Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion

Maree C. Faux[1,*], Janine L. Ross[1], Clare Meeker[1], Terry Johns[1], Hong Ji[2], Richard J. Simpson[2], Meredith J. Layton[2] and Antony W. Burgess[1,3]

[1]Ludwig Institute for Cancer Research and [2]Joint ProteomicS Laboratory and [3]CRC for Cellular Growth Factors, Ludwig Institute for Cancer Research and Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia

*Author for correspondence (e-mail: maree.faux@ludwig.edu.au)

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Summary

The APC tumour suppressor gene is mutated in most colon cancers. A major role of APC is the downregulation of the β-catenin/T-cell factor (Tcf)/lymphoid enhancer factor (LEF) signalling pathway; however, there are also suggestions that it plays a role in the organization of the cytoskeleton, and in cell adhesion and migration. For the first time, we have achieved stable expression of wild-type APC in SW480 colon cancer cells, which normally express a truncated form of APC. The ectopically expressed APC is functional, and results in the translocation of β-catenin from the nucleus and cytoplasm to the cell periphery, and reduces β-catenin/Tcf/LEF transcriptional signalling. E-cadherin is also translocated to the cell membrane, where it forms functional adherens junctions. Total cellular levels of E-cadherin are increased in the SW480APC cells and the altered charge distribution in the presence of full-length APC suggests that APC is involved in post-translational regulation of E-cadherin localization. Changes in the location of adherens junction proteins are associated with tighter cell-cell adhesion in SW480APC cells, with consequent changes in cell morphology, the actin cytoskeleton and cell migration in a wound assay. SW480APC cells have a reduced proliferation rate, a reduced ability to form colonies in soft agar and do not grow tumours in a xenograft mouse tumour model. By regulating the intracellular transport of junctional proteins, we propose that APC plays a role in cell adhesion in addition to its known role in β-catenin transcriptional signalling.

Key words: APC, β-catenin, Colon cancer cells, E-cadherin, Tumour suppressor gene

Introduction

The adenomatous polyposis coli (APC) tumour suppressor gene has been reported to be mutated in most familial and sporadic colorectal cancers (Kinzler and Vogelstein, 1996). Transforming mutations in the *APC* gene, which result in truncation of the APC protein, are thought to be an early, if not an initiating, event in a multistep process involving the successive acquisition of genetic mutations in colon cancer (Fearon and Vogelstein, 1990). Germline mutations in *APC* cause an autosomal, dominantly inherited disease called familial adenomatous polyposis coli (FAP), characterized by the development of hundreds of colorectal adenomas, and ultimately colorectal carcinomas, in which both *APC* alleles are inactivated (Bienz and Clevers, 2000; Groden et al., 1991). APC has been implicated in the regulation of β-catenin signalling through the Wnt pathway (Peifer and Polakis, 2000) and, more recently, in cytoskeletal organization (Rosin-Arbesfeld et al., 2001; Mogensen et al., 2002) (reviewed by Dikovskaya et al., 2001).

The identification of several proteins that interact with APC has provided important insights into its possible functions (reviewed by Polakis, 1997; Bienz, 1999; Dikovskaya et al., 2001). The middle third of APC mediates binding to Wnt signalling pathway effectors, β-catenin, axin and glycogen synthase kinase-3β (GSK-3β). Phosphorylation of β-catenin in this complex by GSK-3β targets β-catenin for ubiquitination and proteasome-mediated destruction (Peifer and Polakis, 2000). Truncations of the APC protein that delete the β-catenin and/or axin binding sites prevent β-catenin degradation, resulting in abnormally high levels of cytoplasmic and nuclear β-catenin in colon tumour cells (Munemitsu et al., 1995). In the nucleus, β-catenin interacts with members of the T-cell factor (Tcf)/lymphoid enhancer factor (LEF) family of transcription factors to activate transcription of Wnt target genes, including cyclin D1 and myc (Korinek et al., 1997), which promote proliferation and are associated with cellular transformation.

