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Phosphorylation and Regulation of β-Catenin by Casein Kinase Iε

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β-Catenin transduces cytosolic signals to the nucleus in the Wnt pathway. The Wnt ligand stabilizes cytosolic β-catenin protein, preventing its phosphorylation by inhibiting glycogen synthase kinase 3 (GSK3). Serine-33 and -37 of β-catenin are GSK3 phosphorylation sites that serve as recognition sites for the β-TRCP–ubiquitin ligase complex, which ultimately triggers β-catenin degradation. Mutations at those two sites, as well as in Ser-45, stabilize β-catenin. Recently, casein kinase Iε (CKIε) has been shown to be a positive regulator of the Wnt pathway. Its action mechanism, however, remains unknown. Here I show that Ser-45 is phosphorylated not by GSK3 but by CKIε. Axin, a scaffold protein that binds CKIε and β-catenin, enhances this CKIε-mediated phosphorylation. Overexpression of CKIε in cells increases the amount of β-catenin phosphorylated at Ser-45. Ser-45 phosphorylated β-catenin is a better substrate for GSK3, which suggests that CKIε and GSK3 may co-operate in destabilizing β-catenin. In spite of the fact that CKIε was found as a positive regulator of the Wnt pathway, mutational analysis suggests that mutation of Ser-45 regulates β-catenin stability by inhibiting the ability of GSK3 to phosphorylate Ser-33 and -37, thereby disrupting the interaction between β-catenin, β-TRCP and Axin. I propose that phosphorylation of Ser-45 by CKIε plays an important role in regulating β-catenin stability.

Key words: β-catenin, β-TRCP, CKIε, GSK3, Wnt.

β-Catenin is a molecule that plays an important role both in cell adhesion and in the Wnt pathway (*1, 2*). At the plasma membrane, β-catenin is found within a cadherin complex involved in cell adhesion, while cytosolic β-catenin is normally turned over quickly by the ubiqitin-proteasome degradation pathway (*3, 4*). In the Wnt pathway, cytosolic β-catenin acts as a signaling molecule that transduces intracellular signals to the nucleus.

Glycogen synthase kinase 3 (GSK3)–mediated phosphorylation of β-catenin results in the creation of a binding site for the F-box protein, β-transducin repeat-containing protein (β-TRCP), which is a part of the ubiquitin ligase complex (*5–7*) involved in β-catenin degradation. GSK3 is a constitutively active kinase, and upon stimulation by a Wnt ligand, GSK3 activity is inhibited by Dishevelled (Dvl) protein, then β-catenin is stabilized. The stabilized cytosolic β-catenin translocates to the nucleus, forms a complex with the lymphoid enhancer binding factor–1 (Lef-1)/T cell factor (Tcf) family of DNA binding molecules, which in turn induces target gene transcription (*8, 9*). The interaction between β-catenin and β-TRCP depends on the GSK3-mediated phosphorylation of specific amino terminal serine residues in β-catenin. Interestingly, this site-specific phosphorylation requires Axin, a scaffold molecule that binds both β-catenin and GSK3, suggesting that β-catenin degradation/stabilization is spatially regulated by the Axin/GSK3 complex.

Based on the sequence similarity between β-catenin and IkB, which has similar phosphorylated sites that bind the ubiquitin ligase complex, Ser-33 and Ser-37 in β-catenin have been proposed to be the target sites of GSK3-mediated phosphorylation of β-catenin (*10*). Mutations at Ser-33 and -37 result in the stabilization of β-catenin and activation of β-catenin/Lef-1-Tcf–dependent gene transcription. Moreover, mutations in β-catenin at Ser-33 and Ser-37 are found in various human cancers as oncogenic mutations (*11*). Other mutations in the amino-terminal region of β-catenin, including those at positions Thr-41 and Ser-45, are also found in human cancers (*11*); however, it is not known whether these sites are directly phosphorylated by GSK3 or other kinases. Here I show that Ser-45 is phosphorylated by a casein kinase Iε (CKIε)/Axin complex, not by GSK3, and that Ser-45 has a role in regulating β-catenin stability.

CKIε has been identified as a positive regulator of the Wnt pathway (*12, 13*), but its mode of action is not yet clear. Overexpression of CKIε mimics Wnt stimulation in that β-catenin is stabilized and β-catenin/Lef-1-Tcf–dependent gene transcription is activated. Kinase-inactive CKIε or antisense oligonucleotides for CKIε attenuate Wnt signaling, suggesting that CKIε kinase activity is required for full activation of the Wnt pathway. Kinase-inactive CKIε also blocks Dvl or β-catenin–induced β-catenin/Lef-1-Tcf–dependent gene transcription (data not shown). Hence, I hypothesize that CKIε can act at a step that is downstream of Dvl. Since CKIε is a component of the Axin complex, I also examined the other molecules in the complex as candidate substrates. My findings suggest that phosphorylation

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Abbreviations: GSK, glycogen synthase kinase; β-TRCP, β-transducin repeat-containing protein; Dvl, dishevelled; Lef-Tcf, lymphoid enhancer binding protein-T cell factor; CKIε, casein kinase Iε.

of Ser-45 in β-catenin is important in regulating β-catenin stability, providing an insight into the mechanism by which CKIε regulates the transduction of Wnt signals.

