Down-Regulation of β-Catenin TCF Signaling Is Linked to Colonic Epithelial Cell Differentiation[1]

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ABSTRACT

The β-catenin TCF pathway is implicated in the regulation of colonic epithelial cell proliferation, but its role in the regulation of cell differentiation is unknown. The colon carcinoma cell line, Caco-2, spontaneously undergoes G_0/G_1 cell cycle arrest and differentiates along the absorptive cell lineage over 21 days in culture. In parallel, we show that β-catenin-TCF activity and complex formation are significantly down-regulated. The down-regulation of β-catenin-TCF signaling was independent of APC, which we characterized as having a nonsense mutation in codon 1367 in Caco-2 cells, but was associated with a decrease in TCF-4 protein levels. Total β-catenin levels increased during Caco-2 cell differentiation, although this was attributable to an increase in the membrane, E-cadherin-associated, fraction of β-catenin. Importantly, down-regulation of β-catenin-TCF signaling in undifferentiated Caco-2 cells by three different mechanisms, ectopic expression of E-cadherin, wild-type *APC*, or dominant negative TCF-4, resulted in an increase in the promoter activities of two genes that are well-established markers of cell differentiation, alkaline phosphatase and intestinal fatty acid binding protein. These studies demonstrate, therefore, that in addition to its established role in the regulation of cell proliferation, down-regulation of the β-catenin-TCF pathway is associated with the promotion of a more-differentiated phenotype in colonic epithelial cells.

INTRODUCTION

Mutations in the *APC*[3] gene are the initiating event in the onset of the majority of colorectal tumors. The evidence for this includes the observation that *APC* is mutated in 80–90% of sporadic colorectal tumors (1) and in all cases of the inherited form of colon cancer, familial adenomotous polyposis (FAP) (2–5). Furthermore, mice that inherit a targeted mutation of an *APC* allele develop multiple intestinal adenomas within a few months of birth (6, 7).

In normal colonic epithelial cells, APC in combination with glycogen synthase kinase 3β and axin regulates free cytoplasmic β-catenin levels by binding to and targeting β-catenin for degradation by ubiquitination-dependent proteolysis (8–12). This regulates the availability of free β-catenin for binding with the TCF-LEF family of transcription factors (13–15). Mutations in *APC* or β-catenin can result in the failure of β-catenin to be degraded, and subsequently, in an increase in β-catenin-TCF complex formation. This, in turn, results in alterations in gene transcription (16–18).

Because APC is important in homeostasis, a strong hypothesis is

that in causing colon tumor formation, the loss of wild-type APC, and hence altered β-catenin-TCF signaling, affects at least one of three pathways of colonic cell maturation: cell cycle arrest, lineage-specific cell differentiation, and apoptosis, all of which take place as cells migrate from the base of the colonic crypt toward the lumen (19, 20).

A role for β-catenin-TCF in the regulation of apoptosis is not clear, with both pro- and antiapoptotic effects reported. A proapoptotic role is suggested by the induction of apoptosis subsequent to down-regulation of this pathway by the reintroduction of wild-type *APC* into *APC* mutant colon cancer cell lines (21, 22). In contrast, however, overexpression of APC in the intestinal epithelium has no effect on apoptosis (23). Furthermore, the induction of apoptosis in certain instances is associated with the cleavage of APC (24, 25), suggesting that APC may, in fact, be a survival factor for colonic epithelial cells.

A role for β-catenin-TCF signaling in the regulation of colonic cell proliferation is more clear. For example, overexpression of wild-type APC results in the induction of G_0/G_1 cell cycle arrest (26), and the presence of functional β-catenin-TCF binding sites have been identified in the promoters of the key cell cycle regulatory genes, *cyclin D1* (27) and *c-myc* (28). Furthermore, mice with a targeted inactivation of the *TCF-4* gene show the loss of a functional stem cell compartment in the small intestine, and the animals die within 2 weeks of birth (29).

It is important, however, that the loss of the stem cell compartment in the *TCF-4*-null mice was coincident with differentiation of cells in the midvillus compartment, suggesting that a primary affect of a loss of β-catenin-TCF signaling may include premature cell differentiation. The present study, therefore, examines the role of the APC-β-catenin-TCF pathway in the regulation of colonic epithelial cell differentiation along the absorptive cell lineage. We have used the Caco-2 colon cancer cell line, which undergoes cell cycle arrest and differentiation along the absorptive cell lineage with time in culture, modeling the phenotypic changes that absorptive cells undergo as they migrate along the crypt axis toward the lumenal surface (30–32). In the present study, we demonstrate that these changes in cell maturation are linked to down-regulation of β-catenin-TCF complex formation and signaling. This down-regulation was most likely attributable to the decrease in TCF-4 expression. Importantly, the premature down-regulation of β-catenin-TCF signaling in undifferentiated Caco-2 cells by ectopic expression of wild-type APC, E-cadherin, or a dominant negative mutant form of *TCF-4*, results in concomitant premature activation of the promoters of two genes whose expression is characteristic of the absorptive cell lineage. The data, therefore, demonstrate a role for β-catenin-TCF signaling in the regulation of lineage-specific differentiation of colonic epithelial cells.

MATERIALS AND METHODS

Cell Culture. Caco-2 cells (passage 22–32) were obtained from the American Type Culture Collection and maintained in MEM supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, and 10 mM HEPES buffer. For routine maintenance, Caco-2 cells were passaged 1:3 by trypsinization immediately upon reaching confluence. For spontaneous differentiation experiments, the time when cells first reached confluence (by light micros-

copy) was designated "day 0," and cells were transfected at this time point or at 2, 5, 7, 14, or 21 days thereafter.

