AxinΔβcat, Axin ΔS45K, and AxinΔDix correspond to deletions of residues 357–526, 526–722, 619–903, and 903–992, respectively, of mouse Axin (Zeng et al., 1997). Axin(G3+βcat), Axin(βcat+S45K), and Axin(βcat) correspond to mAxin residues 357–724, 526–903, and 526–722, respectively. The corresponding cDNA fragments were cloned in CS2+Myc and/or pGEX for GST-fusion proteins.

Antibodies, Immunoprecipitation, and Immunoblotting

Rabbit polyclonal antibodies (Abs) were generated via standard procedures using the following synthetic phosphopeptides as immunogens: SYLDS*GIHS*GATT*TAPC (S* or T*: phospho-S or -T), generating Ab(33+37)/41; CSYLDS*GLHS*GAT, generating Ab(33+37); CGATT*TAPS*KSGKG, generating Ab41/45 and Ab45; and CGATT*TAPSLSGKGNPE, generating Ab41. Bleeds were passed three times through a nonphosphopeptide column to remove Abs that recognized the peptide without phosphorylation. The final flowthrough was loaded onto a phosphopeptide column, which was washed three times with 0.1 M Tris buffer (pH 8.0). Bound Abs were eluted with 0.1 M Glycine (pH 2.5) and neutralized with 1 M Tris buffer. Ab41/45 was further passed three times through a column composed of phospho-T41 peptide to remove Abs that recognized phospho-T41, and the final flowthrough yielded Ab45.

Other Abs were used according to suppliers' instructions: polyclonal anti-β-catenin (C2206, Sigma); anti-CKIα (Santa Cruz Biotechnology); anti-CKIε (C40520, Transduction Laboratories); monoclonal anti-Flag (M2) (Sigma); anti-Myc (9E10); anti-Arm (7A1); and anti-β tubulin (E7, from Developmental Studies Hybridoma Bank, University of Iowa). GSK-3 Abs were described (He et al., 1995; kindly provided by J. Woodgett). Immunoprecipitation, SDS-PAGE, and immunoblotting were done as described (Liu et al., 1999).

Xenopus laevis Embryo Manipulations

Synthetic RNAs for each tested protein were injected at 1 ng/embryo into the animal pole at the 2-cell stage. Whole embryo extracts were made at stage 8 for analyses. Procedures for embryo staging, injection, and extracts were described (Liu et al., 1999).

Protein Purification and Mass Spectrum Analysis

Adult rat brains (200 grams) were homogenized in 450 ml of homogenization buffer (HB, 50 mM HEPES [pH 7.4], 10 mM NaF, plus the protease inhibitor cocktail [Roche]) using a Polytron Homogenizer. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C. The supernatant yielded the brain extract (BE) and was used as the starting material for purification, depletion, or in vitro kinase assays. S45 kinase assay was performed as described (Liu et al., 1999), using GST-N3 as the substrate and Ab45 for phospho-S45 detection. The primed GS peptide was used as the substrate for GSK-3 in BE with [^{32}P]ATP (He et al., 1995). BE (10 μl) was used in a kinase reaction (40 μl final volume). For depletions, 200 μl BE were incubated at 4°C for 2 hr with 50 μl of glutathione-agarose beads bound by 5 μg GST-fusion proteins. After precipitation, 10 μl supernatant was used for kinase assays.

For purification, BE was applied onto a 25 cm × 2.5 cm SP-Sepharose (Amersham Pharmacia) column equilibrated with HB. A S45 kinase activity was eluted when NaCl concentration was above 0.15 M, with maximal elusion at 0.3 M. Fractions 88–97 had the highest specific kinase activity and were pooled together (total 100 ml) for storage at −80°C. A volume of 12 ml was further fractionated via a 1 ml Blue-Sepharose column (Amersham Pharmacia) preequilibrated with 0.3 M NaCl in HB. Bound proteins were eluted with stepwise increases of NaCl with or without ATP. Mass spectrum analysis was done at the Taplin Mass Spectrometry Facility at Harvard Medical School according to standard procedures.

Mammalian Cell Transfection, Extracts, and RNAi

Transfections were done with Lipofectamine (Invitrogen) on 293T cells in 6-well plates. Expression plasmids were transfected each at 1 μg/well. ds RNAi oligos and single-strand sense oligos were transfected at 2.5 μg/well. These were designed as described (Elbashir et al., 2001) and synthesized by Integrated DNA Tech., Inc. These were CKIα (human, sense), 5′-CCAGGCAUCCCCAGUUG CUTT-3′; CKIε (human, sense), 5′- UGGCCAAGAAGUACCGGGATT-3′. ds RNA oligos were annealed in vitro before transfection. For cytosolic extracts, cells were washed in cold PBS, collected in 1 ml PBS (with protease and phosphotase inhibitors), and homogenized by a Dounce homogenizer. Lysates were centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant was collected.

RNAi in *Drosophila* S2 Cells and Embryos

RNAi in S2 cells was performed as described (Clemens et al., 2000). Following DNA primers were used: 5′-GAATTAATACGACTCACTA TAGGGAGAGGCCATCAAGATGGAGAGC-3′ and 5′-GAATTAATAC GACTCACTATAGGGAGACATGTAATCTGGCTGCTCC-3′ (*Drosophila* CKIα); 5′-TAATACGACTCACTATAGGGAGACCACGTCCGGACA GATTCTCTTTG-3′ and 5′-TAATACGACTCACTATAGGGAGACCA CAGCAAGTCCTGGGTCGTTG-3′ (*Xenopus* β-Trcp). Briefly, in vitro transcribed RNA complementary strands were annealed and added at 15 μg/well to S2 cells in 1 ml serum-free medium for 30 min. Regular medium with serum (2 ml) were then added, and cells were cultured for 3 days. Total cell lysates were made by spinning 0.5 ml cells at 1,000 × g and dissolving the pellet in 50 μl 1× SDS loading buffer. Cytosolic extracts were made as for mammalian cells.

For RNAi in *Drosophila* embryos, the wild-type precellular embryo was injected with 3 μM CKIα dsRNA or buffer as described (Kennerdell and Carthew, 1998). Embryos were incubated at 18°C under oil for 2 days and were mounted in Hoyer's medium/lactic acid for

cuticle preparations. Whole-mount in situ hybridization was done as described (Lehmann and Tautz, 1994).

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