EXPERIMENTAL PROCEDURES

Constructs—For GST–β-catenin fusion protein, mouse β-catenin cDNA (a gift from A. Nagafuchi, Kyoto University) was subcloned into a pGEX vector (Amersham Pharmacia Biotech) in frame, then expressed in *Escherichia coli*. GST–β-catenin protein was purified using GST purification modules (Amersham Pharmacia Biotech). For *in vitro* translation, mouse β-catenin cDNA was subcloned into a pcDNA3.1 vector (Invitrogen) with a C-terminal KT3 epitope tag. Site-directed mutagenesis of β-catenin (T41E, T41A, S45E, and S45A) was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). His6-tagged Axin was expressed and purified from baculovirus-infected Sf9 cells.

Reagents—Phospho-epitope-specific β-catenin antibodies (phospho-Ser33/37/Thr41 and phospho-Thr41/Ser45) were from Cell Signaling Technology. KT3 antibody was obtained from L. Conroy (Chiron Corporation). β-Catenin antibody was from Transduction Laboratory, β-tubulin antibody was from Sigma, and GSK3β enzyme was from Upstate Biotechnology. Recombinant mouse CKIε was expressed and purified from baculovirus-infected Sf9 cells. Human β-TRCP cDNA was a gift from R. Benarous (INSERM, Paris, France). Human TCF-4 cDNA was a gift from F. McCormick (UCSF, San Francisco, CA). *In vitro* translated proteins were made in reticulocyte lysates using TNT T7 coupled system from Promega according to the manufacturer's instructions. S^{35}-labeled *in vitro* translated proteins were made by adding S^{35}-methionine in the reaction. Biotinylated β-catenin N-terminal peptides were synthesized and purified by Mimetopes (Australia).

In Vitro Phosphorylation—GST–β-catenin (200 ng) was incubated for 20 min at 30°C in kinase buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$) containing 100 μM ATP and 1 mCi ^{32}P-ATP (for radioactive reaction) or 2 mM ATP (for cold reaction) and either GSK3β or CKIε (0.1 μg each). His-Axin (100 ng) was added to the reaction as indicated in figures. Alternatively, *in vitro* translated β-catenin protein was incubated for 10 min at 30°C in the presence of 2 mM ATP, 0.5 μM okadaic acid, and 10 ng His-Axin with either GSK3β or CKIε, then immunoprecipitated by the KT-3 antibody. The reaction mixture was separated by SDS-PAGE using 4–12% Bis-Tris NuPAGE gel (Invitrogen), transferred to the nitrocellulose membrane and visualized by autoradiography or immunoblotting. The biotinylated peptides (2 μg each) were incubated in the kinase buffer with GSK-3β and ^{32}P-ATP for 20 min at 30°C, separated by SDS-PAGE using 16% Tricine–gel (Invitrogen), transferred to the nitrocellulose membrane, and visualized by the autoradiography.

Cell Culture and Transfection—293 cells were cultured in DMEM/10% fetal bovine serum with antibiotics. Transfection was performed using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instructions. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 300 mM NaCl) for further analysis.

In Vitro Degradation—β-Catenin degradation assay in *Xenopus* extracts was carried out as previously described by Salic *et al.* (*14*). In brief, *in vitro* translated S^{35}-labeled β-catenin was incubated in *Xenopus* egg extracts at room temperature. At the indicated times, aliquots were removed, separated by SDS-PAGE using 4–12% Bis-Tris NuPAGE gel, transferred to nitrocellulose membrane, and visualized by autoradiography. Quantification of β-catenin bands was performed using a GS-700 Imaging Densitometer and Multi-Analyst software (Bio-Rad). The amount of β-catenin protein at each incubation time was normalized to the amount at the 0-h point.

In Vitro Binding—*In vitro* translated proteins were mixed and incubated in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% TritonX-100, 150 mM NaCl) for 2 h at 4°C, then the β-catenin complexes were isolated using the KT3 antibody. The isolated complexes were washed in washing buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% TritonX-100, 300 mM NaCl) several times, then separated by SDS-PAGE using 4–12% Bis-Tris NuPAGE gel, transferred to nitrocellulose membrane, and visualized by autoradiography.

