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Acetylation of β-Catenin by CREB-binding Protein (CBP)*

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Acetylation controls the activity of numerous proteins involved in regulating gene transcription as well as many other cellular processes. In this report we show that the CREB-binding protein (CBP) acetyltransferase acetylates β-catenin protein *in vivo*. β-Catenin is a central component of the Wnt signaling pathway, which is of key importance in development as well as being heavily implicated in a variety of human cancers. We show that the CBP-mediated acetylation of β-catenin occurs at a single site, lysine 49. Importantly, this lysine is frequently found mutated in cancer and is in a region of importance to the regulation of β-catenin. We show that mutation of this site leads specifically to an increase in the ability of β-catenin to activate the c-*myc* gene but not other β-catenin-regulated genes. This suggests that acetylation of β-catenin is involved in regulating Wnt signaling in a promoter-specific fashion.

The Wnt signaling pathway is involved in numerous developmental processes (1), and its deregulation is implicated in the development of cancer (2). A key component in the Wnt signaling pathway is the β-catenin protein, which is responsible for transducing the Wnt signal from the cytoplasm to the nucleus where it results in the activation of Wnt-responsive genes. β-Catenin was originally identified as a component of cell-cell adhesion complexes, connecting cadherins to the cytoskeleton (3). Subsequently, a large proportion of β-catenin was shown to be localized to the cell membrane. This pool of β-catenin is thought not to be involved in Wnt signaling. The signaling pool of β-catenin is relatively small and is found free in both the cytoplasm and the nucleus where it activates transcription by binding and co-activating the TCF/LEF-1[1] family of transcription factors.

One of the key aspects of the regulation of β-catenin is control of its stability, which in turn is thought to regulate its translocation into the nucleus (4). β-Catenin is a target of the ubiquitin-dependent proteasome degradation pathway (5). When the Wnt signaling pathway is in the "off" state β-catenin is continuously ubiquitinated and degraded. It is the phosphorylation of β-catenin by the GSK3β kinase that targets it for ubiquitination. Phosphorylation occurs at three serines and a threonine residue in the N terminus of β-catenin (6) and potentiates β-catenin binding to the F-box protein β-TrCP, which is a part of the SCF ubiquitin ligase complex responsible for the ubiquitination of β-catenin (7). Upon activation of the Wnt pathway GSK3β no longer phosphorylates β-catenin due, at least in part, to the antagonizing function of the disheveled protein.

Deregulation of the Wnt pathway occurs in many types of cancer and is caused by mutation of many of the Wnt signaling components (2). In the case of β-catenin it has been found that most of the mutations occur in the N terminus at or adjacent to the phosphorylated serine and threonine residues (see Fig. 1). Many of these mutations have been shown to stabilize β-catenin and thus lead to overactivation of the Wnt pathway. This overactivation is thought in part to promote oncogenesis through overexpression of TCF/LEF target genes, such as c-*myc* and cyclin D1 (8, 9).

CBP/p300 are distinct but related members of a protein family that participate in many physiological processes including proliferation, differentiation, and apoptosis (10). They are transcriptional co-activators (11) and have an intrinsic histone acetyltransferase activity (12, 13). CBP/p300 are thought to activate transcription by relieving chromatin-dependent repression by the acetylation of histones, by acting as a scaffold for recruiting other co-activator proteins (10), and by acting as a bridge to the basal transcription machinery (14). Recently it was reported that β-catenin is co-activated by CBP/p300, but this effect seems to be promoter-dependent (15–17).

Histone acetyltransferases are being recognized increasingly as modifiers of non-histone proteins, and there is a growing body of evidence supporting the theory that acetylation, like phosphorylation, is an important regulatory protein modification (18). Acetylated proteins include p53 (19), high mobility group I(Y) (20), and E2F1 (21), and these acetylation events have been shown to directly affect protein function. For instance, p53 acetylation stimulates the p53 pathway following a cell's exposure to UV radiation (22). Here we demonstrate that β-catenin is acetylated by CBP at a lysine that is often found mutated in thyroid cancer. A β-Catenin protein, mutated at this lysine, can no longer be acetylated by CBP. In addition, we find that mutation at this site leads to an increase in the ability of β-catenin to specifically activate the c-*myc* gene promoter.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—Myc-tagged mammalian expression constructs of β-catenin K49R (pCS2$^+$MMBCmycK49R) and K19R (pCS2+MMBCmycK19R) have been described previously (5). The 5× Myc-tagged construct WT β-catenin and β-catenin mutated to alanine at serines 33, 37, and 45 and threonine 41 were gifts from Hans Clevers. Wild type Myc-tagged β-catenin in the same background as pCS2$^+$-MMBCmycK49R was constructed by inserting *Nco*I-digested human

