Lysine Residues Lys-19 and Lys-49 of β-Catenin Regulate Its Levels and Function in T Cell Factor Transcriptional Activation and Neoplastic Transformation*

Received for publication, May 3, 2006 Published, JBC Papers in Press, July 18, 2006, DOI 10.1074/jbc.M604217200

Ira S. Winer1, Guido T. Bommer1, Nathan Gonik1, and Eric R. Fearon1,5,*,†,‡

From the 1Department of Molecular Medicine and Genetics, Department of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, Michigan 48109

Wnt signaling regulates cell fate determination, proliferation, and survival, among other processes. Certain Wnt ligands stabilize the β-catenin protein, leading to the ability of β-catenin to activate T cell factor-regulated genes. In the absence of Wnts, β-catenin is phosphorylated at defined serine and threonine residues in its amino (N) terminus. The phosphorylated β-catenin is recognized by β-transducin repeat-containing protein (βTrCP) and associated ubiquitin ligase components. The serine/threonine residues and βTrCP-binding site in the N-terminal region of β-catenin constitute a key regulatory motif targeted by somatic mutations in human cancers, resulting in constitutive stabilization of the mutant β-catenin proteins. Structural studies have implicated β-catenin lysine 19 as the major target for βTrCP-dependent ubiquitination, but Lys-19 mutations in cancer have not been reported. We studied the consequences of single amino acid substitutions of the only 2 lysine residues in the N-terminal 130 amino acids of β-catenin. Mutation of Lys-19 minimally affected β-catenin levels and functional activity, and mutation of Lys-49 led to reduced β-catenin levels and function. In contrast, β-catenin proteins with substitutions at both Lys-19 and Lys-49 positions were present at elevated levels and had the ability to potently activate T cell factor-dependent transcription and promote neoplastic transformation. We furthermore demonstrate that the K19/K49 double mutant forms of β-catenin are stabilized as a result of reduced βTrCP-dependent ubiquitination. Our findings suggest that Lys-19 is a primary in vivo site of βTrCP-dependent ubiquitination and Lys-49 may be a secondary or cryptic site. Moreover, our results inform understanding of why single amino acid substitutions at lysine 19 or 49 have not been reported in human cancer.

Wnts are a family of secreted proteins with critical roles in embryonic development and adult tissues, including functions in regulating cell fate specification, proliferation, survival, and motility (1–5). The “canonical” Wnt pathway is the best characterized Wnt-dependent signaling pathway, and mutations altering essential factors in this pathway have been most clearly implicated in cancer. In the canonical pathway, the β-catenin protein is a key downstream mediator, with certain Wnts (e.g. Wnt-1) acting to stabilize β-catenin. In the absence of activating Wnt signals, glycosen synthase kinase 3β functions in concert with the Axin and adenomatous polyposis coli tumor suppresor proteins and other factors, such as casein kinases, to phosphorylate β-catenin at defined serine and threonine residues in its N-terminal domain. The phosphorylated β-catenin is recognized and ubiquitinated by a complex containing a β-transducin repeat-containing protein (βTrCP),2, and β-catenin is then degraded by the proteasome (6–11). Wnt binding to the Frizzled low density lipoprotein-related protein-5/6 co-receptor complex on the cell surface leads to inhibition of the AXIN/glycosen synthase kinase 3β complex, reduced phosphorylation of β-catenin, and stabilization of the “free” cytosolic and nuclear pools of β-catenin (12–16). In the nucleus, β-catenin can bind to members of the T cell factor (TCF) transcription factor family, and β-catenin-TCF complexes modulate transcription of an array of genes, several of which have been shown to play roles in effecting changes in cell fate, proliferation, and other processes.

Mutational mechanisms with major contributing roles to dysregulation of β-catenin in human cancer include inactivation of the adenomatous polyposis coli, AXIN1 or AXIN2 tumor suppressor proteins, or activating (oncogenic) mutations in a key regulatory motif in the N-terminal domain of β-catenin. This regulatory motif consists of the apparent casein kinase I α phosphorylation site at serine 45; the glycosen synthase kinases 3β phosphorylation sites at threonine 41, serine 37, and serine 33; and the 6-residue destruction motif encompassing amino acids 32–37 of β-catenin, to which βTrCP1 shows Ser-33 and Ser-37 phosphorylation-dependent binding. In cancer cells with inactivation of the adenomatous polyposis coli or AXIN proteins or oncogenic β-catenin mutations, the β-catenin protein is constitutively stabilized in the absence of Wnt signals, and altered transcription of downstream β-catenin/TCF-regulated target genes ensues, including proto-onco-
genes such as c-myc and cyclin D1 (reviewed in Refs. 3–4, 14, 17).