RESULTS AND DISCUSSION

CKIε Phosphorylates β-Catenin in the Axin Complex—CKIε is a positive regulator of the Wnt pathway, and overexpressed CKIε binds both Dvl and Axin in mammalian cells (*12, 13*). CKIε binds Axin directly in yeast-two hybrid systems (*15, 16*). The Axin complex is known to include both positive and negative regulators of the Wnt pathway (*i.e.*, Dvl, GSK3, β-catenin, protein phosphatases, and APC). Since GSK3 and CKIε binding sites on Axin were mapped in separate regions (*17, 18*), Axin could simultaneously bind these two kinases (GSK3β and CKIε) (*15, 19*), and β-catenin. CKIε did not phosphorylate GSK3β or inhibit its activity *in vitro* in the presence of Axin (data not shown, Ref. *19*). Among several molecules phosphorylated by CKIε *in vitro*, including Axin and Dvl, I found that β-catenin is a particularly good substrate for CKIε, especially in the presence of Axin. As shown in Fig. 1A, CKIε phosphorylates β-catenin, and the phosphorylation is potentiated by purified Axin protein *in vitro*. This is an interesting analogy to the phosphorylation of β-catenin by GSK3, which is also potentiated by the Axin scaffold (*17*, Fig. 1A). I hypothesize that both of these kinases phosphorylate β-catenin in this complex, but do so at distinct sites. In addition, as shown in Fig. 1A, Axin was phosphorylated by both GSK3 and CKIε *in vitro*. While it has been previously shown that the GSK3-mediated phosphorylation of Axin stabilizes the Axin protein (*20*), in the present study, I did not obtain conclusive evidence that CKIε regulates Axin stability in a similar fashion.

GSK3β and CKIε Phosphorylate Distinct Residues on β-Catenin—The amino-terminal region of β-catenin contains clusters of serines and threonines, which regulate β-catenin protein stability. Deletions or mutations in this region stabilizes β-catenin protein (*21*). This region also contains the sequence motif for the binding of F-box protein β-TRCP, a component of the ubiquitin-proteasome complex (*3, 4*). Ser-33 and Ser-37, which are located in this region, are required for the recognition of β-catenin by the β-TRCP–ubiquitin ligase complex in a phosphorylation dependent manner (*10*). Mutations at Ser-33 and/or Ser-37 prevent β-

catenin from binding β-TRCP, thereby resulting in the stabilization of β-catenin. Such mutations are found in various human cancers in which accumulated β-catenin activates Tcf/Lef-1–dependent transcription of genes relevant to cancer, including c-myc and cyclin D1 (*22, 23*). Phosphorylation of Ser-33 and Ser-37 is regulated by the GSK3/Axin complex, which is in turn controlled by Wnt signaling. Upon Wnt stimulation, GSK3 activity is inhibited, and cytosolic β-catenin is stabilized. I used antibodies that recognize phosphorylated amino acids at the specific sites of β-catenin to determine the phosphorylation sites either by GSK3 or CKIε. The first antibody recognizes phosphorylation either at Ser-33, Ser-37, or Thr-41, and the second antibody recognizes phosphorylation either at Thr-41 or Ser-45. As shown in Fig. 1B, I confirmed that GSK3β phosphorylated Ser-33 and Ser-37 more efficiently in the presence of Axin. While GSK3 phosphorylates β-catenin at multiple sites more than at the N-terminus *in vitro* (data not shown), Axin appears to help GSK3 in phosphorylating the critical sites in β-catenin when it binds GSK3 and β-catenin.

Interestingly, GSK3-mediated phosphorylation of Thr-41 or Ser-45 as assessed by use of phospho-Thr-41/Ser-45 specific antibody was extremely low, even when GSK3 and β-catenin were complexed with Axin. In addition to Ser-33 and Ser-37, Thr-41, and Ser-45 in the amino-terminal region of β-catenin are also thought to be involved in regulating β-catenin stability. Thr-41 and Ser-45 residues are conserved in β-catenin proteins among different species. Importantly, mutations at Thr-41 and Ser-45 are found in human cancers (*11*). Therefore, it is entirely feasible that Thr-41 and Ser-45 could be the sites for phosphorylation and subsequently have an important regulatory role in β-catenin stability within the context of the adjacent residues. However, as Fig. 1B shows, the Western blots stained with phospho-epitope–specific antibodies demonstrate that GSK3 did not phosphorylate Thr-41 or Ser-45 either alone or in combination with Axin. In contrast, CKIε phosphorylated Thr-41 and/or Ser-45, and the phosphorylation was enhanced by the presence of Axin (Fig. 1B). Since the sites phosphorylated by CKIε were not detected by phospho-Ser-33/37/Thr-41 antibody, it is more likely that CKIε phosphorylates Ser-45, not Thr-41.

Axin is a scaffold molecule for GSK3 and β-catenin in the Wnt pathway (*2*). In the Axin complex, GSK3 selectively phosphorylates the serine residues located at positions 33 and 37, which are critical for the regulation of β-catenin stability. I also showed here that, in the Axin complex, CKIε specifically phosphorylates another residue that is important for β-catenin regulation, Ser-45. My findings in the context of others suggest that Axin serves as a Wnt-specific scaffold for kinase-substrate interaction for both GSK3 and CKIε.