Transfections. The p*l*asmids pTOPFLASH, pFOPFLASH (17), pGL3-iFABP (33), IAP$_{2,4}$CAT (34), pGL3-SI (35), pGL3-CEA (36), CMV-APC (21), pBAT-EM2 (37), CMV-ΔN-TCF-4 (17), and −163CD1LUC (27) have been described previously. In all cases, DNA was purified by the use of the Qiagen maxi-prep kit (Qiagen, Valencia, CA). Cells were grown and transfections done in 24-well plates using the Fugene (Boehringer-Mannheim) transfection reagent according to the manufacturer's instructions. Cells were transfected with 0.1–0.5 μg of reporter plasmid, 0.1–1 μg of test plasmid, and 0.167 μg of CMV-β-GAL, β-actin-β-GAL, or TK-Renilla as a control for transfection efficiency. Appropriate amounts of pBluescript were added to ensure that all cells received equivalent amounts of DNA. β-catenin-TCF activity was determined by calculating the ratio of luciferase activity obtained from pTOPFLASH relative to pFOPFLASH.

Gel Shift Analysis. Nuclear extracts were prepared as reported previously (38) with the addition of 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) to the STKM lysis buffer [30% sucrose (w/v), 40 mM Tris (pH 7.5), 37 mM KCL, 12 mM MgCl$_2$, and 0.8% Triton X-100; Sigma Chemical Co.]. Binding reactions were performed as reported (17), except that the poly [I, C] concentration was adjusted to 400–600 ng per reaction. The double-stranded wild-type (GCACCCTTTGATCTTACC) and mutant (GCACCCTTTGGCCTTACC) TCF oligonucleotides (Promega Co.) were labeled with Promega's 5′-end labeling kit and γ-[^{32}P]dATP (6000 Ci/mmol; NEN, Boston, MA). Anti-β-catenin antibody was obtained from Transduction Laboratories (Lexington, KY) and anti-CD4 (control) antibody from Quidel Corp. (San Diego, CA). Gel shifts were analyzed in 4% polyacrylamide, 1× Tris-borate EDTA gels, dried, and the data were analyzed using a PhosphorImager:425 (Molecular Dynamics, Sunnyvale, CA).

Western Blot Analysis, Immunoprecipitation. Total cellular protein was isolated in immunoprecipitation buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 1 mM EDTA, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, and 0.7 μg/ml pepstatin]. Membrane and cytosolic fractions were prepared as described elsewhere (39). The association of β-catenin with E-cadherin and APC was assessed in IP experiments, and in all cases 300 μg of total cell protein were used. β-catenin-E-cadherin complex formation was determined by IP with anti β-catenin (Transduction Labs, Lexington KY) and detection with anti-E-cadherin (Transduction Labs). APC-β-catenin complexes were detected by IP with anti-APC (Ab-1; Oncogene Research Products, Cambridge, MA) and then with anti-β-catenin (Transduction Labs).

Antibodies directed against β-catenin (1:4000; Transduction Labs), E-cadherin (1:8000; Transduction Labs), TCF-4 (4 μg/ml; Upstate Bioscientific, Lake Placid, NY), APC (1 μg/ml, Oncogene Research Products), and actin (1:2000; Sigma Chemical Co.) were used in Western blot analyses. Proteins (2–100 μg) were resolved in prepoured Tris-glycine SDS gels (Bio-Rad, Richmond CA), and transferred to a nitrocellulose membrane overnight (Bio-Rad). Blots were blocked in 5% nonfat milk in PBS, and incubated with the primary antibody and appropriate secondary antibody for 1 h each. Antibody-binding was detected using enhanced chemiluminescence reagent according to the manufacturer's instructions.

Immunofluorescence. Subcellular localization of β-catenin and E-cadherin was examined by immunofluorescence. Caco-2 cells grown on 0.05% gelatin-coated coverslips were harvested at confluence (day 0) or 21 days thereafter. Monolayers were washed in HBSS and fixed in 4% paraformaldehyde for 20 min at room temperature. Before staining, cells were washed in PBS/5 mM MgCl$_2$, permeabilized in 0.3% Triton X-100/50 mM Tris/150 mM NaCl, for 10 min, washed in Tris/glycine buffer (200 mM Tris/100 mM glycine) for 5 min, and blocked in 2% BSA/2% FBS, for 1 h at 37°C. For undifferentiated Caco-2 cells, monolayers were incubated with anti-E-cadherin (1:2000) or anti-β-catenin (1:2000) for 1 h at 37°C, washed, and incubated with a Cy3-conjugated antimouse antibody (1:750) for 1 h at 37°C. For colocalization studies, monolayers were initially probed for E-cadherin as described above, after which monolayers were washed and then incubated with a FITC-conjugated anti-β-catenin antibody (1:2000; Transduction Laboratories). Monolayers were postfixed in 0.1% paraformaldehyde, and were nuclei stained with 1 μg/ml DAPI. Cells were visualized using a BX60 fluorescence microscope (Olympus America, Melville, NY) equipped with a DAPI and High Q (for detection of FITC and Cy3 dyes) filter set (Chroma Technology, Brattleboro,

VT) and a ×60 Plan Apo 1.4 numerical aperture objective. Images were acquired in grayscale with a SPOT RT-cooled CCD camera (Diagnostica Instruments, Sterling Heights, MI) and SPOT RT software (Diagnostica Instruments).