Though much is known about factors regulating β-catenin in normal and cancer cells, gaps in our knowledge remain. For example, elegant structural studies from Wu et al. (11) suggested that binding of the F-box protein βTrCP1, via its WD repeat domain, to amino acids 32–37 of β-catenin directs ubiquitin conjugation to lysine 19 of β-catenin by the βTrCP1-associated E3 ubiquitin ligase known as SCF (Skp1, Cullin, F-box). Yet, assuming Lys-19 is the critical lysine for SCF-mediated ubiquitination of β-catenin, it is curious that mutational analyses of β-catenin in cancer have not uncovered Lys-19 mutations. Although the studies of Wu et al. indicated β-catenin Lys-19 was the probable site for SCF-βTrCP1-mediated ubiquitination, the authors did not define in vivo sites of ubiquitination on the full-length β-catenin protein. The authors did, however, note that in β-catenin and other proteins with a destruction motif the location of the ubiquitinated lysine relative to the destruction motif was likely a critical factor for optimal efficiency of SCF-βTrCP1-mediated ubiquitination. The optimal distance was judged to be 9–14 amino acids N-terminal to the destruction motif. While Lys-19 is 13 residues N-terminal to the destruction motif, β-catenin also has another lysine, Lys-49, located roughly a similar distance carboxyl (C)-terminal to the destruction motif. Of interest, one prior study of mutant β-catenin proteins carrying missense substitutions of Lys-19 and/or Lys-49 had suggested mutation of Lys-19 or Lys-49 on their own had little effect on β-catenin ubiquitination and both Lys-19 and Lys-49 needed to be mutated to reduce β-catenin ubiquitination even minimally (18). These results were not investigated further.

In light of uncertainties surrounding the significance of Lys-19 and Lys-49 in regulation of β-catenin stability and function, we have pursued studies to address the biological activity of β-catenin proteins carrying missense substitutions of Lys-19 and Lys-49. We show here that single amino acid substitutions of either Lys-19 or Lys-49 minimally affect the biological activity of β-catenin and in the case of K49R even reduce expression levels. In contrast, β-catenin proteins with substitutions at both Lys-19 and Lys-49 have the ability to activate TCF transcription and promote neoplastic transformation in a fashion akin to that of a cancer-derived mutant form of β-catenin in which serine 33 is mutated to tyrosine (i.e. S33Y).

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The β-catenin cDNAs utilized in these studies were subcloned into the pBMN-Z retroviral vector. The wild type and S33Y (S33Y) mutant β-catenin constructs have been previously described (19–21). The β-catenin K19R (19R), K19E (19E), K49R (49R), K49E (49E), K19R/K49E (RE), K19E/K49E (EE), and K19R/K49R (RR) constructs were constructed via two-step PCR site-directed mutagenesis using the WT construct as the template. All constructs incorporated a FLAG tag on the C terminus. Sequences of all constructs were confirmed via automated sequencing. The reporter constructs pTOPFLASH, which contains three copies of an optimal TCF binding motif (CCTTGGATC) upstream of the luciferase gene, and pFOPFLASH, which contains three copies of a mutated motif (CCTTGGCC) upstream of the luciferase gene, have been previously described (22). The LacZ/pBMN plasmid was kindly provided by Dr. G. Nolan. The pCDNA3 LacZ construct carries the β-galactosidase open reading frame driven by a cytomegalovirus immediate early promoter.