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[1] The abbreviations used are: TCF, T-cell factor; LEF, lymphoid enhancer factor; CBP, CREB-binding protein; GSK, glycogen synthase kinase; WT, wild type; P/CAF, p300/CBP-associated factor.

wild type β-catenin sequence into *Nco*I-digested pCS2$^+$MMBCmycK49R. This produces a construct that expresses full-length mouse β-catenin protein (pCS2$^+$MMBCmycWT). Full-length C-terminally His-tagged β-catenin constructs and mutants were constructed by PCR from the relevant mutant background and cloned into pET30z (Novagen). pTOPFlash pRc/RSVmCBP-HA, Del-1 c-*myc* in pBV-luc, −962 cyclin D1 in pGL3Basic, and pCXP/CAF-FLAG are all previously described (8, 9, 23–25).

Transfections, Immunoprecipitations, and Immunoblotting—For endogenous β-catenin immunoprecipitation HEK293T cells were lysed in IPH buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 0.5% Nonidet P-40, 0.5 mM EDTA), and a specific anti-β-catenin antibody (4 μg) (Santa Cruz, H-102) was added for 16 h. Lysate was then incubated for 2 h with a mixture of protein A/G-Sepharose (Roche Molecular Biochemicals). Beads were washed five times in IPH buffer, and immunoprecipitated proteins were resolved in a 8% SDS-PAGE gel. Control immunoprecipitation was performed with an anti-Myc antibody (Roche Molecular Biochemicals, 9E10). For the overexpression of β-catenin and mutants, 10-cm dishes of HEK293T cells (70–80% confluent) were transfected using standard calcium phosphate transfections with 5 μg of appropriate β-catenin expression plasmid and either 5 μg of pRc/RSVmCBP-HA or an empty vector as control. Cell medium was changed 8–16 h after transfection, and cells were harvested 24 h later. Cells were lysed, and protein was immunoprecipitated as for endogenous β-catenin except that an anti-Myc antibody was used (Roche Molecular Biochemicals, 9E10). Western blot analysis was performed on the immunoprecipitates using a specific anti-acetylated lysine antibody (1:1000) (Cell Signaling Technology).

In Vitro Acetylation Assay and Production of Recombinant Proteins—Recombinant His-tagged β-catenins were produced by transforming BRL21 *Escherichia coli* with the appropriate plasmid. Bacteria were grown to mid log phase (OD 0.6), and protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 0.1 mM. Bacteria were harvested and lysed 5 h postinduction in phosphate-buffered saline containing 1% Triton X-100 by sonicating. Proteins were purified using nickel-agarose beads. Bound proteins were eluted from the beads using imidazole (400 mM) and subsequently dialyzed into Tris-buffered saline and 20% glycerol. Purified β-catenins were then added to a standard acetylation reaction (12) using a modified incubation buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol) containing CBP/p300 immunoprecipitated from HeLa nuclear extract using a specific antibody (PharMingen 14991A) and ^{14}C-radiolabeled acetyl-CoA. The products of the reaction were resolved in a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and exposed to film. β-Catenin protein levels were shown to be equal by Ponceau S staining of nitrocellulose.

Transient Transfection Reporter Assays and Cell Culture—HEK 293T were transformed using the standard calcium phosphate co-precipitation technique. A β-galactosidase-expressing plasmid (0.3 μg) was included in each transfection to monitor transfection efficiency. After 48 h, the cells were lysed, and luciferase and β-galactosidase activities were determined by enzyme assay kits from Promega. Luciferase activity was normalized to β-galactosidase activity as an internal transfection control. Each experiment was performed in triplicate and repeated at least three times. Results shown are single experiments representative of results obtained. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium with 4.5g/liter glucose and 10% heat-inactivated fetal bovine serum and glutamine in 5% CO_2 atmosphere.