Characterization of APC Truncation Mutation. Protein truncation mutations within codons 657-1693 of the *APC* gene were identified by PCR and IVTT. Two overlapping segments of the *APC* gene covering codons 657-1284 and 1099–1693 were amplified from genomic DNA using two pairs of specific PCR primers. The primers were based on those described by Levy *et al.* (40), but were modified to contain suitable restriction sites as follows: (*a*) codons 657-1284: forward primer, 5′ GCGGATCCTAATACGACTCACTATAGGAACAGAC-CACCATGGGAGAGAACAA CTGTCTACAAACT-3′; reverse primer, 5′ GGAATTCAGCTGATGACAAAGATGAT A-3′; and (*b*) codons 1099–1693: forward primer, 5′-GCGGATCCTAATACGACTCACTATAGGAACAGACC-ACCATGGTTTCTCCATACAGGTCACGG-3′; reverse primer, 5′-GGAAT-TCTGTAGGAATGGTAT CTCGT-3′. PCR was performed in 50-μl reactions containing 100 ng of genomic DNA, 0.2 μM primers, 0.2 mM dNTPs, 2.5 units of PfuTurbo (Stratagene) in 1× Pfu buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH$_4$)SO$_4$, 2 mM MgSO$_4$, 0.1% Triton X-100, and 0.1 mg/ml BSA]. Cycling conditions for both segments were as follows: 94°C for 5 min and then 35 cycles of 94°C for 1 min; 57°C for 1 min; 72°C for 5 min; and finally one cycle of 72°C for 10 min. Reaction products were purified using a QIAquick PCR purification kit (Qiagen) and then used as templates in IVTT assays performed with the TNT Quick coupled reticulocyte lysate system (Promega) according to the manufacturer's protocol. [^{35}S]-methionine-labeled polypeptides were analyzed by 12% SDS-PAGE and fluorography. *APC* truncation mutation in Caco-2 cells was characterized further by sequence analysis of the PCR products. Internal sequencing primers used were selected from those reported previously (2).

RESULTS

Down-Regulation of β-Catenin-TCF Activity and Complex Formation during Caco-2 Cell Differentiation. We and others have previously demonstrated that Caco-2 cells maintained in culture for 21 days postconfluence undergo a spontaneous G_0/G_1 cell cycle arrest and differentiate along the absorptive cell lineage (30–32), modeling the phenotypic changes that occur as colonic epithelial cells migrate along the crypt axis toward the lumenal surface. To determine the role played by β-catenin-TCF in inducing these phenotypic changes, cells at progressive stages of maturation were transfected with pTOPFLASH or pFOPFLASH, which directly assays β-catenin-TCF activity (17). β-catenin-TCF activity was maximal in rapidly dividing, undifferentiated Caco-2 cells and progressively decreased with increasing time subsequent to confluence in parallel with the cells undergoing cell cycle arrest and differentiation. Compared with cells that had just reached confluence (day 0), β-catenin-TCF activity was reduced ~10-fold in fully differentiated cells (day 21; Fig. 1*A*). As a control, we performed the same experiment in the SW480 cell line, which has high levels of β-catenin-TCF signaling because of a mutation in the *APC* gene, but which does not undergo differentiation with time in culture. In contrast to Caco-2 cells, no change in β-catenin-TCF activity was observed in SW480 cells cultured for 0, 2, 5, 7, or 21 days postconfluence (data not shown).

