β- and γ-Catenin Mutations, but not E-Cadherin Inactivation, Underlie T-Cell Factor/Lymphoid Enhancer Factor Transcriptional Deregulation in Gastric and Pancreatic Cancer

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Abstract

Adenomatous polyposis coli (APC) mutations are present in >70% of colon cancers. The APC protein binds to β-catenin (β-cat), a protein first identified because of its role in E-cadherin (E-cad) cell adhesion. In some colon cancers lacking APC defects, mutations in presumptive glycogen synthase kinase 3β phosphorylation sites near the β-cat NH₂ terminus appear to render β-cat resistant to regulation by APC and glycogen synthase kinase 3β. In cells with APC or β-cat defects, β-cat is stabilized and, in turn, binds to and activates T-cell factor (Tcf)/lymphoid enhancer factor (Lef) transcription factors. To further explore the role of APC, β-cat, Tcf, and E-cad defects in gastrointestinal cancers, we assessed gastric and pancreatic cancers for constitutive Tcf transcriptional activity (CTTA). Two of four gastric and two of eight pancreatic cancer lines showed CTTA. One gastric and one pancreatic cancer had mutations in the NH₂-terminal phosphorylation sites of β-cat. The other gastric cancer with CTTA had a missense mutation at serine 28 of γ-cat, a potential phosphorylation site in this β-cat-related protein. Although E-cad is an important binding partner for β-cat and γ-cat, E-cad inactivation did not result in CTTA. The β-cat and γ-cat mutant proteins identified in our studies strongly activated Tcf transcription in vitro, whereas β-cat mutant proteins with large NH₂-terminal deletions had only modest effects on Tcf. Our results suggest a role for Tcf deregulation in gastric and pancreatic cancer, resulting from β-cat and γ-cat mutations in some cases and, in others, from yet to be defined defects. Furthermore, these data imply that the consequences of APC and β-cat mutations are distinct from the effects of E-cad inactivation.

Introduction

Germ-line inactivating mutations in the APC6 tumor suppressor gene are responsible for familial polyposis. Somatic mutations in APC are present in ~70% of colorectal adenomas and carcinomas but are rare in other cancers (reviewed in Ref. 1). The APC protein has been found to bind to a number of proteins, including β-cat (2, 3), γ-cat (also known as plakoglobin; Refs. 4 and 5), GSK3β (6), EB1 (7), hDLG (8), microtubules (9, 10), and the related proteins axin and conductin (11–14). With the exception of β-cat, GSK3β, and the conductin and axin proteins, the significance and role of APC’s interactions with its various binding partners is not well understood.

β-cat, initially identified as a coprecipitating protein with E-cad, was subsequently shown to link E-cad to α-cat, a vinculin-like protein, which, in turn, links the E-cad/cat cell-cell adhesion complex to the cortical cytoskeleton (15–19). Molecular cloning revealed that β-cat is a member of the armadillo (arm) family. The prototype molecule Arm functions in the wingless signaling pathway in Drosophila, with β-cat functioning in the conserved wnt pathway in vertebrates (12, 15). The γ-cat protein is closely related to β-cat and appears to have similar functions in cell-cell adhesion and the wnt pathway (16–19).

The identification of Tcf/Lef transcriptional factor proteins as downstream targets of β-cat (20–22), together with the observation of CTTA in colorectal cancer cell lines with APC defects (23), supported the hypothesis that APC had a critical role in regulating β-cat (24). Further support was provided by data, indicating that a subset of the colorectal

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6 The abbreviations used are: APC, adenomatous polyposis coli; β-cat, β-catenin; γ-cat, γ-catenin; GSK3β, glycogen synthase kinase 3β; E-cad, E-cadherin; Tcf, T-cell factor; Lef, lymphoid enhancer factor; CTTA, constitutive Tcf transcriptional activity; GI, gastrointestinal; RT-PCR, reverse transcriptase-PCR ECL, enhanced chemiluminescence.
cancers lacking APC mutations had CTTA as a result of mutations altering potential GSK3β phosphorylation sites in the β-catenin NH2-terminus (25). Other studies have reported mutations of the NH2-terminal phosphorylation sites of β-catenin in melanoma cell lines (26) and medulloblastoma (27) as well as carcinomas of the ovary (28), endometrium (29), liver (30), and prostate (31). The mutant β-catenin proteins that are expressed are presumed to be oncogenic because they cannot be appropriately regulated by the protein complex containing APC, GSK3β, and axin or conductin (11–15). Mutant β-catenin accumulates in the cell and deregulates expression of downstream Tcf-/Lef-regulated target genes, including perhaps c-myc (32), via β-catenin’s role as a coactivator of Tcf/Lef transcription.