Overexpression of CKIε Increases the Phosphorylation of Thr-41/Ser-45 of β-Catenin—I next examined whether CKIε is capable of phosphorylating β-catenin *in vivo*. As Fig. 1 shows, CKIε phosphorylates the sites recognized by the phospho-Thr-41/Ser-45 antibody in β-catenin *in vitro*. Consistent with this finding, the amount of Thr-41/Ser-45 phosphorylated β-catenin detected by phospho-epitope–specific antibodies was increased in 293 cells overexpressing CKIε (Fig. 2). This result supports my hypothesis that CKIε phosphorylates Ser-45 in β-catenin *in vivo*. Overexpressed CKIε forms a complex with Axin (*13*), which could facilitate the CKIε-mediated phosphorylation of Ser-45 of β-catenin as shown in Fig. 1. Therefore, the phosphorylation of Ser-45 may participate in regulating β-catenin stability.

Mutation of Ser-45 Stabilizes β-Catenin—To ascertain whether phosphorylation of Ser-45 and/or Thr-41 contributes to the mechanism underlying β-catenin stabilization, the stability of mutant β-catenin was assessed in *Xenopus* egg extracts, as previously described (*14*). Because the regulation of β-catenin stability can be reconstituted in cytoplasmic extracts prepared from *Xenopus* eggs, I used this

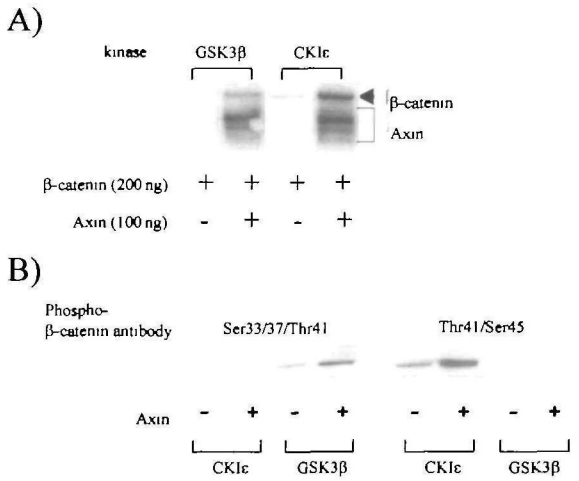

Fig. 1. **β-Catenin is phosphorylated by GSK3β and CKIε.** (A) Axin enhances the GSK3β- and CKIε-mediated phosphorylation of β-catenin. GST-β-catenin was incubated with either GSK3β or CKIε *in vitro*. In the presence of Axin, the phosphorylation of β-catenin by GSK-3 or CKIε was enhanced. The reaction mixture was separated by SDS-PAGE, transferred to nitrocellulose membrane and visualized by autoradiography. The β-catenin band is indicated by the arrow. (B) Axin enhances the CKIε- and GSK3β-mediated phosphorylation of N-terminal residues in β-catenin. GST-β-catenin was incubated with either GSK-3 or CKIε *in vitro* with or without Axin protein. The reaction mixture was separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with phospho-β-catenin antibodies as indicated. GSK3β-phosphorylated sites are recognized by phospho-Ser-33/37/Thr-41 antibody, whereas CKIε phosphorylated sites are recognized by phospho-Thr-41/Ser-45 antibody. The phosphorylation of those sites was enhanced by the presence of Axin.

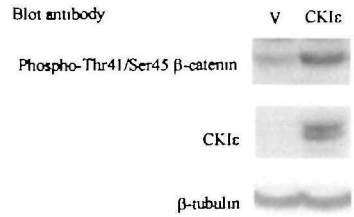

Fig. 2. **Overexpression of CKIε increases the phosphorylation at Thr41/Ser45 in 293 cells.** 293 cells were transiently transfected with CKIε or empty vector plasmid. After 48 h of transfection, cells were lysed in the lysis buffer, and the aliquots of the lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with the antibodies as indicated. The phosphorylated β-catenin detected by phospho-Thr-41/Ser-45 antibody was increased in cells transfected with CKIε. The expression of transfected CKIε was detected by HA antibody. Anti-β-tubulin blot is shown for the loading control.

system to determine the stability of different mutant forms of β-catenin in which Thr-41 was substituted with either alanine (T41A) or glutamate (T41E), and Ser-45 with either alanine (S45A) or glutamate (S45E). Consistent with the overexpression studies in mammalian cells and *Xenopus* embryos, exogenous CKIε stabilized WT-β-catenin in *Xenopus* egg extracts (data not shown, Ref. *24*). The data presented in Fig. 3 indicate that S45A-β-catenin was stabilized in *Xenopus* egg extracts, whereas T41A-β-catenin was degraded with a time course similar to that of wild type β-catenin (WT). S45E-β-catenin was also stabilized in *Xenopus* egg extracts, and T-41E-β-catenin was not. Alteration in Ser-45 may cause structural changes in the N-terminus of β-catenin, rendering it inert to GSK3-mediated phosphorylation and subsequent degradation. This result also suggests that the intact ser-45 residue is required for the stability of β-catenin.