Consistent with the reduction in β-catenin-TCF activity, gel shift analysis demonstrated a decrease in β-catenin-TCF complex formation in Caco-2 cells with time in culture. The specificity of the complex was demonstrated by the ability of an anti-β-catenin antibody to supershift the complex, and by elimination of the detected band using unlabeled TCF consensus oligonucleotides in excess. In contrast, a nonspecific oligonucleotide, or one in which the TCF consensus sequence was altered at two residues (Mut-TCF), failed to compete with the TCF consensus sequence for binding to the β-catenin-TCF complex (Fig. 1*B*). Finally, statistical analysis demonstrated a significant correlation between the decrease in β-catenin-TCF ac-
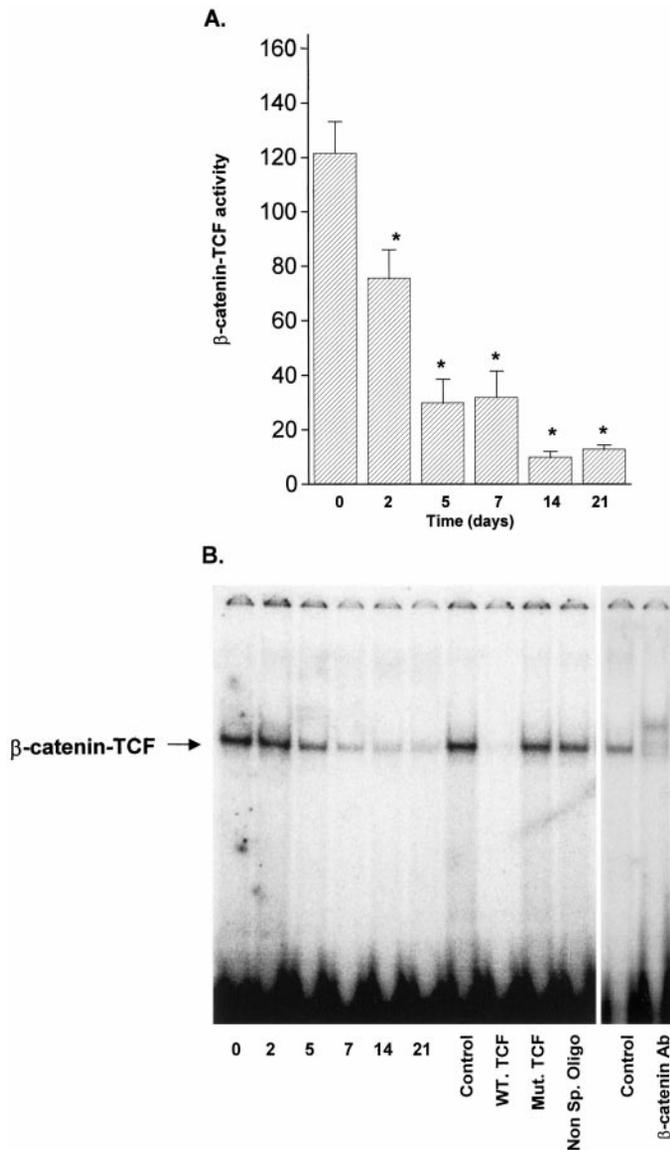
Fig. 1. Effect of spontaneous Caco-2 cell differentiation on (*A*) β-catenin/TCF activity and (*B*) complex formation. *A*, Caco-2 cells at confluence (*day 0*) or 2–21 days thereafter were transiently transfected with pTOPFLASH or pFOPFLASH for 48 h, and luciferase activity was measured in cell lysates. In each case, CMV-GAL was cotransfected as a control for transfection efficiency. Values shown are the mean of four separate experiments, ∗*P* < 0.005; *t* test. *Bars,* ± SE. *B, Lanes* labeled *0, 2, 5, 7, 14,* and *21* are gel shifts produced using nuclear extracts from Caco-2 cells cultured for the respective number of days after confluence. The *lanes* labeled *WT.TCF,* (wild-type TCF), *Mut.TCF* (mutant TCF), and *Non. Sp. Oligo* (non-specific oligonucleotides) included unlabeled oligonucleotides of these sequences as competitors, as described in "Materials and Methods." *Lane* labeled *β-catenin Ab* included this Ab in the reaction mixture.

tivity and complex formation during Caco-2 cell differentiation (*r* = 0.89; *P* = 0.015).

Down-Regulation of β-Catenin-TCF Signaling Is Independent of APC. Caco-2 cells have been reported previously to be *APC* mutant, but the nature of the *APC* mutation has not been characterized (41). IVTT and then DNA sequencing revealed a nonsense mutation at codon 1367, a C→T transition changing Gln (CAG) to a stop codon (TAG). Consistent with these findings and the previous report, we detected low levels of a 170kDa-truncated form of APC in Caco-2 cells, levels of which remained constant during Caco-2 cell differentiation (Fig. 2). Furthermore, an antibody directed against the NH2 terminus of APC was able to immunoprecipitate β-catenin in Caco-2 cells. The amount of APC-β-catenin complex, however, remained unchanged during Caco-2 cell differentiation (Fig. 2), demonstrating

that down-regulation of β-catenin-TCF signaling was independent of APC.

Expression of TCF-4 and β-Catenin. In parallel with the down-regulation of β-catenin-TCF signaling, TCF-4 protein levels decreased steadily during Caco-2 cell differentiation (Fig. 3). In contrast, total β-catenin protein levels increased significantly during Caco-2 cell differentiation (Fig. 3).

Subcellular fractionation studies, however, demonstrated that the increase in β-catenin was attributable to an increase in the membrane fraction of β-catenin, whereas cytosolic levels remained unchanged. As a control for the efficiency of fractionation, E-cadherin levels in membrane and cytosolic fractions were also compared. As expected, E-cadherin was only detected in the membrane fraction (Fig. 4, *top panel*).

Role of E-Cadherin. Inasmuch as β-catenin is known to bind to the cytoplasmic tail of E-cadherin, we examined whether the increase in the membrane fraction of β-catenin reflected an increase in E-cadherin-β-catenin complex formation. First, Western blot analysis demonstrated very high levels of E-cadherin expression in Caco-2 cells, which increased ~2-fold as the cells underwent differentiation (Fig. 4, *middle panel*). Furthermore, immunoprecipitation experiments demonstrated a significant (10-fold) increase in the amount of E-cadherin-β-catenin complex over the same time course (Fig. 4, *bottom panel*). The increase in total β-catenin levels, therefore, most likely reflects an increase in the E-cadherin-associated membrane fraction of β-catenin.

To confirm this further, we examined the subcellular distribution of β-catenin by immunofluorescence staining. As shown in Fig. 5, and consistent with the subcellular fractionation studies, β-catenin staining was exclusively localized to the cell membrane in fully differentiated Caco-2 cells (*panel B*). Similarly to β-catenin, staining for E-cadherin was also exclusively found at the cell membrane (*panel C*), and merging of the β-catenin and E-cadherin images (*panel D*) confirmed the colocalization of the two proteins to the cell membrane. Similar results were observed in undifferentiated Caco-2 cells, although the intensity of β-catenin staining was considerably less (data not shown). No nuclear β-catenin staining was observed at any stage during Caco-2 cell differentiation.
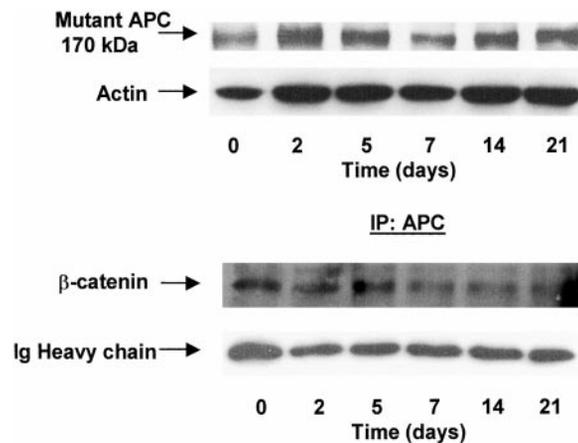