As noted above, in addition to their role in Wnt signaling, β-catenin and γ-catenin are key components in E-cad cell-cell adhesion, a function that is critical in development, cell differentiation, and maintenance of tissue architecture (15–19). In many epithelial cancer types, loss of E-cad expression has been frequently observed (18). The mechanisms underlying loss of E-cad are rather poorly understood in the majority of cases. However, in some cancers, including diffuse-type gastric carcinoma, somatic mutations in the E-cad gene (CDH1) underlie loss of its expression (18, 33, 34). CDH1 germ-line mutations have recently been found in several families with inherited predisposition to gastric cancer (35, 36), further highlighting the significance of E-cad alterations in GI cancer. In contrast to the extensive body of data in colorectal cancer, there have been only limited reports of APC mutations and no reports of CTNNB1 or CTNNG1 mutations in tumors of the upper GI tract or pancreas (37–41). Decreased expression of E-cad and/or β-catenin, however, has been seen in a significant fraction of esophageal, gastric, and pancreatic cancers, and altered expression is correlated with high grade or advanced stage (42–45).

To explore defects in the APC/β-catenin/Tcf and E-cad/catenin pathways in cancers of the GI tract further, we examined gastric and pancreatic cancer cell lines for CTTA, using Tcf reporter gene constructs. Our studies indicate the presence of Tcf deregulation in gastric and pancreatic cancer, resulting from CTNNB1 or CTNNG1 mutations in some cases and from yet to be defined defects in other cases. Our findings also imply that the consequences of APC or catenin mutations in cancer development are distinct from those that are attributable to E-cad inactivation.

Results

CTTA in Gastric and Pancreatic Cancer. As reviewed above, previous studies revealed CTTA in colorectal cancer lines with APC inactivation, such as SW480 cells, and in lines with missense mutations or deletions of single amino acids in the potential GSK3β phosphorylation sites at the NH2-terminus of β-catenin, such as HCT-116 cells (23, 25). Hence, we assayed Tcf transcriptional activity as an initial strategy for detecting alterations in the regulation or function of APC, β-catenin, or Tcf/Lef family members. Two reporter gene constructs, pTopflash and pFopflash, used in prior studies (23, 25), were also used for our studies. The pTopflash construct contained consensus Tcf binding sites cloned upstream of a minimal promoter element and luciferase reporter gene, and pFopflash contained mutated Tcf binding sites cloned upstream of luciferase. In any given cell line, comparison of the luciferase activity of the pTopflash construct versus that of the control pFopflash construct proves a measure of the relative Tcf activity. As expected, the SW480 and HCT-116 colorectal cancer lines showed clear evidence of CTTA (Fig. 1). We then assessed Tcf transcriptional activity in four gastric and eight pancreatic cancer lines. The majority of the lines displayed no evidence of CTTA, with mean relative Tcf activities between 0.25 and 2.0. However, CTTA was found in two gastric (NCI-N87 and AGS) and two pancreatic (ASPC and HS766T) lines, with mean relative Tcf activities ranging from ~5 in the NCI-N87 line to 50 in the ASPC line (Fig. 1). The specific Tcf/Lef factor(s) responsible for the observed Tcf reporter gene activity are not known, although RT-PCR studies indicated that Tcf-4 was expressed in all cell lines studied (data not shown).

