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Advances in Brief

Phosphorylation of β-Catenin at S33, S37, or T41 Can Occur in the Absence of Phosphorylation at T45 in Colon Cancer Cells[1]

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Abstract

Several previous studies in a variety of systems have suggested that phosphorylation at S45 of β-catenin is essential for subsequent phosphorylation at more NH_2-terminal residues. Using genetic models expressing β-catenin under endogenous regulation, we find that phosphorylation of β-catenin at S45 is not required for phosphorylation at residues S33, S37, or T41 in human colon cancer cells, in contrast to prevailing models. These findings suggest that there are important cell- and organism-specific differences in even the most highly conserved signaling pathways and emphasize the importance of examining these pathways in the cancer cell types in which the pathways are actually deregulated.

Introduction

Regulation of β-catenin plays a critical role in both normal development and neoplasia. This regulation is particularly important in colorectal cancers, wherein its disruption is the rate-limiting step for tumorigenesis (1). Phosphorylation of β-catenin at a cluster of serine/threonine residues near the NH_2-terminus by glycogen synthase kinase 3β is known to play a critical role in this process (2, 3). Several studies have recently suggested that phosphorylation of S45 by casein kinase I plays an essential role in this negative regulation (4–6). In particular, a specific model was proposed wherein a priming phosphorylation event at S45 by casein kinase I was required for subsequent glycogen synthase kinase 3β-mediated phosphorylation of β-catenin at residues 33, 37, and 41 (the S45 primer model). This S45 primer model has now gained widespread acceptance (2, 7, 8). However, the studies described below suggest that this model is not applicable in general, as in particular, it does not apply to colorectal cancer cells, the major cell type in which this pathway leads to neoplasia.

Materials and Methods

Cell Lines. LS 174T cells were obtained from the American Type Culture Collection, and the derivation of β-catenin somatic cell knockouts were described previously (9). Cells were maintained in McCoy 5A media plus 10% fetal bovine serum for all assays described.

Sequencing. Exon 3 of β-catenin, which contains the NH_2-terminal regulatory region, was amplified by PCR using primers 5′-TTTGATGGAGTTGGACATGG-3′ and 5′-CAGGACTTGGGAGGTATCCA-3′ and directly sequenced as described (9).

Immunoprecipitation and Western Blot Analysis. Attached cells from a confluent T75 flask were rinsed with PBS and incubated in 1.5 ml of IP buffer [50 mM HEPES, (pH 7.4), 50 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.3 mM Na Orthovanadate, 50 mM NaF, 80 μM β-glycerophosphate, 20 mM Na PP_I, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor cocktail (Roche catalogue no. 1697498) for 10 min on ice. Cells were scraped and centrifuged at 10,000 × *g* for 30 min at 4°C. Proteins were then incubated with antibody for 1 h at 4°C and precipitated using protein A agarose beads, which had been blocked with 3% powdered milk. Beads were washed four times with 1 ml of IP buffer and then mixed with 2× Laemelli sample buffer. Western blots were performed essentially as described (9). The anti-phospho-S33/S37/T41 β-catenin (catalogue no. 9561) and the anti-phospho-T41/S45 β-catenin antibody (catalogue no. 9565) used for Western blot analyses were obtained from Cell Signaling Technology. The phosphorylation insensitive anti-β-catenin antibody (catalogue no. C19220) used for immunoprecipitation and Western blot analyses were obtain from Transduction Labs.

Results

The most cogent studies supporting the S45 primer model used exogenously overexpressed β-catenin mutants and did not examine cells of intestinal epithelial origin, the major cell type in which disruption of this pathway results in neoplasia. We reasoned that naturally occurring mutations of the β-catenin gene in colorectal cancers could provide a unique opportunity to address this model in cells of colorectal epithelial origin under near physiological conditions (*i.e.,* without overexpression of exogenous proteins). It was reported previously that the colorectal cancer cell line LS 174T harbors a mutation of β-catenin at codon 45 (S45F). We confirmed the presence of the S45F mutation in LS 174T and further determined that these cells lacked a WT[3] allele (Fig. 1*A*). Accordingly, the S45 primer model would suggest that phosphorylation of β-catenin at S33, S37, or T41 should be absent or greatly diminished in these cells. Indeed, previous studies using overexpressed mutant S45F β-catenin in human kidney or *Xenopus* cells demonstrated that this mutant protein was not detectably phosphorylated at S33/S37/T41, unlike overexpressed WT β-catenin. As expected, Western blot analysis of LS 174T cells with an antibody that recognized β-catenin only when phosphorylated at T41/S45 failed to detect any β-catenin, because the S45 epitope was absent in these cells (Fig. 1*B*). Surprisingly, phosphorylation of β-catenin was readily detected by Western blot analysis using an antibody that recognizes β-catenin only when phosphorylated at S33/S37/T41 (Fig. 1*B*). This is the same phospho-specific antibody that was used in previous studies using overexpressed proteins (6). That the detection of β-catenin in LS 174T cells by this antibody was dependent on phosphorylation was confirmed by the complete elimination of signal by treatment with alkaline phosphatase (Fig. 1*B*). In contrast, phosphatase treatment had no effect on the detection of β-catenin with an antibody whose recognition site was not dependent on phosphorylation (Fig. 1*B*).