Pre-Phosphorylation at Ser-45 Enhances the Phosphorylation by GSK3 at the N-Terminal Residues in β-Catenin—The stabilization of S45A-β-catenin may be due to an absolute requirement for an unphosphorylated serine at site 45. The mutation of Ser-45 to either alanine or glutamate simply blocks the phosphorylation by CKIε or by another unknown kinase, which leads to degradation of β-catenin. Thr-41 and Ser-45 could create a consensus GSK3 phosphorylation site, -SXXXpS/pT- (*25*), especially when Ser-45 is phosphorylated. Phosphorylation of both Thr-41 and Ser-45 may be required for the degradation of β-catenin. In spite of the cancer mutation data, my data in Fig. 3 suggest that a mutation at Thr-41 alone is not sufficient, yet it may be necessary, to stabilize β-catenin. To test this hypothesis, I compared the Ser-45 pre-phosphorylated N-terminal peptides of β-catenin with the non-phosphorylated peptides in their ability to be phosphorylated by GSK3. As shown in Fig. 4, Ser-45 pre-phosphorylated peptides were phosphorylated better than non-phosphorylated peptides. Although there are other serines and threonines in the peptides (Fig. 4), Thr-41 could be the best site for GSK-3 when Ser-45 is phosphorylated. Therefore, as opposed to the known function of CKIε as a positive regulator in the Wnt pathway, it is possible that CKIε and GSK3 co-operate to phosphorylate both Thr-41 and Ser-45. The mutational data in Fig. 3 also suggest that phosphorylation at Thr-41 and Ser-45 may induce degradation of β-catenin.

Mutation of Ser-45 Affects Phosphorylation of β-Catenin by GSK3—To determine whether Ser-45 is critical for regulating β-catenin stability, I examined the extent of GSK3β- and CKIε-mediated phosphorylation of amino terminal residues in mutant β-catenins using phospho-epitope–specific antibodies. Consistent with my finding that Ser-45 is specifically phosphorylated by CKIε, S45A-β-catenin and S45E-β-catenin proved to be poor CKIε substrates (Fig. 5A). The Thr-41 mutant, T41E-β-catenin, was also not phosphorylated by CKIε (Fig. 5A), although Thr-41 is unlikely to be a direct phosphorylation site for CKIε. Mutations at Thr-41 may prevent the phosphorylation at Ser-45 by CKIε. CKIε is an "acidotrophic" kinase which preferentially targets negatively charged residues at the minus position and isoleucine or leucine at the +1 position (*26*). From examination of the amino acid sequence of β-catenin, it is possible that Ser-45 is a favored phosphorylation site for CKIε, especially when minus positions are pre-phosphorylated. Kinases other than CKIε may pre-phosphorylate β-catenin to create a favaorable site for CKIε at Ser-45. As discussed above, phosphorylation at both Thr-41 and Ser-45 may leads degradation of β-catenin. CKIε phosphorylation at Ser-45 may create a pre-phosphorylated site at Thr-41 for GSK3. Conversely, elimination of Thr-41 may cause significant conformational change and block the phosphorylation of Ser-45 by CKIε.

Next, I assessed GSK3β-mediated phosphorylation of Ser-33/37 in the mutant β-catenins. Although Ser-45 is not directly phosphorylated by GSK3β, as shown in Fig. 1B, mutation of Ser-45 to alanine inhibits the ability of GSK3β to phosphorylate Ser-33/37. As discussed above, phosphorylation at Ser-45 may be required for the sequential phosphorylation at Thr-41, Ser-37, and Ser-33 by GSK3. This result is consistent with the *in vitro* β-catenin degradation

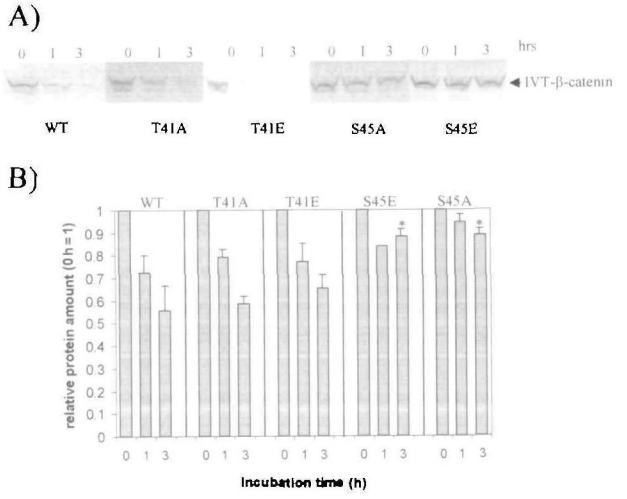

Fig. 3. **S45E-β-catenin is stable in *Xenopus* egg extracts.** *In vitro* translated S^{35}-labeled WT-β-catenin or mutant β-catenin (T41E, S45E, or S45A) was incubated in *Xenopus* egg extracts for the indicated times to monitor β-catenin degradation *in vitro*. Representative data for each β-catenin construct are shown in (A). The relative amounts of the β-catenin protein compared to the time 0 (no incubation) for each β-catenin construct are shown in (B). The mean and SEM of three independent experiments is shown. After 3 h of incubation, the relative protein amount of S45A and S45E β-catenin was significantly increased ($p < 0.001$, indicated as *) compared to that of the WT.