Reverse Transcription-PCR—Total cellular RNA was extracted with TRIzol (Invitrogen) from 293T cells transiently transfected with pCS2+ as a control, wt, and mutant K49R β-catenin using FuGENE 6 (Roche Molecular Biochemicals). The samples were then treated with DNase I (Ambion Co.). 5 μg of RNA from each sample was incubated with random primers and Superscript reverse transcriptase (Invitrogen) to yield cDNA. For each gene, PCRs were performed to determine the linear range of amplification that would permit a quantitative assessment of expression levels. Primers specific for c-*myc* are: forward 5′-CTT CTG CTG GAG GCC ACA GCA AAC CTC CTC-3′, reverse 5′-CCA ACT CCG GGA TCT GGT CAC GCA GGG-3′, 308-bp product; for cyclin D1: forward 5′-GAG ACC ATC CCC CTG ACG GC-3′, 483-bp product; and for highly conserved ribosomal protein L7: forward L7 5′-GGG GGA AGC TTC GAA AGG CAA GGA GGA AGC-3′, reverse L7 5′-GGG GGG TCG ACT CCT CCA TGC AGA TGA TGC-3′, 475-bp product. Each primer set was amplified at 95 °C for 30 s, 61 °C (c-*myc*), 65 °C (cyclin D1), or 55 °C (L7) for 30 s and 72 °C for 30 s for the indicated number of cycles followed by a 10-min extension at 72 °C. Amplified PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μg/ml). Bands were analyzed by Fluor-S MultiImager (Bio-Rad).

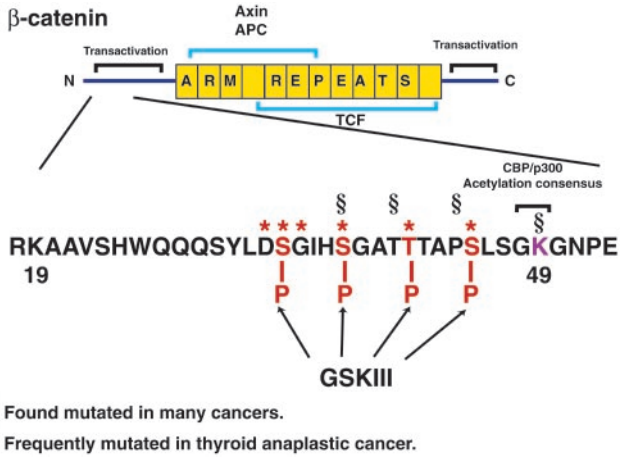

FIG. 1. **β-Catenin lysine 49 is in a region of functional importance and is frequently mutated in thyroid anaplastic carcinoma.** Lysine 49 lies in close proximity to four sites of GSKIII phosphorylation that are frequently mutated in many types of carcinoma. *APC*, adenomatous polyposis coli.

RESULTS

A large proportion of anaplastic thyroid carcinomas contain somatic mutations in the β-catenin gene (26), which lead to changes in the primary amino acid sequence of the β-catenin protein (Fig. 1). Many of these mutations occur at, or adjacent to, the well characterized regulatory serine and threonine residues in the N terminus. Interestingly, the site with the highest prevalence of mutation, lysine 49 (Lys-49), lies very close to the sites of phosphorylation. Because Lys-49 has no characterized function (2) we investigated whether mutation at this residue has physiological relevance. To address this we decided to compare the ability of wild type β-catenin to activate target promoters to a β-catenin mutant containing a single lysine to arginine substitution at position 49 (K49 Mut). The TCF-responsive promoters, TOP-FLASH, cyclin D1, and c-*myc* (Fig. 2), were used in our analysis. Transient transfection experiments in human 293T cells showed that the K49 Mut has no significant effect upon the ability of β-catenin to activate the TOP-FLASH or cyclin D1 promoter-driven reporter constructs (Fig. 2, *A* and *B*). However, in the case of the c-*myc* promoter β-catenin K49 Mut shows an increased ability to activate this reporter compared with the wild type protein (Fig. 2*C*). To confirm that these transient transfection experiments relate to the effect of β-catenin on endogenous genes, we performed reverse transcription-PCRs to determine the relative expression levels of the endogenous c-*myc* and cyclin D1 genes in cells that had been transiently transfected with the different β-catenin constructs (Fig. 2*D*). Again we observed that the K49R mutation increased the ability of β-catenin to up-regulate the c-*myc* gene and not the cyclin D1 gene. We do note that in this experiment WT β-catenin is able to activate the c-*myc* promoter unlike in the transient transfection experiments. This discrepancy is probably due to differences in either size or chromatin context of the c-*myc* promoter in these two experiments. These observations are consistent with the finding that the c-*myc* oncogene is often found highly expressed in thyroid anaplastic cancer (27, 28), and it possibly explains the prevalence of this mutation in this type of carcinoma.