β- and γ-catenin Mutations in Gastric and Pancreatic Cancers. We sought to determine the basis for CTTA in the gastric and pancreatic cancers. Prior studies indicate that APC mutations are rare or absent in gastric and pancreatic cancer (1, 39, 41), and we failed to find evidence of APC inactivation in the cell lines through Western blot studies of APC (data not shown). Because of the contribution of CTNNB1 mutations to CTTA in a subset of colorectal cancers, we carried out RT-PCR studies to determine whether CTNNB1 mutations were present in any of the 12 gastric and pancreatic cancer lines. The entire open reading frame of β-catenin was amplified, and no evidence of large deletions or insertions was found. Analysis of the sequences encoding the NH2-terminal region of β-catenin (corresponding to codons 1–86) revealed that the AGS gastric cancer cell line had a missense mutation of glycine to glutamic acid at codon 34, and the HS766T pancreatic cancer cell line had an in-frame deletion of serine codon 45 (Fig. 2). Although CTNNG1 mutations have not been previously reported in cancer, given the very high sequence conservation between the γ-catenin and β-catenin proteins, we analyzed the sequences encoding the NH2-terminal region of γ-catenin in all 12 gastric and pancreatic cancer cell lines. In NCI-N87, we found a missense mutation at CTNNG1 codon 28, resulting in a serine-to-leucine substitution and alteration of a potential phosphorylation site (Fig. 2). No CTNNB1 or CTNNG1 mutations were found in ASPC or the eight lines that failed to manifest CTTA. Sequencing studies of exons 2–4 of CTNNB1 and the corresponding region of CTNNG1 in 36 primary gastric cancers, using genomic DNA microdissected from regions enriched in neoplastic cells, failed to identify any CTNNB1 or CTNNG1 mutations, suggesting that mutations in β-catenin and γ-catenin are likely to be present in only a minority of gastric cancers.

Tcf Transcriptional Activity in the ASPC Line Is Not Responsive to APC. Our sequencing-based studies did not identify CTNNB1 or CTNNG1 mutations in the ASPC pancreatic cancer line, and our Western blot studies failed to provide evidence of APC inactivation. To address the possibility that the ASPC line might have a subtle mutation in APC, we sought to determine whether Tcf transcriptional activity in ASPC was inhibited following overexpression of
SW480 (APC mutation) and HCT116 (SD. CTTA is seen in the colorectal cancer lines three or more transfection experiments; lactosidase activities, determined following activated activities are not shown to scale. Tcf activities for the four lines with very elevated activities are not shown to scale.}

Intriguingly, although Tcf activity was not affected by APC overexpression in cancer cell lines with mutant β-catenin (e.g., HCT-116; Fig. 3), we found that overexpression of APC decreased Tcf activity in a cell line with mutant γ-catenin (i.e., NCI-N87; Fig. 3). The ability of APC to suppress Tcf activity in a cell line expressing a mutant γ-catenin protein is consistent with the proposal that mutant γ-catenin may affect Tcf activity indirectly. Perhaps mutant γ-catenin protein interferes with the degradation machinery regulating β-catenin protein levels, causing β-catenin protein levels to rise, with resultant Tcf activation. Support for this potential mechanism of Tcf activation by mutant γ-catenin is provided by the studies of Miller and Moon (46), who demonstrated that expression of ectopic (aberrantly localized) β-catenin and γ-catenin proteins mimicked Wnt pathway activation in *Xenopus*.

**Mutant β-catenin and γ-catenin Proteins Activate Tcf Transcriptional Activity.** Mutant β-catenin proteins with missense mutations or in-frame deletions of single amino acids in the NH₂-terminal phosphorylation sites have been shown previously to strongly activate Tcf transcriptional activity when expressed in heterologous cell types, such as the 293 kidney cell line (21). We sought to confirm that the mutant CTNNB1 and CTNN1 alleles that we had identified did, in fact, encode proteins that activated Tcf transcription. Consistent with prior results, a mutant CTNNB1 allele encoding a β-catenin protein with the codon 33 serine-to-tyrosine substitution had only a modest ability to activate Tcf transcription, although wild-type β-catenin had readily detectable but more modest effects (Fig. 4A). We found an in-frame deletion of serine codon 45 (ΔS45) in the HS766T pancreatic line; this mutation had previously been found in the HCT-116 colorectal line. The ΔS45 mutant CTNNB1 allele strongly activated Tcf transcription, as did the G34E mutant allele from the AGS gastric cancer line (Fig. 4B). In contrast to the strong activity of these selected mutant CTNNB1 alleles, the mutant CTNN1 allele in the NCI-N87 gastric line (serine-to-leucine mutation at codon 28: S28L) had only a modest ability to activate Tcf transcription, although the mutant γ-catenin protein was 2-fold more potent than wild-type γ-catenin (Fig. 4C). The differing strength of the mutant β-catenin and γ-catenin proteins in our assay may reflect true
differences in their in vivo function, or it may be attributable, at least in part, to the heterologous 293 cell line used for the assay. Nevertheless, the data demonstrating the S28L mutant CTNNNG1 allele had less activity than the CTNNNB1 alleles with point mutations and small deletions (i.e., S33Y, ΔS45, and G34E) are consistent with the observation that the NCI-N87 line (i.e., the line from which the S28L CTNNNG1 allele was derived) had less CTTA relative to lines with mutant β-catenin (e.g., AGS and HS766T; see Fig. 1).