Although these experiments suggested that phosphorylation of β-catenin at S33/S37/T41 can occur in the absence of phosphorylation at S45, we could not rule out the possibility that the level of this phosphorylation was attenuated in LS 174T or that the phenylalanine

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[3] The abbreviation used is: WT, wild type.

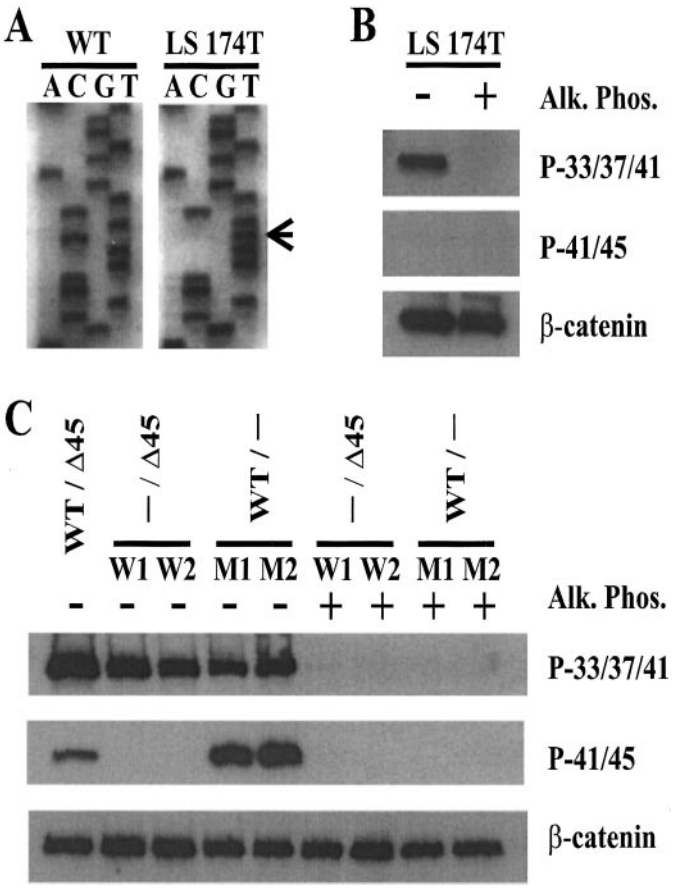

Fig. 1. Phosphorylation of β-catenin in human colorectal cancer cells. *A*, status of β-catenin in LS 174T cells. Exon 3 of β-catenin, which contains the NH_2-terminal regulatory region, was amplified by PCR and directly sequenced. LS 174T displayed a C to T transition (indicated by *arrow*) resulting in an S45F coding change and a complete lack of WT sequence. *B*, phosphorylation of S45F β-catenin in LS 174T cells. β-catenin was immunoprecipitated from LS 174T cells using a monoclonal anti-β-catenin antibody and then detected by Western blot analysis using the anti-phospho-S33/S37/T41 antibody to β-catenin, the anti-phospho-T41/S45 β-catenin antibody, or a phosphorylation insensitive anti-β-catenin antibody (β-catenin) antibody. Samples were treated with alkaline phosphatase as indicated. *C*, phosphorylation of Δ45 β-catenin in HCT116 cells. Phosphorylation was assessed as described in *B* except that extracts from either parental HCT116 cells (WT/Δ45) or derivatives expressing only WT (WT/−, clones M1, M2) or mutant (−/Δ45, Clones W1, W2) β-catenin were used.

at codon 45 in LS 174T cells could somehow substitute for phospho-S45. To rigorously address this issue, we used HCT116 cells, a human colorectal cancer cell line that contains one β-catenin allele with a deletion of codon 45 (Δ45) and one WT allele. As described previously, clones of this cell line that lacked either the WT allele (−/Δ45, clones WT1 and WT2) or the mutant allele (WT/−, clones M1 and M2) were produced by targeted homologous integration (9). Both the −/Δ45 and WT/− clones expressed robust and similar amounts of β-catenin (Fig. 1*C*) despite a dramatic reduction in catenin-regulated transcription in the WT/− clones (9–11). As in LS 174T, Western blot analysis with an anti-phospho-T41/S45 antibody failed to detect any β-catenin in cells with only the mutant β-catenin allele (Fig. 1*C*). However, phosphorylation of β-catenin at residues S33/S37/T41 was readily detected using the anti-phospho-S33/S37/T41 antibody (Fig. 1*C*). Both −/Δ45 and WT/− cells expressed indistinguishable amounts of phosphorylated β-catenin, which was completely eliminated by treatment with alkaline phosphatase (Fig. 1*C*).

Discussion

These results unambiguously demonstrate that phosphorylation of β-catenin at residues NH_2 terminal to S45 can occur regardless of the status of S45 residue in human colorectal cancer cells. The discrepancy between our findings and those published previously may relate to differences in regulation of β-catenin in different species or tissues or different experimental approaches (*i.e.*, examination of exogenously introduced *versus* endogenous β-catenin). In any event, these experiments support the important concept that even the most fundamental features of highly conserved signaling pathways cannot be assumed to function identically in all cell types. Such differences will markedly complicate the large scale efforts currently underway in systems biology and reveal the power of genetic technologies, particularly those using site-directed changes in endogenous proteins, to illuminate these issues.

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