Fig. 2. Mutant APC expression and β-catenin-APC complex formation during Caco-2 cell differentiation. *Top,* expression of mutant APC protein levels was determined by Western blot in Caco-2 cell lysate harvested at confluence (*day 0*) or at 2–21 days thereafter. A band of ~ 170kDa corresponding to the mutant form of APC expressed in these cells is shown. *Bottom,* β-catenin-APC complex formation was determined in Caco-2 cells lysates harvested at confluence (*day 0*) or at 2–21 days thereafter. APC was immunoprecipitated using an antibody directed against the NH2 terminus of APC, the immunocomplexes was resolved by PAGE, and blots were probed with anti-β-catenin. The immunoglobulin heavy chain is also shown as a control for loading.

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Fig. 3. Expression of components of the β-catenin-TCF pathway during Caco-2 cell differentiation. TCF-4 and β-catenin protein levels were measured by Western blot in Caco-2 cell lysates harvested at confluence (*day 0*) or at 2–21 days thereafter.
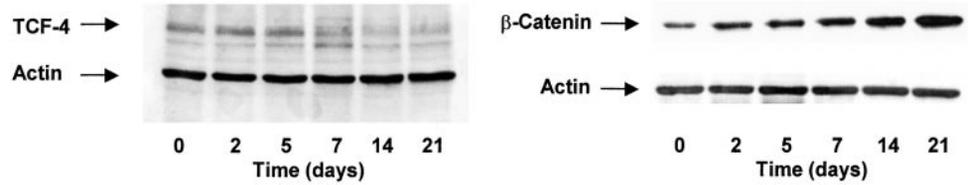
Effect of Down-Regulation of β-Catenin-TCF Activity in Undifferentiated Caco-2 Cells on Genes Linked to Cell Differentiation. It has been shown that both the *cyclin D1* and *c-myc* genes, important regulators of cell cycle progression, are transcriptionally up-regulated by β-catenin-TCF (27, 28, 42). The down-regulation of β-catenin-TCF activity in Caco-2 cells as a function of time, therefore, is consistent with the spontaneous G_0/G_1 cell cycle arrest that the cells undergo simultaneously (32).

The effect of the β-catenin-TCF pathway on the regulation of differentiation in colonic epithelial cells, however, is unknown. To test this, we examined the effect of down-regulation of β-catenin-TCF signaling on the promoter activities of four genes linked to cell differentiation: *ALP*, *iFABP*, *CEA*, and *SI*.

First, we tested whether the promoter activities of these genes were regulated during spontaneous Caco-2 cell differentiation. Vectors containing the promoters of the *ALP*, *iFABP*, *CEA*, and *SI* genes linked to chloramphenicol acetyltransferase (CAT) or luciferase reporters were transfected into Caco-2 cells at different stages during the spontaneous maturation of these cells, and their activity was determined. As shown in Fig. 6, the promoter activities of all markers increased 2- to 4-fold during Caco-2 cell differentiation. Thus, transcriptional up-regulation of these genes accompanied the decrease in β-catenin-TCF signaling.

Next, to determine whether down-regulation of β-catenin-TCF signaling would alter the promoter activities of these four genes, first we established conditions for down-regulation of this pathway in undifferentiated Caco-2 cells. Ectopic expression of ΔN-TCF-4 (a dominant negative mutant of TCF-4), wild-type APC, or E-cadherin, in undifferentiated Caco-2 cells (day 0 postconfluence), in each case, and as expected, resulted in significant down-regulation of β-catenin-TCF signaling (Fig. 7, *A–C*). Also, and consistent with previous reports (27, 42), down-regulation of the pathway by these mechanisms resulted in a decrease in cyclin D1 promoter activity in undifferentiated Caco-2 cells (data not shown).

Finally, cotransfection of each modulator of β-catenin-TCF signaling, with reporter constructs driven by the alkaline phosphatase or iFABP promoters, in each case resulted in a significant increase in promoter activity (Fig. 7, *A–C*). In contrast, in each case, the down-regulation of β-catenin-TCF activity failed to induce any significant change in CEA or sucrase-isomaltase promoter activities (Fig. 7, *A–C*).

DISCUSSION

We and others have demonstrated previously that, with time in culture, Caco-2 cells undergo spontaneous cell cycle arrest, with cells accumulating in the G_0/G_1 phase of the cell cycle (31, 32, 43). Simultaneously, Caco-2 cells undergo spontaneous differentiation along the absorptive cell lineage, as shown by the increased activities of alkaline phosphatase, maltase, dipeptidyl peptidase IV, and carcinoembryonic antigen expression, mimicking the phenotypic changes that absorptive cells undergo as they migrate up the crypt axis toward the colonic lumen (30, 32). It is important to note that Caco-2 cells do not undergo apoptosis with time in culture (32), but as we have recently reported, only 1% of colonic epithelial cells undergo detect-

able apoptosis *in vivo* (44). The Caco-2 cell line, therefore, is an excellent model for the study of the molecular pathways that regulate cell cycle and differentiation programs of colonic epithelial cells.