In addition to missense mutations and single amino acid deletions in the NH2-terminal phosphorylation sites of β-catenin, larger in-frame deletions of β-catenin NH2-terminal sequences have been reported in a few cancer cell lines and primary tumors (22, 26, 47). The deletions are presumed to promote cancer growth through the same mechanisms as do mutant β-catenin proteins with localized mutations. We found, however, that mutant β-catenin proteins with substantial NH2-terminal deletions, such as deletion of β-catenin amino acids 1–90 (ΔN90) or deletion of amino acids 5–80 (Δex3), had activities in the Tcf assay that were indistinguishable from those of wild-type β-catenin (Fig. 4A). We confirmed expression of Flag epitope-tagged wild-type β-catenin and selected mutant β-catenin proteins in 293 cells. Like the S33Y β-catenin protein, the ΔN90 β-catenin protein accumulated to higher levels than did wild type β-catenin (Fig. 4B). Despite being expressed from the same vector as the β-catenin proteins, wild-type and mutant γ-catenin proteins were rather poorly expressed in 293 cells (Fig. 4B).

On the basis of our data, at least two explanations for the reduced transcriporal activation potential of NH2-terminal truncated forms of β-catenin can be considered. (a) It is entirely possible that the in vitro assay of Tcf transcriptional activity in 293 cells does not accurately reflect the in vivo activity of all mutant β-catenin proteins. (b) A second, albeit less likely, explanation is that the assay does, in fact, reflect the ability of mutant β-catenin proteins to activate Tcf transcription, but β-catenin proteins with larger NH2-terminal deletions contribute to cancer through mechanisms that are independent of Tcf activation. Our rationale for favoring the former explanation is that mutant β-catenin proteins with substantial deletions of the NH2 terminus were previously shown to activate Tcf transcription in certain cell types (48).

Loss of Endogenous E-cad Expression Does Not Lead to CTTA. In normal cells, β-catenin is a highly abundant protein, complexed primarily with E-cad at the plasma membrane. As reviewed above, E-cad expression is often very reduced or absent in many epithelial cancers, including gastric cancer. To examine the possibility that E-cad inactivation might lead to increased levels of β-catenin and CTTA, we first examined the expression of E-cad and then that of β-catenin, γ-catenin, and GSK3β in the four gastric and eight pancreatic cancer cell lines. Three of the four gastric cancer lines and one pancreatic cancer line had very reduced or absent E-cad expression (Fig. 5). Comparison of the pattern of E-cad expression in the lines (Fig. 5) to their Tcf transcriptional activity (Fig. 1) revealed no clear association. Specifically, of the three gastric cancer lines that had loss endogenous E-cad expression, CTTA was found only in the AGS line, in which a mutant β-catenin protein was also present. No evidence of CTTA was found in the SNU-5 or RF-1 lines. Similarly, despite the fact that the MIA PaCa-2 pancreatic line lacks endogenous E-cad expression, CTTA was not seen. Comparison of the levels of β-catenin and γ-catenin in the four gastric cancer lines revealed modest elevations of β-catenin and γ-catenin in NCI-N87 (AGS has a β-catenin mutation and NCI-N87 has a γ-catenin mutation; Fig. 5). However, no clear change in β-catenin levels was seen in the pancreatic line with a β-catenin mutation (HS766T; Fig. 5). Our failure to find a strong correlation between CTNNB1 and CTNNNG1 mutations and the relative abundance of the respective protein products may be largely attributable to the fact that we assessed total levels of β-catenin and γ-catenin in the cells, rather than “free” cytosolic pools. The cell lines expressed roughly equivalent levels of GSK3β, with only modest changes in abundance observed (Fig. 5).