**RK3E Focus Formation Studies**—RK3E and Phoenix cells were grown in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum/1% penicillin-streptomycin (Invitrogen). The amphotrophic Phoenix cells retroviral packaging cell line was obtained from Dr. G. Nolan (Stanford University School of Medicine). Transfection of Phoenix cells was carried out at ~60% confluency, using FuGENE 6 (Roche Applied Science) according to protocol. The following day the medium was changed. 48 h post-transfection, the medium was collected and filtered through a 0.4-μm filter. RK3E cells were obtained from American Type Tissue Culture Collection (Rockville, MD). When the cells had reached ~50% confluence, they were transduced with one of the previously collected supernatants mixed 1:1 with normal culture medium containing polybrene (4 μg/ml final concentration): pBMN/WT β-catenin (×2)-negative control, pBMN/S33Y (×2)-positive control, pBMN/β-catenin mutant constructs. The medium was changed the next day to growth medium, and the cells were allowed to grow for 25 days. The plates were washed with 1× PBS and fixed using a solution of 1.6% glutaraldehyde, 0.06% methylene blue in Hank’s Balanced Salt Solution (Invitrogen) for 3 h at room temperature. Plates were destained in multiple changes of 1× PBS. Foci numbers were quantified from the plates by manual counting.

**Luciferase Assays**—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum/1% penicillin-streptomycin (Invitrogen). Cells were transfection using FuGENE 6 transfection reagent using 2 μl of reagent/2 μg of DNA. Cells were plated in 35-mm dishes 12 h prior to transfection, and 0.85 μg of plasmid DNA encoding a mutant or WT form of β-catenin were transfected along with 0.75 μg of either the pTOPFLASH or pFOPFLASH reporter construct and 0.4 μg of LacZ/PCDNA-3 in duplicate. 48 h post-transfection cells were washed once with 1× PBS and suspended in reporter lysis buffer. Luciferase activities were measured in a luminometer after adding luciferase assay reagent (Promega) to the cell lysates. β-galactosidase activities were determined according to standard methods and were used to control for transfection efficiency.

**Transfection of Cells and Analysis of the Ubiquitination or Phosphorylation Levels**—HEK293 cells were plated in 100-mm dishes 12 h prior to transfection. Transfections were performed using FuGENE 6. 48 h post-transfection, cells from all of the plates were harvested into 1 ml of cold 1× PBS and lysed using a 1% Triton-based lysis buffer with addition of 1 mm sodium orthovanadate and 200 mm sodium fluoride. Western blot analysis was performed as previously described (19). Protein expression levels for WT, S33Y, and other mutant forms of β-catenin were detected with a monoclonal anti-FLAG antibody (Stratagene). Expression of β-actin was used to control for the loading of the lanes and was detected utilizing an anti-β-actin polyclonal antibody (Sigma). Fractionation of cells into cytoplasmic and nuclear fractions was performed using the “NE-PER” extraction reagent (Pierce). Total cellular extracts were subcloned into the pBMN-Z retroviral vector. The wild type and S33Y (S33Y) mutant β-catenin constructs have been previously described (19–21). The β-catenin K19R (19R), K19E (19E), K49R (49R), K49E (49E), K19R/K49E (RE), K19E/K49E (EE), and K19R/K49R (RR) constructs were constructed via two-step PCR site-directed mutagenesis using the WT construct as the template. All constructs incorporated a FLAG tag on the C terminus. Sequences of all constructs were confirmed via automated sequencing. The reporter constructs pTOPFLASH, which contains three copies of an optimal TCF binding motif (CCTTGGATC) upstream of the luciferase gene, and pFOPFLASH, which contains three copies of a mutated motif (CCTTGGCC) upstream of the luciferase gene, have been previously described (22). The LacZ/pBMN plasmid was kindly provided by Dr. G. Nolan. The pCDNA3 LacZ construct carries the β-galactosidase open reading frame driven by a cytomegalovirus immediate early promoter.
were produced as described previously (19). Antibodies used for confirmation of cell fractions were a rabbit polyclonal against the nuclear protein TIP-49 (23) and a monoclonal against β-galactosidase (Promega), which was expressed from the cotransfected plasmid pCDNA3 LacZ to demonstrate equal transfection efficiency. For the ubiquitination studies, cells were transfected according to the above protocol with β-catenin constructs and a HA epitope-tagged ubiquitin. 60 h post-transfection, MG-132 (AG-Scientific) at a final concentration of 40 μM was added to the cells with fresh medium for 6 h. The cells were harvested in the Triton-based lysis buffer described above. Lysates were immunoprecipitated using monoclonal M2-FLAG-linked agarose beads (Sigma) according to the manufacturer’s protocol. Immunoprecipitated lysates were run on a low percentage SDS-PAGE gel and blotted utilizing an anti-HA rat monoclonal antibody (Roche Applied Science) and M2-anti-FLAG antibody as a loading control. For the phosphorylation studies, HEK293 cells were transfected with β-catenin constructs and lysed/immunoprecipitated as above. Following SDS-PAGE, β-catenin phosphorylation was assessed using an anti-phospho-β-catenin-33/37/41 antibody (Cell Signaling Technologies). The M2-anti-FLAG was used to measure total ectopic β-catenin levels. Western blots were quantified using the ImageJ analysis software (NIH).