The subcellular distribution of APC to punctate clusters near the ends of microtubules provided the initial evidence for a role for APC in cell migration and adhesion (Nathke et al., 1996). APC binds to microtubules both directly and indirectly through association with Eb1 (Munemitsu et al., 1994; Su et al., 1995) and stabilizes microtubules in vivo (Zumbrunn et al., 2001). APC appears to accumulate in clusters near the plus ends of microtubules at the leading edge of migrating cells (Nathke et al., 1996) and has been shown to move along a subset of microtubules towards their distal ends (Mimori-Kiyosue et al., 2000). In polarized cells, APC has been found at sites of cell-

cell adhesion and at the basal membrane (Rosin-Arbesfeld et al., 2001). Disruption of either actin or microtubule networks has been used to show that the association of APC with basal membranes requires an intact actin cytoskeleton, and that clustering of APC at the microtubule tips requires intact microtubules (Rosin-Arbesfeld et al., 2001). By contrast, truncated APC in colon cancer cell lines does not appear to associate with either the plasma membrane or microtubule tips (Rosin-Arbesfeld et al., 2001).

The association of APC with the cytoskeleton and the plasma membrane at cell-cell junctions has led to suggestions of a role for APC in cell-cell adhesion (Dikovskaya et al., 2001; Mimori-Kiyosue and Tsukita, 2001). Isolation of a loss-of-function allele of *Drosophila* E-APC that caused defects in adherens junction formation has also provided evidence that *Drosophila* APC functions in cell adhesion (Hamada and Bienz, 2002). Here, we report the generation of a novel cell line derived from the human colon carcinoma cell line SW480 (Leibovitz et al., 1976), in which full-length, recombinant APC is stably expressed. SW480 cells normally express only truncated APC (Nishisho et al., 1991) but, upon restoration of full-length APC (SW480APC), the cells exhibit a downregulation in β-catenin/Tcf/LEF transcriptional signalling. Although stable expression of full-length APC in this cell line did not lead to marked decreases in total cellular β-catenin, it did result in translocation of β-catenin from the nucleus to the cell periphery. Restoration of full-length APC also caused an increase in total cellular levels of E-cadherin and in the translocation of E-cadherin to the plasma membrane, where it assembled into functional adherens junctions, with associated changes in cell morphology and the actin cytoskeleton. These results suggest that full-length APC plays a role in cell-cell adhesion and maintenance of the epithelial phenotype in normal cells and that truncation of APC might initiate transformation by more than one mechanism, including effects on cell adhesion as well as the known effects on β-catenin signalling.

Materials and Methods

Antibodies

The following mouse monoclonal antibodies (mAbs) were used: anti-β-catenin (clone 14 C 19920/610153; BD Transduction Laboratories), anti-APC (Ab-1, clone FE-9; Oncogene Research Products), anti-E-cadherin (clone 36 C20820/610181; BD Transduction Laboratories), anti-myc (9E10) (Evan et al., 1985), anti-actin (clone AC-40; Sigma), HRP-conjugated goat anti-mouse IgG (H+L) secondary antibodies (170-6516; Bio-Rad Laboratories) and fluorescently labelled Alexa488 goat anti-mouse and Alexa546 goat anti-mouse secondary antibodies (Molecular Probes).