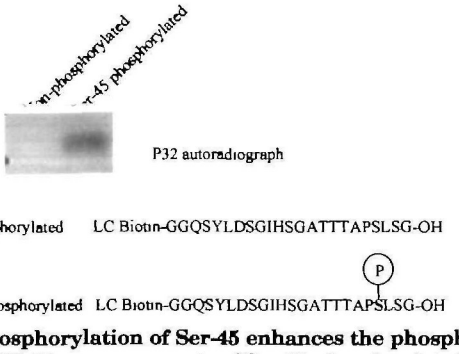

Fig. 4. **Pre-phosphorylation of Ser-45 enhances the phosphorylation by GSK3.** The same amounts of Ser-45–phosphorylated and non-phosphorylated β-catenin peptides (corresponding residues AA28–48, peptides sequences are shown) were phosphorylated by GSK3β *in vitro*. Ser-45 pre-phosphorylated peptide was phosphorylated better by GSK3β.

assay data shown in Fig. 3, as well as mutations found in human cancer. T41E- and S45E-β-catenins were phosphorylated by GSK3β at Ser-33/37. However, S45E-β-catenin was a poor substrate for GSK3β when compared to WT or T41E β-catenin, as shown in Fig. 5B. This explains why S45E-β-catenin was stable in the *Xenopus* egg extracts while T41E-β-catenin was not (Fig. 3), and suggests that an unphosphorylated serine at position 45 is a definite requirement for GSK3-mediated phosphorylation of Ser-33/37.

Mutation of Ser-45 in β-Catenin Reduces Axin and β-TRCP Binding—β-Catenin stability is regulated by the phosphorylation at its N-terminus and its binding to other molecules. Axin binds β-catenin directly and facilitates its subsequent phosphorylation at Ser-33 and Ser-37, as shown in Fig. 1. Depending on the phosphorylation at Ser-33 and Ser-37, β-catenin binds β-TRCP, which in turn recruits ubiquitin-proteasome complex (*3–5*). β-Catenin also directly binds Tcf/Lef-1, a family of DNA-binding proteins that act as a transcriptional activator when complexed with β-catenin (*8, 9*). To assess the role of N-terminal serine/threonine residues of β-catenin in its activity, I examined the interaction of β-catenin mutants with its binding partners, Axin, β-TRCP and Tcf-4, *in vitro*. As shown in Fig. 6A, S45A-β-catenin and S45E-β-catenin bound less β-TRCP than WT-β-catenin. This is consistent with the reduced GSK3β-mediated phosphorylation of Ser-33/37 in S45-mutated β-catenins (Fig. 5B), since phosphorylation at Ser-33/37 is required for successful β-TRCP binding to β-catenin (*10*). These data suggest that elimination of Ser-45 can cause β-catenin to dissociate from its negative regulators, β-TRCP and Axin/GSK3, sufficiently to result in β-catenin stabilization. In addition, S45A- and S45E-β-catenin bound less Axin than WT and T41E-β-catenin (Fig. 6B). This was unexpected, since the N-terminal region of β-catenin was shown not to be involved in the direct binding to Axin (*18*). Reduced phosphorylation at Ser-33 and Ser-37 as shown in Fig 5, therefore, probably underlies these changes in β-catenin binding to its partners.

These observations can be explained by my earlier findings that a mutation resulting in the substitution of Ser-45 with alanine or glutamate blocks not only CKIε-mediated phosphorylation of Ser-45 but also GSK3β-mediated phosphorylation of Ser-33/37 (Fig. 5). Phosphorylation at Ser-33/37 is critical for the degradation of β-catenin through β-TRCP binding, and changes in Ser-45 may cause significant conformational alteration to affect the phosphorylation of Ser-33/37 by GSK3β. Ser-45 phosphorylation could create a sequence of GSK3 phosphorylation sites at Thr-41, then Ser-37 (by Thr-41 phosphorylation), and Ser-33 (by Ser-37 phosphorylation). Both S45A- and S45E-β-catenins are unable to bind Axin and β-TRCP, and this is consistent with the result in Fig. 4. The data further support my hypothesis that an unphosphorylated serine at residue 45 is required for β-catenin degradation.