**RESULTS**

β-catenin proteins show greatest conservation in their central roughly 560-amino acid region, which consists of the 12 armadillo repeats that mediate β-catenin binding to adenomatous polyposis coli, AXIN, and TCFs. The N-terminal region of β-catenin also has some highly conserved motifs in vertebrate homologues and the *Drosophila* β-catenin homologue armadillo. In addition to conservation of the serine and threonine residues that are phosphorylated by casein kinase 1α and glycogen synthase kinase 3β and the βTrCP1-binding/destruction motif at amino acids 32–37, the lysines corresponding to Lys-19 and Lys-49 in the human sequence are conserved (Fig. 1A). In fact, though most vertebrate β-catenin homologues have 26 lysine residues, only two lysines are located in the first 130 amino acids, with the remainder in the central armadillo repeat region.

To address the role of the Lys-19 and Lys-49 residues in β-catenin function, we generated β-catenin alleles containing missense mutations at these positions. Both charge-conservative (e.g. substitution of arginine (R), K19R, also termed 19R) and charge-non-conservative (e.g. substitution of glutamic acid (E), K19E also termed 19E) mutant alleles were generated, together with selected double mutation alleles (e.g. K19R/K49R; also termed RR; Fig. 1B). In prior published work, we have shown that use of the RK3E cell line for focus formation assays is a robust strategy for assessing the biological activity of wild type and mutant β-catenin alleles (18–20). In brief, RK3E is an adenovirus E1A-immortalized rat kidney epithelial line that grows in a contact-inhibited and anchorage- and serum-dependent manner. β-catenin proteins that are stabilized by mutations affecting the conserved N-terminal domain serine/threonine residues or the destruction motif will readily induce large numbers of morphologically transformed neoplastic foci in a TCF-dependent fashion. Wild type β-catenin does not induce transformed foci in RK3E. Consistent with our prior published results, we found the cancer-derived S33Y mutant β-catenin protein induced large numbers of transformed foci and wild type β-catenin or the empty expression vector did not generate foci (Fig. 2). Single mutations at codon 19 (19R and...
Lysines 19 and 49 of β-Catenin Regulate Ubiquitination

A

| Species      | Sequence 19 | Sequence 33 | Sequence 37 | Sequence 41 | Sequence 45 | Sequence 49 |
|--------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Human        | MATQADLMEDMAMEP | DRKAAVSHQQYSYL | DSGIHSATTAPSLSGK |          |            |             |
| Mouse        | MATQADLMEDMAMEP | DRKAAVSHQQYSYL | DSGIHSATTAPSLSGK |          |            |             |
| Rat          | MATQADLMEDMAMEP | DRKAAVSHQQYSYL | DSGIHSATTAPSLSGK |          |            |             |
| Zebrafish    | MATQSDLMEEMMDP | DRKAAVSHQQYSYL | DSGIHSATTAPSLSGK |          |            |             |
| Drosophila   | MSYPAQNRMTSHNQNYNPDLFPFMVSAKETLIMWQQNYSLYGDSIHSATTAPSLSGK |          |            |             |             |

FIGURE 1. The N terminus of β-catenin is conserved from Drosophila to man. A, ClustalW alignment of the N-terminal 49 amino acids of human, rat, mouse, and zebrafish β-catenin and Drosophila armadillo. Presumptive sites for phosphorylation (numbers 33, 37, 41, and 45) and conserved lysine residues at positions 19 and 49 are indicated. B, the mutant β-catenin proteins generated for study are shown. Charge was conserved by mutation of the lysine (K) to an arginine (R); charge was reversed by mutation of the lysine (K) residues to glutamic acid (E).