Having determined that the Lys-49 to Arg mutation leads to activation of the c-*myc* promoter we next investigated possible biochemical mechanisms that may underlie this observation. One possibility is that Lys-49 is a site of ubiquitination, and its mutation leads to stabilization of β-catenin. However, this seems unlikely as it has previously been shown that Lys-49 is not a major target of the ubiquitination machinery (5). We

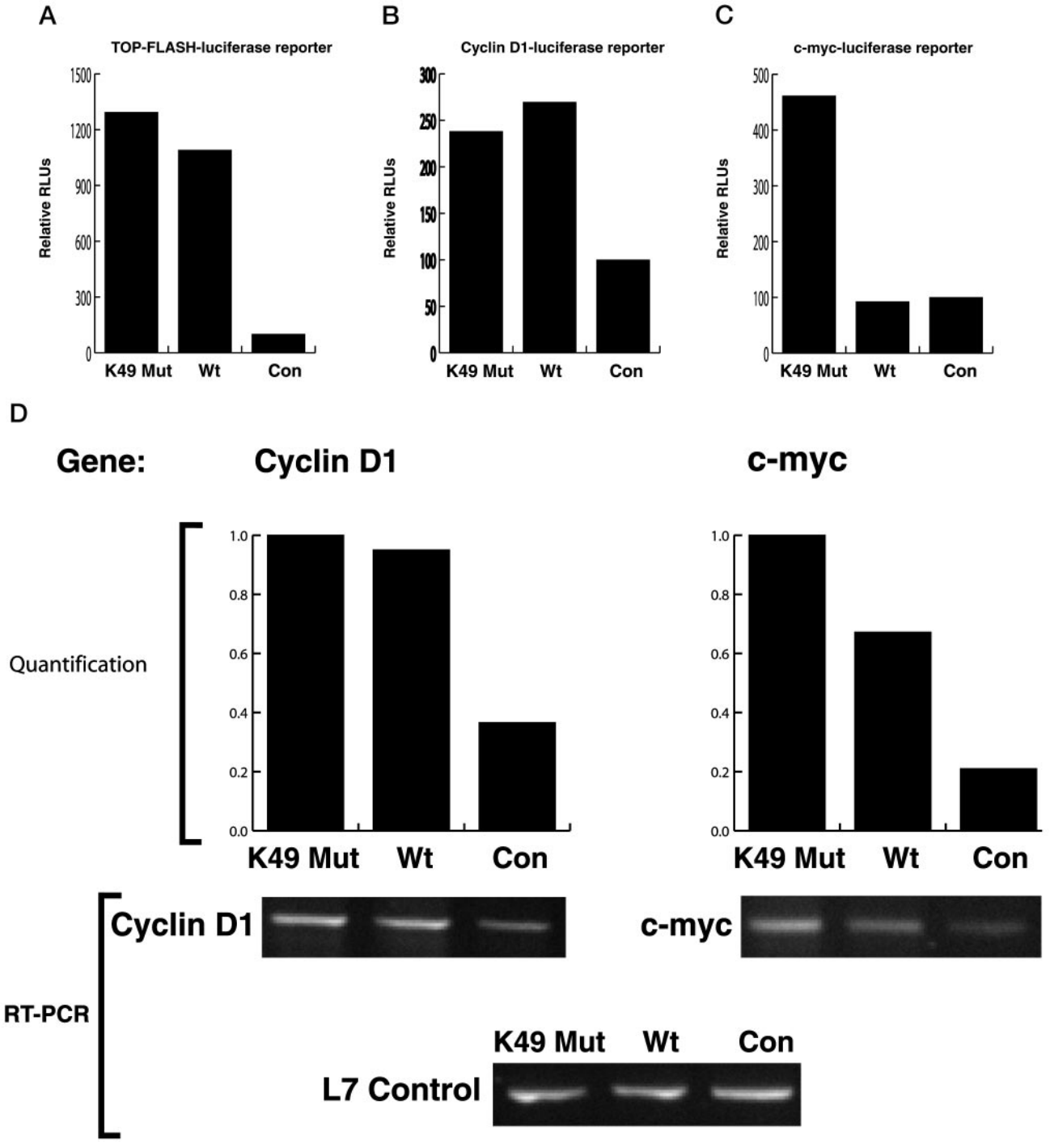

FIG. 2. **Comparison of WT β-catenin and β-catenin mutated at Lys-49 transcriptional activation properties on different promoters.** HEK 293T cells were transfected with 1 μg of luciferase reporter constructs driven by the TOP-FLASH (*A*), cyclin D1 (*B*), or c-*myc* (*C*) promoters and co-transfected with either a control plasmid (pCS2$^+$), wild type β-catenin (pCS2$^+$MMBCmycWT) (*WT*), or β-catenin with lysine 49 mutated to arginine (pCS2$^+$MMBCmycK49R) (*K49 MUT*). Representative examples of each experiment are shown. Levels of β-catenin were confirmed to be equal by Western blot (data not shown). *D*, reverse transcription-PCR analysis of c-*myc*, cyclin D1, and the *L7* ribosomal protein genes in HEK 293T cells transiently transfected with WT, K49R β-catenin, or control plasmid (pCS2$^+$). Quantification of the gels normalized against loading control of L7 ribosomal protein is shown in bar graphs.