To demonstrate that the lack of a relationship between loss of endogenous E-cad expression and Tcf transcriptional activity was a general feature in cancer cells, we assessed Tcf transcriptional activity in eight breast cancer lines, including five lines lacking E-cad expression. No evidence for CTTA was found in any of the lines (data not shown). To determine whether the signaling pathways for responding to
deregulated β-catenin or γ-catenin proteins were intact in breast cancer, we assessed the ability of wild-type and mutated forms of β-catenin and γ-catenin to activate Tcf transcription in BT549 breast cancer cells, a line lacking E-cad expression. As shown in Fig. 6, mutated β-catenin and γ-catenin proteins were able to stimulate Tcf transcriptional activity more potently than their wild-type counterparts. The stronger activation of Tcf by the S28L mutant γ-catenin in BT549 cells (Fig. 6) versus 293 cells (Fig. 4A) may reflect increased stability of γ-catenin and selected mutant forms of the proteins. Western blot analysis with an anti-Flag antibody was carried out on whole cell lysates prepared 48 h after transfection of 293 cells with pcDNA3 constructs encoding the indicated β-catenin and γ-catenin proteins. To control for loading, we stripped the blot and performed ECL-Western with an anti-actin antibody.

Discussion

Recent observations have greatly advanced our understanding of the significance of the interaction between the APC tumor suppressor protein and β-catenin. Together with other proteins, such as the GSK3β kinase and axin, APC functions to regulate the stability and abundance of β-catenin in the cytosol, presumably in response to signals from upstream pathways, such as those from the Wnt pathway. If β-catenin accumulates in the cytosol, it can bind to Tcf/Lef transcription factors, and following its translocation to the nucleus, the Tcf/β-catenin complex activates expression of cellular genes, perhaps including c-myc (32). In colorectal cancers, APC inactivation has been seen in nearly 70–75% of cases, and a subset of the cancers that lack APC mutations harbors activating mutations in NH2-terminal phosphorylation sites of
β-cat (25, 47, 49, 50). Several other cancer types have also been reported to have β-cat mutations (26–31).

The studies presented here were undertaken with the intent of further defining the role of defects in the APC/β-cat/Tcf and E-cad cat pathways in cancer. Our findings indicate a role for Tcf deregulation in gastric and pancreatic cancers, resulting from mutations in β-cat and γ-cat in some cases and from yet to be defined defects in others. Mutations in β-cat were found in one gastric and one pancreatic cancer cell line, and we confirmed that the mutant β-cat proteins activate Tcf transcription in vitro. Like most other previously described CTNNB1 mutations in colorectal and other cancers, the mutations affect presumptive GSK3β phosphorylation sites of β-cat. Our studies also identified a missense mutation of a potential GSK3β phosphorylation site in the NH₂-terminal region of γ-cat, and we found that this mutant protein had clearly detectable but more modest effects on Tcf transcription than comparable mutant β-cat proteins. To the best of our knowledge, our studies are the first to identify activating mutations in the CTNNB1 gene in cancer, and the findings imply that missense mutations and small in-frame deletions of the NH₂-terminal phosphorylation sites of γ-cat are likely to have effects in cancer cells that are analogous to those of β-cat mutations. We failed to find CTNNB1 or CTNN1 mutations in studies of a panel of 36 primary gastric cancers. Hence, similar to colorectal cancer, in which β-cat mutations are only found in ~2–5% of primary tumors (47, 49, 50), mutations in β-cat and γ-cat appear to be rare in primary gastric cancers. Nevertheless, our data imply that Tcf transcriptional deregulation may be important in the pathogenesis of a subset of pancreatic and gastric cancers. In some cases, such as the ASPC pancreatic cancer line, it may result from defects other than those in the APC, CTNNB1, or CTNN1 genes.

We had hoped to reconcile the large body of data demonstrating frequent inactivation of E-cad by mutational or epigenetic mechanisms in various cancers with the recent findings implicating β-cat deregulation and Tcf transcriptional activation in colon and other cancers. E-cad is a major binding protein for β-cat in cells, and β-cat has an important role in E-cad’s cell adhesion function, via its ability to link E-cad to α-cat and the cytoskeleton. We initially predicted that loss of endogenous E-cad might lead to a significant increase in free cytosolic β-cat and Tcf deregulation. However, this prediction was not borne out by our studies. Loss of endogenous E-cad expression in gastric, pancreatic, and breast cancers was not associated with obvious increases in β-cat levels, and no evidence of CTTA was found in any of the cancer lines lacking endogenous E-cad expression. A likely explanation for the observations is that the proteins and pathways responsible for regulating β-cat cytosolic levels, such as GSK3β, APC, and axin, remain fully functional in cancer cells with E-cad defects.