B

19E) very minimally activated β-catenin-transforming activity relative to the S33Y mutant, and single mutations at Lys-49 (49R and 49E) did not activate the transforming activity of β-catenin. In contrast to the effect of the single mutations at Lys-19 and Lys-49, β-catenin alleles carrying mutations at both Lys-19 and Lys-49 had potent ability to neoplastically transform RK3E. Alleles carrying charge-conservative substitutions at Lys-19 (i.e. RR and RE alleles) showed greater activity than a double mutant allele carrying charge-non-conservative substitutions at both Lys-19 and Lys-49 (Fig. 2, EE).

Consistent with the ability of the double mutant β-catenin alleles to promote neoplastic transformation in RK3E cells, we found the RR mutant allele strongly activated the TCF-dependent TOPFLASH model reporter gene construct in HEK293 cells (Fig. 3). Whereas the 19R allele had roughly twice the activity of wild type β-catenin in the TCF reporter gene assays and the 49R allele was no more active than wild type β-catenin, the RR allele had roughly 5-fold the activity of wild type β-catenin and roughly half the activity of the S33Y mutant allele (Fig. 3). The RK3E transformation and TCF reporter gene assay results, taken together with our prior published results (19), imply that a threshold level of β-catenin/TCF transcriptional activity is needed for neoplastic transformation in RK3E cells. But, beyond that level, only minimal enhancement of neoplastic transformation in RK3E is seen.

To address the basis for the potent transforming and TCF activation properties of the double mutant RR β-catenin proteins and the lack of such activities in the single mutant proteins, we sought to assess the abundance of the free pool of β-catenin in cells expressing these proteins. Previous studies have shown that a recombinant GST fusion protein in which GST sequences are fused in-frame to the cytoplasmic tail of E-cadherin can readily be used to monitor the abundance of the free pool of β-catenin, i.e. the pool of β-catenin that is stabilized in response to activating Wnt signals (24–27). As shown in Fig. 4A, following ectopic expression of the S33Y mutant form of β-catenin in HEK293 cells or LiCl treatment of HEK293 cells to inhibit glycogen synthase kinase 3 β-mediated phosphorylation and degradation of endogenous β-catenin, very substantial increases in the levels of the free pool of β-catenin were seen, as demonstrated by recovery of β-catenin proteins and the lack of such activities in the cell lysates following incubation with the GST-E-cadherin protein (Fig. 4B). Using the GST-E-cadherin recombinant protein, we found that abundance of the RR double mutant β-catenin protein in the free pool was nearly on par with that of the S33Y mutant. The 19R single mutant protein showed reduced free pool levels relative to the RR and S33Y mutants but clearly increased abundance compared with the 49R or wild type β-catenin proteins.

To confirm that increases in the free pool of β-catenin were associated with increased cytoplasmic and nuclear levels of β-catenin, we analyzed the relative abundance of wild type and mutant forms of β-catenin in whole cell extracts and cytoplasmic and nuclear fractions. As shown in Fig. 5, the whole cell, cytoplasmic, and nuclear levels of the RR double mutant were nearly equivalent to those seen for the S33Y mutant. The 19R
Lysines 19 and 49 of β-catenin Regulate Ubiquitination

![FIGURE 3. Mutation of Lys-19 and Lys-49 synergistically increases the capacity to activate TCF transcription. TCF reporter activity was assessed by using the β-catenin-responsive TOPFLASH reporter and the mutant control FOPFLASH reporter. The mean and S.D. of the TOP/FOP ratio, as compared with the empty vector control, for four independent experiments performed in duplicate are shown.](image)

![FIGURE 4. Mutations at Lys-19 and/or Lys-49 differentially affect the free pool of β-catenin. A, the GST-E-cadherin (GST-Ecad) fusion protein detects increases in the free pool of β-catenin. HEK293 cells were harvested 48 h after transfection with S33Y β-catenin (S33Y) or wild type β-catenin (WT) and precipitated with GST-Ecad beads. Independent experiments are denoted “a” and “b.” B, GST-E-cadherin precipitation of the free pool of different β-catenin mutants. 50 μg of total cell lysate was analyzed in Western blot analysis to assess whole cell levels of exogenous FLAG-tagged β-catenin proteins (upper panel). Free β-catenin levels were assessed by analyzing FLAG-tagged proteins bound to GST-E-cadherin beads from 250 μg of total cellular lysate (lower panel).](image)