Recombinant APC construction

The cDNA encoding full-length human APC (pcDNA3-APC) was provided by Dr Paul Polakis. An N-terminally myc-tagged full-length human APC was constructed in three parts, assembled in the pET30a(+) vector (Novagen) then subcloned into the pEF/myc/cyto (pEF) mammalian expression vector (pShooter; Invitrogen). A PCR product encoding a myc-tagged N-terminal APC fragment (APCN) was generated from pcDNA3-APC using Primer 1 (5′-ATCCGG-TCGACATGGAACAAAAACTCATCTCAGAAGAGGATCTGAAT-GGGGCCGCAATGGCGCAGCTTCATAT-3′) and Primer 2 (5′-TTGCTGAATTCTGGCTATTCTTCGCTGTGCTCG-3′), cloned into the pCR®2.1-TOPO vector (Invitrogen) and subcloned by digestion with *Sal*I and *Eco*RI into pET30a(+). The C-terminal fragment of APC (6498-8532 bp) (APCC) was amplified from pcDNA3-APC using Primer 3 (5′-GGCCCACGAATTCTAAAACCAGGG-3′) and Primer 4 (5′-GACTGGATCCGTCGACTTAAACAGATGTCACAA-GGTA-3′), cloned into the pGEX4T-1 vector (Pharmacia Biotech) then subcloned by digestion with *Eco*RI and *Bam*HI into pET30a(+)-APCN. The remainder of APC cDNA (660-6497 bp) was subcloned into pET30a(+)-APCN-APCC by digestion with *Eco*RI. This internal fragment of APC was derived from a cDNA provided by Dr Bert Vogelstein, as sequencing of pcDNA3-APC revealed that it differed from the reported sequence of human APC (GenBank accession number M74088) in having a 53 bp insert after nucleotide 1410. The assembled APC cDNA was then digested with *Sal*I and subcloned into pEF.

Cell culture and transfections

SW480 (Leibovitz et al., 1976) and LIM1215 (Whitehead et al., 1985) human epithelial colon cancer cell lines were grown in RPMI supplemented with 1.08% 10-2 thioglycerol, 100 U/ml insulin, 50 mg/ml hydrocortisone, 10% foetal calf serum (FCS) and 1% penicillin/streptomycin. For stable transfections, 10^6 SW480 cells were plated per 100 mm tissue culture plate. After 24 hours, 10 μg of either pEF-mycAPC or pEF was transfected using FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. After 48 hours, 1.5 mg/ml Geneticin (neomycin) (GIBCO BRL) was added. Cells were then cultured for 2 weeks in the presence of 1.5 mg/ml Geneticin, and Geneticin-resistant colonies were selected and plated into individual tissue culture wells. Cells were screened for expression of ectopic APC by reverse-transcription PCR (RT-PCR), immunostaining with anti-β-catenin antibodies, Tcf/LEF (TOPflash) reporter assays, and western blot analysis for full-length APC.

Immunocytochemistry and drug treatments

Cells were seeded on 1.5 mm optical glass coverslips in growth media 24-48 hours prior to fixation for microscopy. The coverslips were washed twice in PBS fixed in 3.7% formaldehyde/PBS for 5 minutes, permeabilized using 0.2% Triton X-100/PBS for 5 minutes, then washed with PBS and blocked in 0.2% BSA/PBS for 30 minutes. Coverslips were incubated with primary antibodies diluted in 0.2% BSA/PBS (1:400 for β-catenin, 1:200 for E-cadherin) for 1 hour followed by washing in 0.2% BSA/PBS, then incubated for 1 hour with fluorescent secondary antibodies and/or Rhodamine-Phalloidin (Molecular Probes) diluted in 0.2% BSA/PBS followed by washing in 0.2% BSA/PBS. Immunofluorescent staining was detected in successive focal planes using a laser-scanning confocal microscope (Bio-Rad) at excitation wavelengths of 488 nm for Alexa488 or 514 nm for Alexa546 and Rhodamine.

For cytochalasin D treatment, cells were grown on glass coverslips, washed with PBS and incubated with 10 μM cytochalasin D or dimethyl sulfoxide (DMSO) vehicle control for 30 minutes or 4 hours at 37°C. Cells were then washed with PBS, fixed, permeabilized and immunostained as described. Adherens junction disassembly was triggered by chelation of calcium. Cells were serum-starved overnight and incubated in serum-free RPMI containing 4 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and 1 mM $MgCl_2$ for 1 hour. Cells were washed twice with PBS and either fixed, permeabilized and immunostained, or incubated in serum-free RPMI containing 10 mM $CaCl_2$ for 2 hours prior to washing twice with PBS and immunostaining as described.