therefore decided to focus on other potential modifications of lysine 49. Recently, the human importin-α protein Rch1 was shown to be acetylated by CBP (29). The importin-α protein has a very similar domain structure to β-catenin (Fig. 3*A*). Both proteins contain armadillo repeats at the N terminus; importin-α has a lysine (Lys-22), which is acetylated by CBP. Preceding lysine 22 is a glycine, which is indicative of a CBP-specific motif (Fig. 3*A*) (29). We noted that Lys-49 in β-catenin is also preceded by a glycine, and therefore it was considered as a candidate for acetylation by CBP. To investigate whether β-catenin is acetylated *in vivo*, endogenous β-catenin was immunoprecipitated from 293T cells and subjected to Western blot analysis using an antibody that recognizes acetylated lysines. Fig. 3*B* shows that endogenous β-catenin is indeed acetylated.

To establish which acetyltransferase is responsible for acetylating β-catenin *in vivo*, a set of candidate acetyltransferases (CBP, hGCN5, and P/CAF) were co-transfected with Myc-

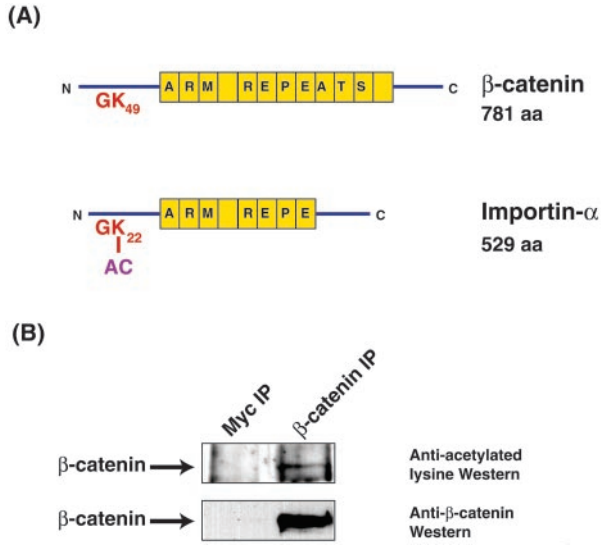

FIG. 3. ***β*-Catenin is acetylated *in vivo*.** *A*, structural similarities between β-catenin and importin-α. *B*, β-catenin is acetylated *in vivo*. Endogenous β-catenin was immunoprecipitated from 293T cells in IPH buffer using a specific anti-β-catenin antibody. Control immunoprecipitation was performed using an anti-Myc antibody. Western blot analysis was performed on the immunoprecipitate using a specific anti-acetylated lysine antibody. Levels of β-catenin in lanes were confirmed by stripping the blot and reprobing with anti-β-catenin antibody.