When dividing or comparing wild type β-catenin with the mutant showed increased levels relative to wild type β-catenin. Despite the fact that the 49R mutant was essentially as active as the wild type β-catenin protein in the TCF reporter gene assay (Fig. 3), the abundance of the 49R mutant in whole cell extracts and cytoplasmic and nuclear fractions was reduced compared with those for wild type β-catenin (Fig. 5A). To exclude that the observed differences in protein levels after transfection of different β-catenin expression constructs were caused by variations in mRNA levels, we performed a Northern blot analysis. As shown in Fig. 5B, all constructs led to equivalent mRNA levels.

We next sought to clarify how mutations at Lys-19 and/or Lys-49 affected the biological activity and levels of β-catenin. We pursued studies with an antibody that reacts with β-catenin when it is phosphorylated at serines 33 and 37 and threonine 41 (i.e. anti-phospho 33/37/41 β-catenin). Because phosphorylation at these residues targets wild type β-catenin for ubiquitination and degradation, we treated cells with the proteasome inhibitor MG-132 to inhibit protein degradation. Following ectopic expression of the FLAG epitope-tagged β-catenin constructs in HEK293 cells and MG-132 treatment of the transfected cells, the extracts were immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were analyzed by immunoblotting with the anti-FLAG antibody or the anti-phospho-33/37/41 antibody. As expected, the S33Y mutant protein showed negligible reactivity with the anti-phospho-33/37/41 antibody (Fig. 6). Although a minimal increase in the relative abundance of phospho-33/37/41 β-catenin forms was seen in cells transfected with the 49R and RR mutant proteins (Fig. 6 and data not shown), there was no evidence that mutations at Lys-19 and/or Lys-49 had marked effects on the ability of the mutant proteins to be phosphorylated at serines 33 and 37 and threonine 41 (Fig. 6).

Because reduced phosphorylation of the regulatory regions of the 19R and RR mutant forms of β-catenin did not appear to play a contributing role in their increased expression levels, we pursued studies to assess the ubiquitination of the mutant proteins. Following co-expression of the FLAG epitope-tagged β-catenin constructs in HEK293 cells together with an expression construct encoding an influenza hemagglutinin (HA) epitope-tagged ubiquitin polypeptide, the transfected cells were treated with MG-132. Extracts were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were analyzed with anti-FLAG and anti-HA antibodies. After normalizing for the abundance of the ectopically expressed proteins via immunoblot analysis with anti-FLAG antibody, substantial differences were seen in the amount of HA-tagged ubiquitin that was linked to β-catenin when comparing wild type and certain mutant β-catenin proteins. The S33Y mutant protein showed minimal evidence of ubiquitinated species, either low molecular weight (LMW) or high molecular weight
Lysines 19 and 49 of β-Catenin Regulate Ubiquitination

we ectopically expressed an F-box-deleted βTrCP1 protein that functions as a dominant negative (dnβTrCP1) together with the FLAG epitope-tagged β-catenin constructs and the HA-tagged ubiquitin construct. The dnβTrCP1 protein potently inhibited the ubiquitination of wild type β-catenin and all mutant forms, essentially rendering the relative levels of ubiquitination of all proteins equivalent. These data indicate that the predominant factor(s) likely to be responsible for the ubiquitination of β-catenin proteins under the conditions studied was either βTrCP1 or βTrCP2.

DISCUSSION

A good deal is known about factors and mechanisms regulating levels and localization of β-catenin in normal and cancer cells. For instance, there is strong evidence that β-catenin levels are tightly regulated as a result of ubiquitin conjugation to β-catenin by the SCF-βTrCP ubiquitin ligase complex. The ability of this complex to regulate β-catenin appears to depend on direct binding of βTrCP1/2 to the 6-residue destruction motif encompassing amino acids 32–37 of β-catenin and to which βTrCP1/2 shows Ser-33 and Ser-37 phosphorylation-dependent binding. Notwithstanding the progress to date, certain aspects of β-catenin regulation remain to be fully clarified. For example, while structural studies had previously implicated Lys-19 as the critical lysine for SCF-βTrCP-mediated ubiquitination of β-catenin, convincing evidence that Lys-19 is ubiquitinated in vivo has not previously been offered. Moreover, if Lys-19 were the unique site for βTrCP-mediated ubiquitination, it is perhaps surprising that mutational analyses of β-catenin in cancer had not uncovered Lys-19 mutations as a means of constitutively stabilizing β-catenin. In addition, prior studies by Aberle et al. (18) had generated data suggesting that neither Lys-19 nor Lys-49 mutations on their own much affected β-catenin ubiquitination and K19/49 double mutations reduced, but did not abrogate, ubiquitination.