Tcf-4, one of the Tcf protein families, directly binds β-catenin (*8, 9*). In the *in vitro* binding assay, I found no significant difference in binding of Tcf-4 with any of the mutant β-catenins compared to the WT β-catenin (Fig. 6C). The N-terminal region of β-catenin is not involved in the interaction between Tcf and β-catenin (*8, 9*). On the other hand, a recent report showed that CKIε phosphorylates *Xenopus* Tcf-3, and the phosphorylation enhances the interaction between Tcf-3 and β-catenin (*14*). CKIε has multiple substrates in the Axin complex, and the combination of these phosphorylation events may contribute to the regulation of β-catenin stability.

In this study, I showed that Ser-45 in β-catenin is the phosphorylation site for CKIε but not for GSK3, whereas Ser-33/37 is the phosphorylation site for GSK3β but not for

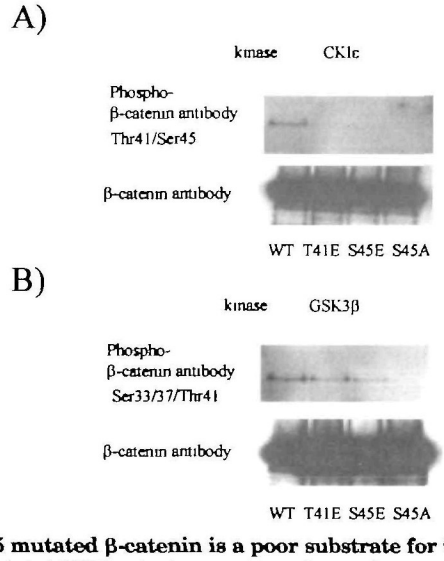

Fig. 5. **S45 mutated β-catenin is a poor substrate for GSK3β.** *In vitro* translated WT-β-catenin or mutant β-catenin was phosphorylated by GSK3β or CKIε *in vitro* in the presence of Axin. The reaction mixture was immunoprecipitated with KT3 antibody, then separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with phospho-β-catenin antibodies as indicated. The total β-catenin amount precipitated by the KT-3 antibody is shown by the β-catenin antibody blot. (A) Phosphorylation at Thr41/Ser45 by CKIε. T41E, S45E, and S45A-β-catenin were defective for phosphorylation by CKIε at Thr41/Ser45. (B) Phosphorylation at Ser33/37/Thr41 by GSK3. S45A-β-catenin was defective for the phosphorylation by GSK3. GSK3 phosphorylated S45E-β-catenin at S33/37/T41 to a lesser degree than WT.

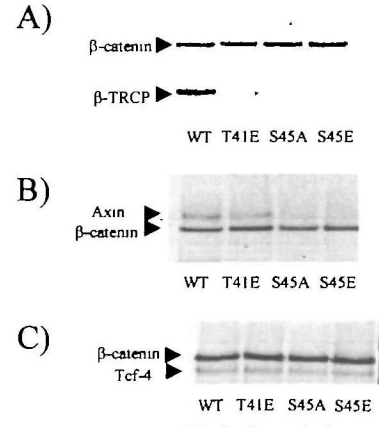

Fig. 6. **S45mutated-β-catenin binds less Axin and β-TRCP.** *In vitro* translated S^{35}-labeled β-catenin (2 μl each) was mixed with either *in vitro* translated S^{35}-labeled (A) Axin, (B) β-TRCP, or (C) Tcf-4 (3 μl each). The resulting β-catenin complexes were isolated by immunoprecipitation using KT3 antibody. The complex was separated by SDS-PAGE, transferred to nitrocellulose membrane and visualized by autoradiography.

CKIε. The site-specific phosphorylation of β-catenin by either CKIε (at Ser-45) or GSK3 (at Ser-33/37) takes place in the Axin complex. Axin binds two kinases, GSK3β and CKIε, and their substrate, β-catenin, facilitating the phosphorylation in the context of the Wnt pathway. Ser-45-phosphorylated β-catenin is a better substrate for GSK-3, suggesting that Thr-41 is also a GSK3 site. The substitution of Ser-45 with alanine or glutamate attenuates the GSK3β-mediated phosphorylation of Ser-33/37, which in turn results in β-catenin dissociation from the β-TRCP-ubiquitin proteasome complex.

Phosphorylation at Ser-45, therefore, may affect β-catenin stability by increasing the phosphorylation by GSK3. Ser-45 is one of the N-terminal serine/threonine residues that are known to regulate the stability of β-catenin. Ser-45 mutations are found in various human cancers. I showed that, under normal circumstances, Ser-45 is not phosphorylated by GSK3 even in the Axin complex. Mutations of Ser-45 attenuate the GSK3-mediated phosphorylation of Ser-33/37, disrupting Axin and β-TRCP binding to β-catenin, and therefore stabilizing β-catenin, suggesting the importance of Ser-45 in regulating β-catenin. This also suggests that even though Ser-45 is not the direct phosphorylation site for GSK3, this kinase is still a key regulator of the β-catenin stability.