Sadot et al. (51) have recently reported that overexpression of full-length N-cadherin or the cytoplasmic (β-cat binding) region of N-cadherin effectively inhibits the ability of β-cat to activate Tcf transcription in Chinese hamster ovary cells and SW480 colorectal cancer cells. Although the findings stand in apparent contradiction to our results, it is difficult to make direct comparisons between studies of the effects on Tcf activation when various forms of N-cadherin are overexpressed and studies of Tcf activation in cancer cell lines lacking endogenous E-cad expression. We believe that our studies better reflect the situation found in primary cancers in which endogenous E-cad expression is lost. As such, our data imply that there is no direct connection between the E-cad cat and APC/cat/Tcf pathways in cancer cells. Moreover, our findings indicate that the functional consequences of inactivating mutations in APC or activating mutations in β-cat or γ-cat are distinct from those attributable to E-cad inactivation. Future studies will provide novel and definitive insights into the means by which β-cat functions in both E-cad cell-cell adhesion and Tcf transcriptional activation as well as the specific means by which E-cad inactivation contributes to altered cell growth control and cancer development and progression. Perhaps then, the apparently differing consequences of catenin and E-cad defects on the cancer cell phenotype can be reconciled.

### Materials and Methods

**Cell Lines.** All cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in the recommended growth conditions. Genomic DNA was isolated by incubating cell pellets for 48 h at 48°C with 0.5 mg/ml proteinase K in 1.0% SDS, followed by phenol-chloroform extractions and ethanol precipitation. Total RNA was isolated from cells using the RNAlater RNA isolation system (Promega, Madison, WI), and protein lysates were prepared in radioimmunoprecipitation buffer with protease inhibitors.

<table>
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<th>Table 1</th>
<th>Oligonucleotide primers for cloning and mutational analysis of β-cat and γ-cat</th>
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<tr>
<td><strong>Oligonucleotide</strong></td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>βCATFL-S</td>
<td>5′-GGACGAGGGAGCGAGGACG-3′</td>
</tr>
<tr>
<td>βCATFL-A</td>
<td>5′-GCCATACCAGAGTGTTCAAGAC-3′</td>
</tr>
<tr>
<td>PLAKFL-S</td>
<td>5′-TCCTCAATCCCTCCAAGGACAC-3′</td>
</tr>
<tr>
<td>PLAKFL-A</td>
<td>5′-AGCTGACCAAGGCGTGCTCAAGACGAGG-3′</td>
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<tr>
<td>βCATDN90-S</td>
<td>5′-AGCGGGAGGTCACATGTGGATAAGTTGAG-3′</td>
</tr>
<tr>
<td>βCATD54-S</td>
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<td>βCATD25-S</td>
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<td>βCATG-S</td>
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<td>5′-CTCTTACCAGCTACTNYCTCTTGTA-3′</td>
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<td>PLAKG-S</td>
<td>5′-GGACGAGGGAGCGAGGACG-3′</td>
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<tr>
<td>PLAKG-A</td>
<td>5′-GCCATACCAGAGTGTTCAAGAC-3′</td>
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DNA Transfections and Reporter Assays. Transfections to assess Tcf transcriptional activation were performed on cells growing at ~70% confluence in six-well plates, using 1 ml of Opti-MEM reduced serum medium (Life Technologies, Inc., Grand Island, NY), 6 µl of Lipofectin reagent (Life Technologies, Inc.), 0.75 µg of pcH110, and 0.75 µg of pTopflash (optimal motif) or pFopflash (mutant motif) in each well (21). To assess the effect of wild-type and mutated forms of β-catenin and γ-catenin on Tcf transcriptional activity, we undertook cotransfections of 293 cells as described above, except that 0.5 µg of pcH110, 0.5 µg of pTopflash or pFopflash, and 0.5 µg of the β-catenin or γ-catenin expression vector were used. To assess the effect of APC on Tcf activity, we cotransfected cells with 0.5 µg of pcH110, 0.5 µg of pTopflash or pFopflash, and 0.25 or 0.75 µg of the wild-type β-catenin expression construct. Varying amounts of an empty control vector (pcDNA3) were used to normalize the DNA mass to 2 µg in each transfection. Cell extracts were prepared 24–30 h posttransfection using reporter lysis buffer (Promega). Luciferase and β-galactosidase assays were carried out as recommended by the manufacturer (Promega). Luciferase activities were measured in a luminometer (model TD-20E; Turner Corp., Mountain View, CA). All transfections were repeated three or more times. For each cell line, luciferase activities in a given series of transfection experiments were first normalized for β-galactosidase activity. Then, the corrected mean luciferase activity of the pFopflash reporter construct was assigned a value of 1, and the luciferase activity with pTopflash was reported relative to the pFopflash activity.