In our studies, we found β-catenin proteins with single missense substitutions at the Lys-19 or Lys-49 positions were minimally (Lys-19) or not at all (Lys-49) stabilized relative to wild type β-catenin. However, the K19/K49 double mutant β-catenin was present at elevated levels and had the ability to potently activate TCF transcription and promote neoplastic transformation. The K19/K49 double mutant β-catenin was stabilized as a result of reduced βTrCP-dependent ubiquitination. Interestingly, the transforming potential of β-catenin mutants was not directly proportional to the transcriptional activation potential observed. In fact, as previously suggested (19), the transformation of RK3E cells seems to require the bypass of a certain threshold of activity. It is tempting to speculate that the modest subthreshold increase in transcriptional activity by the K19R mutation of β-catenin is not sufficient to confer a sufficient selective advantage in vivo that would allow selection of a secondary mutation at Lys-49, thereby producing a double mutant.

Comparison of the relative levels and functional activity of wild type β-catenin to that of each mutant β-catenin protein studied here, along with the data on the relative levels of ubiquitination, offers new insights into the likely in vivo site(s) of βTrCP-dependent ubiquitination for β-catenin. Specifically, although single missense mutations at Lys-19 of β-catenin had...
negligible ability to activate the transforming potential of β-catenin, the 19R mutant protein had clearly detectable increases in its free levels and TCF transcriptional activation potential relative to wild type β-catenin. Additionally, a clear-cut reduction in ubiquitination of the 19R mutant protein was seen. In contrast to the findings for the 19R mutant, no stabilization or enhanced function of the 49R mutant protein was seen and no evidence for reduced ubiquitination was seen for the 49R mutant. These findings suggest the principal site of βTrCP-dependent ubiquitination on wild type β-catenin is likely to be Lys-19 and not Lys-49. However, when the Lys-19 site is mutated, βTrCP-dependent ubiquitination may be directed to secondary sites, perhaps most efficiently at lysine residues within a certain physical distance from the 32–37-amino acid βTrCP binding motif, such as Lys-49. Nevertheless, because the K19R/K49R double mutant β-catenin protein showed residual βTrCP-dependent ubiquitination, whereas the S33Y mutant protein, which fails to bind to βTrCP1/2, shows essentially no ubiquitination by βTrCP, it seems that βTrCP-dependent ubiquitination of other lysines may be somewhat promiscuous following binding of βTrCP1 to the 32–37-amino acid destruction motif, resulting in low levels of ubiquitination at one or more of the 24 lysine residues located outside of the N-terminal 130 amino acids of β-catenin.

Besides clarifying the likely in vivo site(s) of βTrCP-dependent ubiquitination for β-catenin, a few other points of interest have emerged from our studies. Although the Lys-49 residue does not appear to be a principal site for βTrCP-dependent ubiquitination, the fact that the 49R mutant β-catenin protein showed considerably reduced levels in the “free pool” of β-catenin in the nucleus and cytoplasm relative to wild type β-catenin suggests the Lys-49 residue may play some role in regulating the levels, localization, and/or functional activity of β-catenin. Perhaps this reflects the potential for other post-translational modifications of Lys-49, such as acetylation (26) or even conceivably methylation or sumoylation. The possibility that post-translational modifications other than polyubiquitination at Lys-49 might be required for optimal functional activity of β-catenin is not inconsistent with our findings showing that the RR double mutant protein is essentially stabilized to the degree of the S33Y mutant protein but is not as active in RK3E transformation or TCF transcriptional activation. Another issue clarified by our findings showing that both Lys-19 and Lys-49 have to be mutated to demonstrably active transforming activity of β-catenin is why single missense mutations at Lys-19 have not been reported in human cancer. Because an array of single nucleotide substitutions can lead to missense substitutions with dramatic effects on β-catenin phosphorylation by casein kinase 1α or glycogen synthase kinase 3β and/or binding by βTrCP1/2, it is not unexpected that double mutations affecting both Lys-19 and Lys-49 would very rarely, if ever, be seen in human cancer. Further studies of the regulation of β-catenin by post-translational modification at Lys-19 and Lys-49 will undoubtedly shed even greater light on factors and mechanisms regulating β-catenin in normal and cancer cells.