Several previous observations have suggested a role for β-catenin-TCF signaling in the regulation of intestinal epithelial cell proliferation (27–29, 42). Consistent with these observations, the present study demonstrates an additional link between cell proliferation and β-catenin-TCF signaling. β-catenin-TCF signaling and complex formation was greatest in undifferentiated, proliferating Caco-2 cells (day 0) and gradually diminished with time after confluence (days 2–21) as Caco-2 cells underwent G_0/G_1 cell cycle arrest and differentiation along the absorptive cell lineage. Consistent with the down-regulation of β-catenin-TCF signaling, the expression of the *c-myc* and *cyclin D1* genes have been shown previously to be down-regulated as Caco-2 cells undergo these changes (31, 45).

The constitutively high levels of β-catenin-TCF signaling in undifferentiated Caco-2 cells is consistent with the fact that these cells have a mutant *APC* gene. Protein truncation assays and sequencing analysis
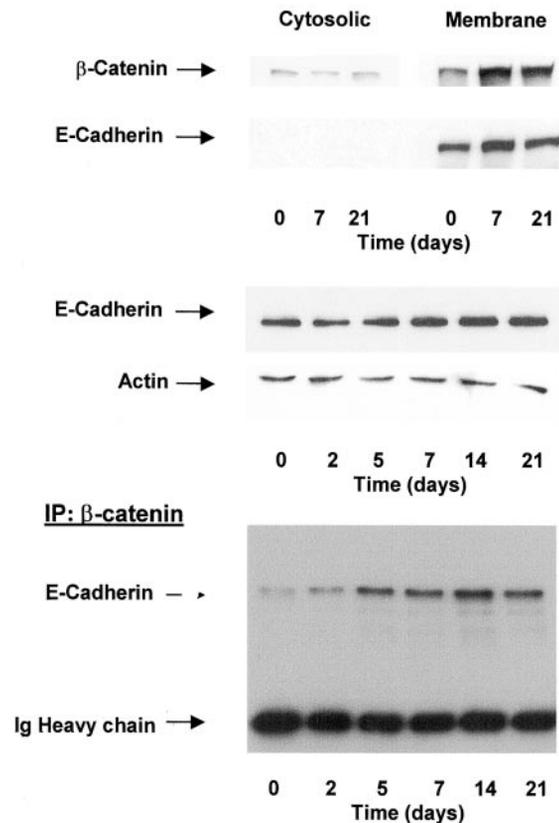

Fig. 4. Increase in β-catenin is attributable to an increase in the E-cadherin-associated membrane fraction. *A,* total cellular protein was isolated from Caco-2 cells at confluence (*day 0*) or at 7 and 21 days postconfluence, and membrane and cytosolic fractions were prepared. β-catenin and E-cadherin levels in the two fractions were determined by Western blot. *B,* determination of E-cadherin protein levels in total cell lysates at various stages during Caco-2 cell differentiation, by Western blot. *C,* β-catenin-E-cadherin complex formation was determined in Caco-2 cell lysates harvested at various stages after confluence by immunoprecipitating with an anti-β-catenin antibody and then by detection with an anti-E-cadherin antibody.
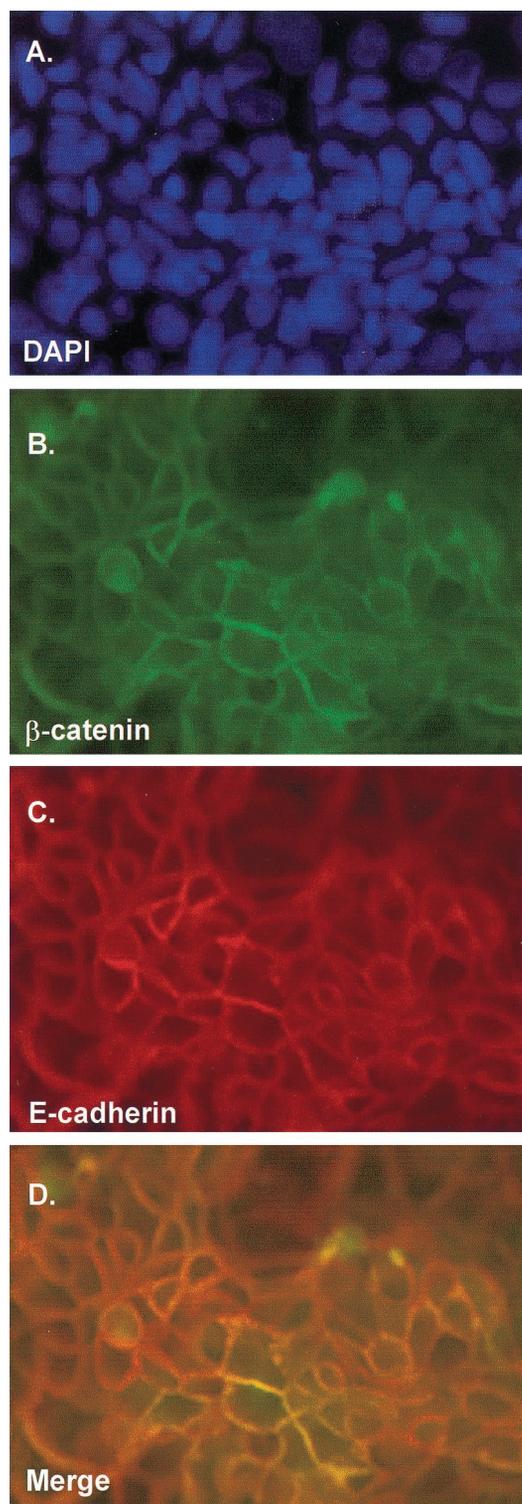
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Fig. 5. Colocalization of β-catenin (B) and E-cadherin (C) in differentiated (21 days after confluence) Caco-2 cells. A, DAPI staining; B, β-catenin; C, E-cadherin; D, merged image of B and C.