Quantitation of fluorescence images

Immunostained β-catenin was scored for subcellular distribution

using a confocal fluorescence microscope. Parental SW480 cells have predominantly nuclear and cytoplasmic β-catenin and display a small degree of peripheral staining. In brightly stained cells, it is difficult to distinguish cytoplasmic and peripheral β-catenin. SW480APC clones expressing APC predominantly demonstrate peripheral staining but there is a varying degree of nuclear β-catenin present. Cells were therefore divided into groups according to whether β-catenin was predominantly nuclear/cytoplasmic (N), nuclear/cytoplasmic and peripheral (N/P), or peripheral (P). Cells were only scored as P if there was no nuclear β-catenin present.

RNA preparation and RT-PCR analysis

mRNA from LIM1215 and SW480 (parental, vector control and APC-transfected) cells was extracted and purified using standard methods (Chomczynski and Sacchi, 1987). The mRNA was converted to cDNA and then amplified using the SuperScript™ One-Step RT-PCR system (Invitrogen) and Primer R (5′-CTTCTGCTTGGTGGCATGG-3′) and Primer F (5′-CAGACGACACAGGAAGCAG-3′). This PCR fragment contains a *Pst*I site surrounding codon 1338 in wild-type APC but not mutated APC in SW480 cells. Amplified PCR products were digested with *Pst*I (MBI Fermentas) at 37°C for 2 hours and products visualized after agarose gel elecrophoresis.

Immunoprecipitation and western blot analysis

Cells were washed twice in PBS prior to harvesting. Cell lysates were prepared using ice-cold Lysis buffer [10 mM Tris pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM DTT and Complete (an EDTA-free protease inhibitor cocktail; Roche)], followed by Dounce homogenization. Lysates were clarified by microcentrifugation at 16,060 ***g*** for 30 minutes at 4°C. Total cell lysates were analysed by SDS-PAGE (4% or 4-20% gradient acrylamide gels; NOVEX), electrotransferred onto nitrocellulose and detected using appropriate primary antibodies. The levels of β-catenin and E-cadherin were quantitated by densitometry, normalized to actin, and analysed using ImageQuaNT software.

2-dimensional gel electrophoresis (2DE)

SW480 (parental, vector control and APC-transfected) cells were washed 3× with PBS and lysed in Urea lysis buffer containing 9 M urea, 4% (v/v) Chaps and 50 mM DTT. First-dimension IEF using precast IPG gel strips (Pharmacia) was performed in a Pharmacia IPGphor apparatus. Briefly, the IPG gel strips (pH 3-10, 7 cm) were rehydrated in 9 M urea, 4% CHAPS, 0.5% IPG buffer and 50 mM DTT containing 150 μg of protein for 12 hours. After focusing for 17,500 Volt-hour, the IPG gel strips were equilibrated (2×20 minutes) in 10 ml of equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, 50 mM DTT, 20% glycerol and 0.001% Bromophenol Blue) and applied to second-dimensional SDS-PAGE (4-20% gradient acrylamide gel; NOVEX). After 2DE, proteins were electrotransferred onto PVDF membranes using a semi-dry blotting apparatus, blocked and immunoblotted as above.

β-catenin-Tcf/LEF reporter assays

SW480 (parental, vector control and APC-transfected) cells were seeded into 24-well tissue culture dishes and transfected with 0.25 μg of TOPflash (containing the binding site for the Tcf/LEF family of transcription factors fused to a luciferase reporter gene) or FOPflash (negative control) reporter plasmids as described (van de Wetering et al., 1997). The cells were lysed and assayed for firefly luciferase activity using STOP and GLO (Promega) and results expressed as a ratio of TOPflash:FOPflash.

Growth assays

SW480 (parental, vector control and APC-transfected) cells were seeded into 24-well plates at 10^4, 5×10^4 or 3×10^5 cells/ml. At each time point, cells were washed twice with PBS and harvested. Single cell suspensions were counted in a haemocytometer (four fields counted in duplicate). Each time point was performed in triplicate.