Acknowledgments—We thank Dr. G. Nolan for providing the pBMN-LacZ plasmid and the phoenix retroviral packaging cell line. We thank Dr. Y. Feng for assistance with the studies and the manuscript.

REFERENCES

1. Peifer, M. (1993) Science 262, 1667–1668
2. Cadigan, K. M., and Nusse, R. (1997) Genes Dev. 11, 3286–3305
3. Bienz, M., and Clevers, H. (2000) Cell 103, 311–320
4. Peifer, M., and Polakis, P. (2000) Science 287, 1606–1609
5. Taipale, J., and Beachy, P. A. (2001) Nature 411, 349–354
6. Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M., and Byers, S. W. (1997) J. Biol. Chem. 272, 24735–24738
7. Liu, C., Kato, Y., Zhang, Z., Do, V. M., Yankner, B. A., and He, X. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6273–6278
8. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) Genes Dev. 13, 270–283
9. Sadot, E., Conacci-Sorrell, M., Zhurinsky, J., Shnider, D., Lando, Z., Zharhary, D., Kam, Z., Ben-Ze’ev, A., and Geiger, B. (2002) J. Cell Sci. 115, 2771–2780
10. Staal, F. J., van Noort, M., Strous, G. J., and Clevers, H. C. (2002) EMBO Rep. 3, 63–68
11. Wu, G., Xu, G., Schulman, B. A., Jeffrey, P. D., Harper, J. W., and Pavletich, N. P. (2003) Mol. Cell 11, 1445–1456
12. Li, L., Yuan, H., Weaver, C. D., Mao, J., Farr, G. H., III, Sussman, D. J., Jonkers, J., Kimelman, D., and Wu, D. (1999) EMBO J. 18, 4233–4240
13. Fraser, E., Young, N., Dajani, R., Franca-Koh, J., Ryves, J., Williams, R. S., Yeo, M., Webster, M. T., Richardson, C., Smalley, M. J., Pearl, L. H., Harwood, A., and Dale, T. C. (2002) J. Biol. Chem. 277, 2176–2185
14. Giles, R. H., van Es, J. H., and Clevers, H. (2003) Biochim. Biophys. Acta 1653, 1–24
15. Cliffe, A., Hamada, F., and Bienz, M. (2003) Curr. Biol. 13, 960–966
16. Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z., and He, X. (2004) Mol. Cell 13, 149–156
17. Logan, C. Y., and Nusse, R. (2004) Annu. Rev. Cell Dev. Biol. 20, 781–810
18. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) EMBO J. 16, 3797–3804
19. Kolligs, F. T., Hu, G., Dang, C. V., and Fearon, E. R. (1999) Mol. Cell. Biol. 19, 5696–5706
20. Kolligs, F. T., Kolligs, B., Hajra, K. M., Hu, G., Tani, M., Cho, K. R., and Fearon, E. R. (2000) Genes Dev. 14, 1319–1331
21. Kolligs, F. T., Niemann, M. T., Finner, I., Hu, G., Van Mater, D., Feng, Y., Smith, I. M., Wu, R., Zhai, Y., Cho, K. R., and Fearon, E. R. (2002) Cancer Cell 1, 145–155
22. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. (1997) Science 275, 1784–1787
23. Feng, T., Lee, N., and Fearon, E. R. (2003) Cancer Res. 63, 8726–8734
24. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996) Mol. Cell. Biol. 16, 2128–2134
25. Muller, T., Choidas, A., Reichmann, E., and Ullrich, A. (1999) J. Biol. Chem. 274, 10173–10183
26. Gottardi, C. J., and Gumbiner, B. M. (2004) J. Cell Biol. 167, 339–349
27. Wolf, D., Rodova, M., Miska, E. A., Calvet, J. P., and Kouzarides, T. (2002) J. Biol. Chem. 277, 25562–25567