In my study, mutations resulting in the substitution of Thr-41 with either alanine or glutamate were not sufficient to stabilize β-catenin when assessed in *Xenopus* egg extracts. Ser-45, therefore, may be much more important than Thr-41 in regulating the stability of β-catenin. Since mutations of Thr-41 have been found in various cancers (*11*), it is possible that Thr-41 is phosphorylated by GSK3 or other kinases and has a role in degrading β-catenin. Phosphorylation at both Thr-41 and Ser-45 may be required for the degradation of β-catenin, and in this circumstance, CKIε may play a role in degrading β-catenin together with GSK3. In fact, Ser-45 phosphorylation creates a GSK3 consensus site at Thr-41, then Thr-41 phosphorylation creates a GSK3 consensus site at Ser-37, and Ser-37 phosphorylation creates a GSK3 consensus site at Ser-33. As I have shown here, Ser-45 phosphorylated peptides are indeed good substrates for GSK3.

CKIε was indially shown to be a positive regulator in the Wnt pathway (*12, 13*). However, recent findings suggest that the negative role of CKIε (*27–29*). Yanagawa *et al.* showed that, in *Drosophila*, the disruption of the CKIε function of CKIε activates the Wnt-β-catenin pathway, resulting in β-catenin stabilization (*27*). Lui *et al.* (*28*) and Amit *et al.* (*29*) also showed that Ser-45 in β-catenin can be phosphorylated by CKI family kinases. At this point, it is not clear how these positive and negative roles of CKIε function in the Wnt pathway. There may be other regulatory molecules involved in regulating those functions.

The Axin complex contains two kinases that regulate β-catenin activity in the Wnt pathway, CKIε and GSK3β, of which one is a positive regulator and the other is a negative regulator. In this paper, I showed that both kinases appear to phosphorylate the N-terminal region of β-catenin in the Axin complex, albeit at different sites, and that phosphorylation by these two kinases may have significant effects on β-catenin stability. In contrast to the previous findings that overexpression of CKIε activates the Wnt pathway, phosphorylation of β-catenin at Ser-45 by CKIε may have the opposite effect, since mutation of Ser-45 stabilized β-catenin and pre-phosphorylation at Ser-45 enhanced the phosphorylation by GSK3 in my study. GSK3 kinase activity is inhibited upon Wnt stimulation, but it is not yet clear whether Wnt regulates CKIε activity. Wnt appears to shift the balance of positive and negative regulators towards the positive side, ultimately resulting in the stabilization of β-catenin. Similarly, when CKIε is overexpressed, even when GSK3 activity is not inhibited, the balance of positive and negative regulators in the Axin complex is also shifted to the positive side, as overexpressed CKIε binds Axin (*13*).

The Axin complex contains other substrates for CKIε that may contribute to β-catenin stabilization. CKIε phosphorylates Axin and Dvl *in vitro* and *in vivo* when co-overexpressed in mammalian cells (data not shown). The phosphorylation of these two β-catenin regulators by CKIε may also modulate the functions of these regulators. Recently, Tcf3 and APC were shown to be phosphorylated by CKIε (*30, 16*). Tcf3 directly binds CKIε. Lee *et al.* showed that, in *Xenopus* egg extracts, Tcf3 stabilizes β-catenin, and CKIε enhances the effect. Phosphorylation of Tcf3 by CKIε increases binding between Tcf3 and β-catenin, and this promotes β-catenin stabilization (*30*). Rubinfeld *et al.* showed that APC is phosphorylated by CKIε in the Axin complex. In contrast to the phosphorylation of Tcf3 or β-catenin, APC phosphorylation by CKIε increases its ability to degrade β-catenin (*16*). In this case, CKIε appears to negatively regulate β-catenin. Again, this contrasts with cases in which CKIε is overexpressed and CKIε acts to stabilize β-catenin.

It is not clear how CKIε phosphorylates multiple substrates, and positively and negatively regulates β-catenin stability. The amount or activity of positive and negative regulators in the Axin complex may determine the function of CKIε in regulating β-catenin stability. Recently Gao *et al.* showed that PP2A plays an important role in regulating β-catenin stability, and that it is also regulated by CKIε activity (*24*).

Although the CKI family of kinases is considered to be constitutively active (*31*), the C-terminal domain of CKIε/δ may function as a regulatory domain (*32*). The regulation of CKIε by upstream signals, including those initiated by Wnt ligands, remains to be elucidated. It would also be interesting to determine whether the CKIε-mediated phosphorylation of Ser-45 has some role in regulating β-catenin activity other than aiding in regulating its stability. In addition to its C-terminal transactivation domain, the N-terminal region of β-catenin has also been shown to possess some transcriptional activity (*33*). It is also possible that CKIε may play a role in activating β-catenin as a transcriptional factor through phosphorylation. All these issues need to be evaluated experimentally in the future.

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