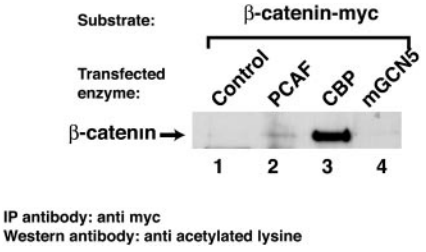

FIG. 4. ***β*-Catenin is specifically acetylated by CBP.** 293T cells were transiently transfected with a construct expressing β-catenin fused to a Myc tag at the N terminus. Cells were co-transfected with constructs expressing the acetyltransferases P/CAF, CBP, and hGCN5. 36 h after transfection cells were lysed in IPH (12), and β-catenin was immunoprecipitated using an anti-Myc antibody. Western blot analysis of precipitates was carried out using an anti-acetylated lysine antibody. β-Catenin levels were assessed by Western blot and shown to be the same in each lane (data not shown).

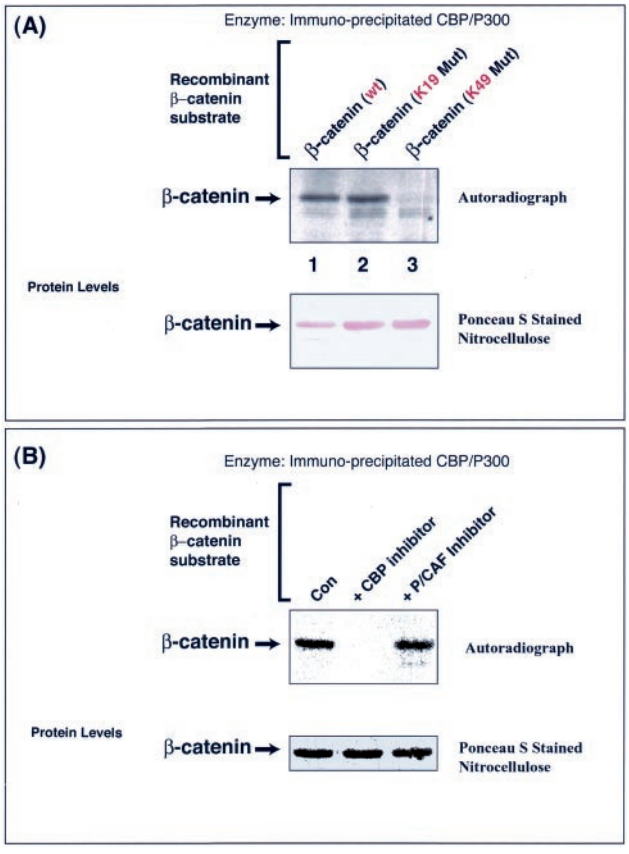

FIG. 5. ***β*-Catenin is acetylated at lysine 49 by CBP *in vitro*.** *A*, wild type full-length mouse β-catenin (*WT*), β-catenin with lysine 19 mutated to arginine (*K19 Mut*), and β-catenin with lysine 49 mutated to arginine (*K49 Mut*) were expressed as C-terminal His tag fusions. Purified β-catenins were then added to a standard acetylation reaction (12) using incubation buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, pH 7.5) containing CBP/p300 immunoprecipitated from HeLa nuclear extract (PharMingen 14991A). The products of the reaction were resolved in a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and exposed to film. β-Catenin levels were shown to be the same by Ponceau S staining of nitrocellulose. *Lane 3* shows that β-catenin mutated at Lys-49 is not acetylated. *B*, acetylation reactions were preformed as above using WT β-catenin and a p300/CBP inhibitor (20 μM LysCoA) in *lane 2* and a P/CAF inhibitor in *lane 3* (20 μM H3-CoA-20)