Soft agar growth assay

SW480 (parental, vector control and APC-transfected) cells were suspended in growth media containing 0.3% agarose at 10^3, 5×10^3 or 10^4 cells/ml and layered over 0.6% agarose in growth media in 35 mm dishes. The agar was allowed to solidify at RT for 20 minutes before incubating the cells at 37°C and 10% CO_2. After 10 days, colonies were stained with Crystal Violet (0.005%) then counted and photographed. Three fields were counted for cells seeded at 1×10^4 cells/ml and >4 fields for cells seeded at 5×10^3 and 10^3 cells/ml. Triplicate samples were counted at each cell density.

Xenograft tumour growth assay

SW480 vector control (SW480control) and APC-transfected (SW480APC) cells (5×10^6) were injected into nude Balb/c mice and tumour volumes of xenografts measured (n=8). Each mouse received two xenografts. Mice with SW480 xenografts were observed for 53 days at which time the xenografts were palpable (mean volume of 1096 mm^3) and the mice sacrificed. Mice with SW480APC xenografts were observed for 60 days.

Wound assays

SW480 (parental, vector control and APC-transfected) cells were grown as confluent monolayers in 6-well plates. The cells were washed twice with PBS and a wound applied with a plastic pipette tip. The cells were washed twice with PBS, photographed (time=0) then incubated in media at 37°C and 10% CO_2. At 24 or 48 hours, cells were gently washed with PBS and the wound area photographed. Assays were performed in triplicate.

Results

Expression of full-length APC in SW480 cells results in translocation of β-catenin and downregulation of β-catenin/Tcf signalling

We generated a construct for expression of full-length APC with an N-terminal myc-tag in the pEF mammalian expression vector (pEF-mycAPC). Transient expression of pEF-mycAPC in SW480 cells resulted in the disappearance of β-catenin from the nucleus and cytoplasm and a decrease in Tcf/LEF reporter activity (data not shown), consistent with the reported function of transiently expressed APC in SW480 cells (Munemitsu et al., 1995).

SW480 cells stably expressing full-length epitope-tagged APC (SW480APC) were generated by transfection with pEF-mycAPC and selection in neomycin. Control cells (SW480 control) were generated by transfection with unmodified pEF vector and selection in neomycin in an identical manner. SW480 cell clones were analysed for expression of full-length APC by RT-PCR (Fig. 1A) and were immunostained to estimate the levels of nuclear β-catenin (Fig. 1B). Stable expression of full-length APC in SW480 cells resulted in a dramatic shift of β-catenin from the nucleus to the cell periphery (Fig. 1B). Several neomycin-resistant cell clones that express APC as demonstrated by RT-PCR analysis (e.g. clones .23, .15 and .24) exhibit little

or no nuclear or cytoplasmic β-catenin staining, but instead β-catenin is concentrated at the cell periphery, particularly at sites of cell-cell contact (Fig. 1B). Scoring of cells revealed that the proportion of peripheral and non-nuclear β-catenin was markedly increased in APC-expressing cells (Fig. 1B). SW480APC cell clones that are negative for APC expression by RT-PCR analysis (e.g. clones .14, .18 and .5) demonstrate levels of nuclear β-catenin comparable with vector-transfected or parental cells. SW480 control clones consistently demonstrated nuclear β-catenin (Fig. 1B). As would be expected, there was a reproducible (n>3) decrease in Tcf/LEF reporter activity in several neomycin-resistant SW480APC clones exhibiting peripheral β-catenin staining compared with parental and control SW480 cells (Fig. 1C). In particular, SW480APC clone .15 had a reproducible (n=10) fivefold decrease in Tcf/LEF reporter activity compared with controls. The presence of recombinant mycAPC in SW480APC clones was confirmed by western blot analysis of cell extracts of SW480APC clones but not in untransfected SW480 cells or SW480 control clones (Fig. 1D).