β-catenin and γ-catenin Mutation Analyses. For the four gastric and eight pancreatic cancer cell lines, first strand cDNA was prepared from total RNA using avian myeloblastosis virus reverse transcriptase (Promega) and random hexamers. The entire open reading frames of CTNNB1 and CTNNG1 were then amplified from cDNA by PCR with Pfu (Stratagene, La Jolla, CA) and primers jCATFL-A and jCATHL-A and PLAKFL-S and PLAKFL-A, respectively (see Table 1 for primer sequences). In 36 primary gastric cancer specimens, DNA was isolated by microdissection of regions of carcinoma from thick, unstained sections of formalin-fixed and paraffin-embedded tumors. To amplify a CTNNB1 genomic DNA fragment containing exons 2–4 (codons 1–86), we carried out PCR with Pfu, using oligonucleotide primers jCATEX2-A and jCATEX4-A. Similarly, PCR with Pfu was carried out to amplify a genomic DNA fragment containing codons 7–70 of CTNNG1, using primers PLAKG-S and PLAKG-A. PCR products were purified following agarose gel electrophoresis and sequenced directly with the following primers: jCATEX2-S and jCATEX4-A for β-catenin RT-PCR products; jCATGS-S and jCATGS-A for CTNNB1 genomic DNA products; and PLAKG-S and PLAKG-A for CTNNG1 RT-PCR and genomic DNA products. Thermo-Sequenase and 33P-labeled ddNTPs were used according to the manufacturer’s instructions (Amer sham, Arlington Heights, IL). Mutations were verified in independent PCR amplification and sequencing studies.

Plasmid Constructs. The vector pcDNA3 (Invitrogen, San Diego, CA) was used to generate β-catenin and γ-catenin expression constructs. Full-length wild-type CTNNB1 and CTNNG1 cDNAs were amplified by PCR from a normal colon cDNA library (Clontech, Palo Alto, CA), using primers jCATHL-S and jCATHL-A for CTNNB1 and PLAKFL-S and PLAKFL-A for CTNNG1 (Table 1). The oligonucleotides were modified to yield BamHI (5′ end) and XbaI (3′ end) recognition sites at the ends of the CTNNB1 cDNA and KpnI (5′ end) and EcoRI (3′ end) sites for the CTNNG1 cDNA. Mutant CTNNB1 and CTNNG1 cDNAs were amplified by PCR from cDNA of selected gastric and pancreatic cancer cell lines with the respective primer pairs. To generate the β-catenin and γ-catenin expression constructs, the forward primers jCATEXH0-S and jCATEX3S-S were used together with the jCATHL-A reverse primer, PCR amplification of CTNNB1 and CTNNG1 cDNAs was carried out with Pfu polymerase, and the sequences of all PCR products was verified by manual or automated DNA sequencing. The pCH110 eukaryotic expression vector (Pharmacia, Piscataway, NJ), containing a functional LacZ gene cloned downstream of a cytomeg alovirus early region promoter/enhancer element was used to control for transfection efficiency in the luciferase assays.

Western Blot Analysis. Approximately 40 µg of each protein lysate in radiomunoprecipitation lysis buffer (Tris-buffered saline, 0.5% deoxycholate, 0.1% SDS, and 1% NP40) buffer was separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Bedford, MA) by semidry electrobottling (Transblot, Bio-Rad, Hercules, CA). ECL Western blot analysis was carried out with the mouse monoclonal antibodies against α-catenin, β-catenin, γ-catenin, and GSK-3β (all from Transduction Laboratories, Lexington, KY), and E-cad (Zymed Laboratories, Inc., San Francisco, CA). A polyclonal antibody against Na+/K+-ATPase (Research Diagnostics, Inc, Flanders, NJ) was used to verify equal loading of Western blots. For studies of the expression of Flag epitope-tagged wild-type and mutated forms of β-catenin and γ-catenin, antibody was used at 1:1000. The secondary antibodies used for ECL-Western studies were horseradish peroxidase-conjugated goat-antimouse IgG or goat-antirabbit immunoglobulin antibodies (Pierce Biochemicals, Rockford, IL). Antibody complexes were detected with the ECL-Western blot kit (Amersham) and exposure to X-Omat film (Kodak, Rochester, NY).

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References