tagged β-catenin into 293T cells. β-catenin was immunoprecipitated and subjected to an anti-acetylated lysine Western blot analysis (Fig. 4). When CBP is co-transfected with β-catenin a dramatic increase in the level of acetylation is observed (compare *lanes 1* and *3*). However, co-transfection of other acetyltransferases caused no appreciable increase in the level of acetylation, indicating that β-catenin is specifically acetylated by CBP. The observation that CBP acetylates β-catenin is consistent with the fact that β-catenin and CBP/p300 interact (15, 16).

To ascertain whether β-catenin is acetylated by CBP at Lys-49, as the presence of the GK motif would suggest, full-length β-catenin with Lys-49 mutated to arginine was expressed recombinantly. As a control, β-catenin with Lys-19 mutated to arginine (K19 Mut) was also expressed. These two mutants, as well as the full-length recombinant wild type protein, were used as substrates in *in vitro* acetylation reactions (modified from Ref. 12). Immunoprecipitated p300/CBP from HeLa nuclear extract was used as a source of CBP/p300 as recombinant CBP and p300 were found to acetylate β-catenin relatively inefficiently *in vitro* (data not shown). Fig. 5*A* shows that the β-catenin Lys-49 mutant is not acetylated by CBP/p300 (*lane 3*), whereas β-catenin mutated at Lys-19 is as good a substrate as WT (*lanes 1* and *2*). These results demonstrate that β-catenin is acetylated exclusively at Lys-49 by CBP/p300. To ensure that the acetyltransferase activity responsible for the acetylation of β-catenin (in Fig. 5*A*) was p300 or CBP (rather than the CBP-associated factor P/CAF) specific inhibitors of CBP/p300 or P/CAF were added to the reactions (30). Fig. 5*B* shows that β-catenin acetylation is abrogated in the presence of the p300/CBP inhibitor, whereas the P/CAF inhibitor had no effect on the acetylation of β-catenin. This result is consistent with the finding that CBP but not PCAF can acetylate β-catenin *in vivo* (Fig. *5*).

To confirm that residue Lys-49 in β-catenin is the sole target for CBP *in vivo*, we co-expressed CBP with various β-catenin mutants to determine which mutation prevented CBP acetylation. Fig. 6 shows that β-catenin mutated at Lys-49 is severely impaired for acetylation by CBP (*lane 3*); in contrast, mutation of Lys-19 does not affect the ability of CBP to acetylate β-catenin (*lane 2*). We conclude that Lys-49 is the major target for *in vivo* acetylation by CBP. To explore the possibility that phosphorylation by GSKIII may affect the acetylation status of β-catenin we used as a substrate a stabilized β-catenin with the four known GSKIII phosphorylation sites (Ser-33, Ser-37, Ser-