β-catenin-APC complex, but levels of both remained constant during Caco-2 cell differentiation, suggesting that APC plays a minimal role in the down-regulation of β-catenin-TCF signaling during the differentiation of these cells.

In parallel with the down-regulation of β-catenin-TCF signaling during Caco-2 cell differentiation, TCF-4 protein levels decreased significantly. As TCF-4 is the DNA-binding component of the β-catenin-TCF complex, its down-reglution most likely mediates the loss of β-catenin-TCF-4 signaling during Caco-2 cell differentiation. In contrast to TCF-4, total β-catenin levels increased significantly during Caco-2 cell differentiation. This increase, however, was attributable to an increase in the E-cadherin-associated membrane fraction of β-catenin, whereas cytosolic β-catenin levels, which is the critical parameter for β-catenin-TCF signaling (46), remained unchanged over the time course. That the increase in β-catenin was attributable to an increase in the E-cadherin-associated membrane fraction was demonstrated, first, by immunoprecipitation experiments, which showed increased β-catenin-E-cadherin complex formation, and, second, by immunofluorescence staining, which showed strong colocalization of β-catenin and E-cadherin in differentiated Caco-2 cells. It is important to note, however, that whereas E-cadherin levels increased ~2-fold during Caco-2 cell differentiation, E-cadherin-β-catenin complex formation increased ~10-fold. This discrepancy may be explained by the fact that E-cadherin levels are very high, even in undifferentiated Caco-2 cells. The greater increase in E-cadherin-β-catenin complex formation, therefore, may reflect the progressive sequestration of β-catenin by the high levels of E-cadherin that are present even before the increase that occurs during differentiation.

In addition to undergoing cell cycle arrest, colonic epithelial cells undergo differentiation along one of three cell lineages as they migrate upwards along the crypt axis (19, 20). Caco-2 cells model differentiation along the absorptive cell lineage as shown by the increased promoter activities of four genes that encode markers of absorptive cell differentiation: ALP, CEA, SI, and iFABP protein. These observations are consistent with previous reports demonstrating that the enzymatic activity and expression of ALP, CEA and SI are increased in Caco-2 cells over this same time course (32), as well as during the upward migration along the crypt axis of colonic epithelial cells in vivo (47).

That down-regulation of β-catenin-TCF signaling may play a role in inducing colonic epithelial cell differentiation is suggested, first, by the observation that the progressive differentiation of Caco-2 cells over time is accompanied by the simultaneous down-regulation of this pathway; and, second, the rapid down-regulation of β-catenin-TCF
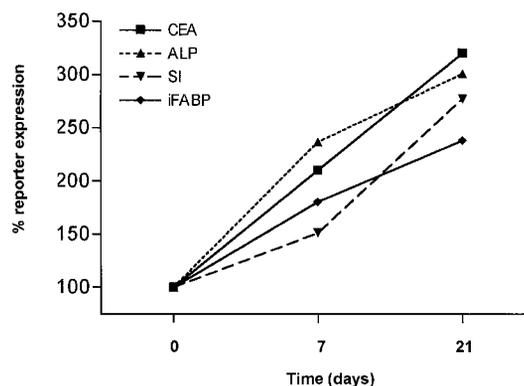
Fig. 6. Promoter activities of markers of cell differentiation during Caco-2 cell differentiation. Caco-2 cells at confluence (day 0), or at 7 or 21 days thereafter were transiently transfected with the CAT or luciferase-linked promoter constructs of four known markers of absorptive cell differentiation for 48 h, and lysates were assayed for luciferase or CAT activity.

demonstrated the presence of a stop mutation in codon 1367 (exon 15), which is located in the mutation cluster region of the APC gene. This mutation would be expected to result in the translation of a truncated APC protein that retains its ability to bind β-catenin, but which would be unable to target β-catenin for degradation. To test this prediction, we examined whether changes in the levels of APC contributed to the down-regulation of β-catenin-TCF signaling. We were able to detect low levels of this truncated APC protein and also of