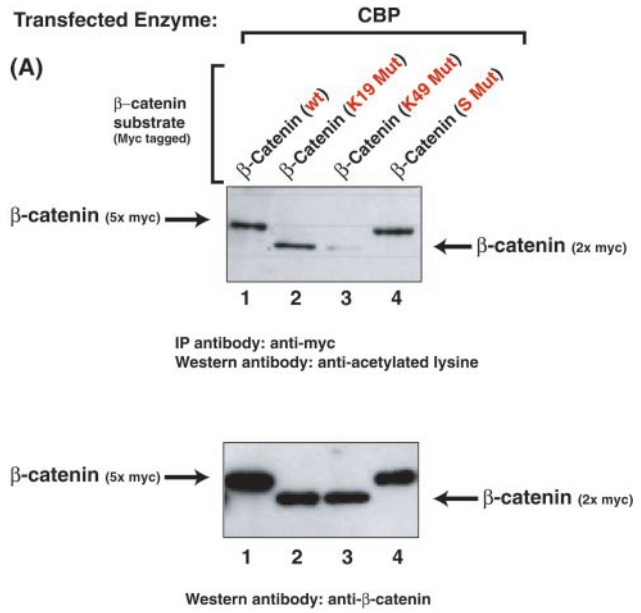

FIG. 6. *A*, β-catenin is acetylated by CBP at lysine 49 *in vivo*. 293T cells were transiently transfected with constructs expressing wild type and mutated β-catenin fused to Myc tags at the N terminus. Mutations within β-catenin are described in Fig. 3 except S Mut, which is β-catenin mutated to alanine at serines 33, 37, and 45 and threonine 41. Cells were co-transfected with a construct expressing CBP. 36 h after transfection cells were lysed in IPH, and β-catenin was immunoprecipitated using an anti-Myc antibody. Western blot analysis of the precipitates was carried out using an anti-acetylated lysine antibody. Precipitated levels of β-catenin were then confirmed by stripping the blot and reprobing with anti-β-catenin antibody. *Lane 3* shows that β-catenin mutated at lysine 49 is severely impaired for acetylation by CBP *in vivo.*

45, and Thr-41 (6)) mutated to alanine. CBP acetylated this stabilized form of β-catenin, suggesting that the phosphorylation of β-catenin does not affect the ability of CBP to acetylate Lys-49 (Fig. 6, *lane 4*).

DISCUSSION

It is now apparent that control of the Wnt signaling pathway is far more complex than β-catenin simply shuttling into the nucleus and activating transcription by binding TCF/LEF DNA binding factors. A number of nuclear regulatory mechanisms of the Wnt signaling pathway have been described recently (for review see Hecht and Kemler (31)). These include regulation by factors such as Xsox17α/β (32), the LIT-1/Nemo-like kinase (33–35), and specific targeting of β-catenin-LEF complexes by Smad-4 (36). Another characterized regulator of the Wnt signaling cascade is CBP/p300, and several recent papers have shown that in mammalian systems CBP/p300 interacts with β-catenin and is able to co-activate TCF-responsive genes. This co-activation is promoter-dependent and has been shown to be independent of the acetyltransferase activity of p300/CBP (16).

We have shown that β-catenin is acetylated by CBP at lysine 49 and that mutation of this site prevents β-catenin being acetylated. We have also shown that this unacetylatable form of β-catenin is a more potent activator of the c-*myc* gene but not of other TCF-responsive genes. Taken together, these facts suggest that acetylation of β-catenin may act negatively to regulate transcription. The suggestion that CBP might be repressive has precedent in *Drosophila* where dCBP is a negative regulator of the Wnt signaling pathway. The mechanism of this repression involves the acetylation of TCF and disruption of the TCF/β-catenin interaction (37). There are also instances where CBP acts positively to co-activate β-catenin-dependent transcription, but in these cases its histone acetyltransferase activity is not necessary for activation (16).

The identification of a molecular mechanism by which acetylation regulates β-catenin activity remains elusive. We have analyzed the differences between wild type β-catenin and K49 Mut in areas such as protein stability, interaction with members of the Wnt signaling pathway, and nuclear localization (data not shown). No differences were detected in these assays. A greater understanding of how β-catenin mediates promoter-specific effects will help define avenues for future study of this effect.

Mutation of Wnt pathway members occurs in a variety of carcinomas. The two genes found most frequently mutated are adenomatous polyposis coli and β-catenin, and these mutations generally lead to an overactivation of the Wnt pathway. This overactivation is thought to promote oncogenesis through derepression and stimulation of TCF/LEF-responsive genes. The K49R mutation in β-catenin represents an example of a mutation that up-regulates specific TCF-responsive genes, namely c-*myc*. This observation is consistent with the finding that the c-*myc* oncogene is often found highly expressed in thyroid anaplastic cancer (27, 28). Moreover c-*myc* overexpression is associated with poor prognosis and thyroid cell de-differentiation (41). There is also a positive correlation between the level of c-*myc* mRNA and histological aggressiveness in thyroid tumors obtained at surgery (42). The block of c-*myc* protein synthesis with Myc-specific antisense oligonucleotides reduces the growth rate of the thyroid carcinoma cell lines significantly, which indicates that c-*myc* overexpression plays a critical role in the growth of thyroid cancer cells. Thus our observation that the β-catenin K49R mutation induces c-*myc* expression fits well with it being the most commonly mutated residue in thyroid anaplastic carcinoma.

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