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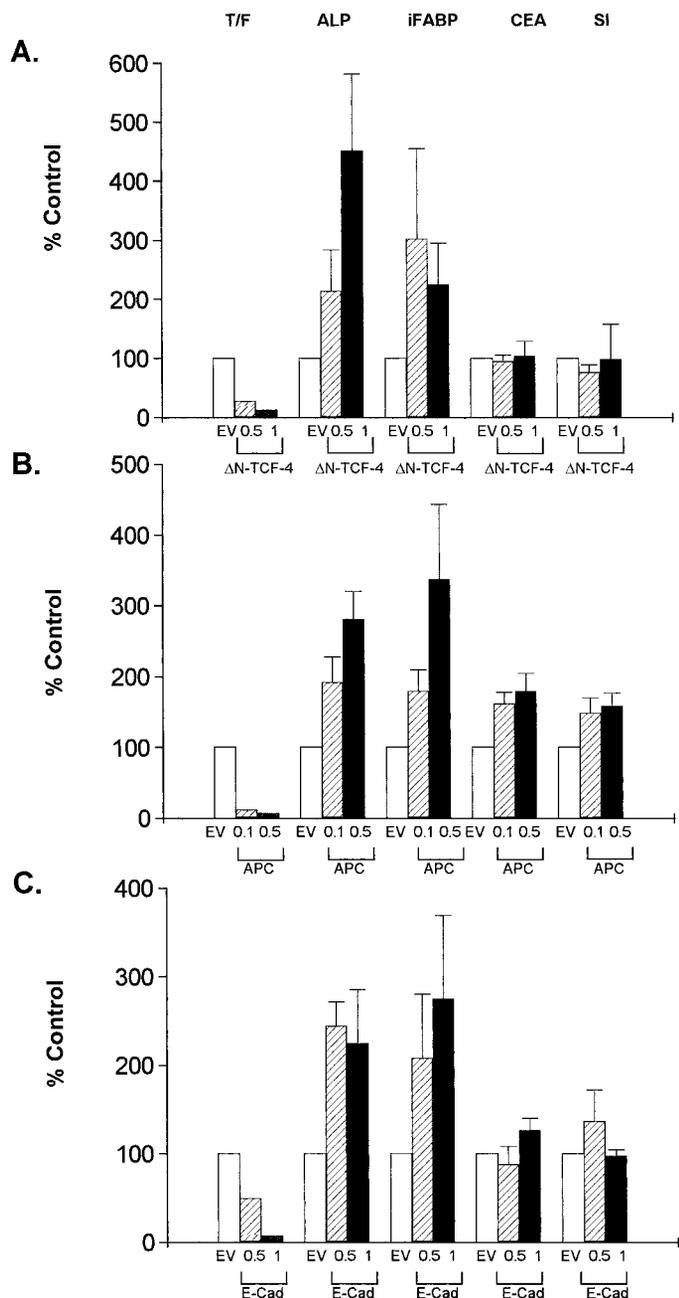Fig. 7. Effect of ΔN-TCF-4, WT-APC, and E-cadherin β-catenin-TCF activity and ALP, iFABP, CEA, and SI promoter activities. Undifferentiated Caco-2 cells were transiently cotransfected with either pTOPFLASH or pFOPFLASH or with promoter constructs from the *ALP, iFABP, CEA,* and *SI* genes in the presence of either (*A*) CMV-ΔN-TCF-4 or CMV-EV (*empty vector*) control, (*B*) CMV-APC or CMV-EV (*empty vector*) control, or (*C*) E-cadherin or pBAT-NEO control for 48 h, and luciferase activity was measured.

naling is not surprising. We have demonstrated previously that the patterns of cell differentiation induced in Caco-2 cells by differentiation-inducing agents, such as sodium butyrate, differs from the pattern of cell differentiation induced during spontaneous Caco-2 cell differentiation (32). Furthermore, we have recently demonstrated by microarray analysis that the extent of gene reprogramming during Caco-2 cell differentiation is extensive and extremely complex (45), representing modulation of multiple pathways. It is likely, therefore, that the complete differentiation of colonic epithelial cells requires the interaction of multiple pathways, with the β-catenin-TCF pathway only one component, albeit an important one, of the maturation program.

The mechanism by which down-regulation of β-catenin-TCF signaling induces the promoter activities of ALP and iFABP requires additional investigation. Increased β-catenin-TCF complex formation results in transcriptional activation, whereas in the present study we demonstrate increased transcriptional activation in response to down-regulation of the pathway. The effects observed, therefore, most likely reflect an indirect effect on β-catenin-TCF signaling, requiring additional transcription factors. For example, down-regulation of β-catenin-TCF signaling may result in the down-regulation of transcription factors whose normal role is to repress the expression of genes associated with the onset of cell differentiation. There is clear precedent for this hypothesis because it was recently shown that APC induces expression of the transcription factor *CDX2* (48), which has been shown to induce differentiation of the IEC-6 cell line (49). Finally, the present findings that link down-regulation of β-catenin-TCF signaling to the induction of absorptive cell differentiation is consistent with observations made in *TCF-4*-null mice. In these animals, down-regulation of the pathway by the targeted inactivation of TCF-4 results in the premature onset of differentiation. In comparison with controls, in which fully differentiated epithelial cells are observed primarily in the villus, fully differentiated cells were observed significantly earlier, in the intervillus region, in mutant mice (29).

In conclusion, these observations suggest that, in addition to its role in regulating cell proliferation, the β-catenin-TCF signaling pathway plays an additional role in regulating colonic epithelial cell differentiation.

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signaling in undifferentiated Caco-2 cells by three independent mechanisms: ectopic expression of a dominant negative mutant form of TCF-4 (ΔN-TCF-4), WT-APC, or E-cadherin, which resulted in consistent increases in the promoter activities of ALP and iFABP. Down-regulation of this pathway, however, had no effect on the promoter activities of the *CEA* or *SI* genes, suggesting that whereas down-regulation of the β-catenin-TCF pathway results in the promotion of a more differentiated phenotype, the effect on cell differentiation is not complete. Pathways in addition to β-catenin-TCF signaling, therefore, must be activated or down-regulated for the complete differentiation of colonic epithelial cells. This incomplete induction of cell differentiation subsequent to down-regulation of β-catenin-TCF sig-

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Down-Regulation of β-Catenin TCF Signaling Is Linked to Colonic Epithelial Cell Differentiation

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