Although stable expression of full-length APC in SW480APC clones decreased the amount of β-catenin in the cytoplasm and nucleus (Fig. 1B), total cellular levels of β-catenin were not markedly reduced (Fig. 1E). Whereas a small decrease in β-catenin is apparent in some SW480APC clones, we did not observe consistent decreases in β-catenin levels in comparison with SW480 cells or SW480 control clones that would be consistent with increased proteasomal degradation of β-catenin. Instead, stable expression of APC in SW480APC clones causes translocation of β-catenin from the nucleus and cytoplasm to the plasma membrane (Fig. 1B). The difference between cells transiently expressing full-length APC, which results in the disappearance of β-catenin, and the SW480APC clones may be due to lower levels of APC expression. Indeed, low expression levels of recombinant APC in SW480APC clones may have allowed the cells to survive, as previous studies have shown that over-expression of APC leads to apoptosis (Morin et al., 1996; Groden et al., 1995).

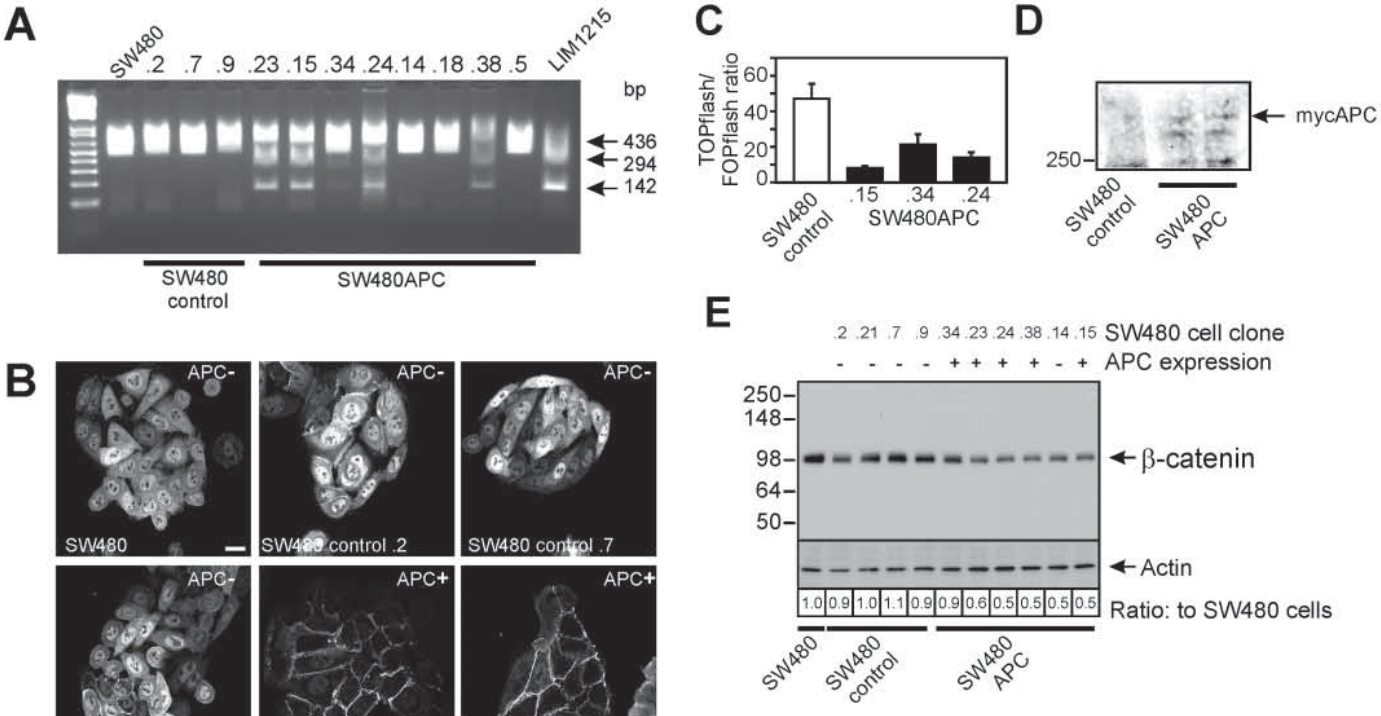
Fig. 1. Stable expression of functional, full-length mycAPC in SW480 cells. (A) Expression of wild-type APC mRNA in SW480APC cells by RT-PCR analysis. SW480 cells were transfected with pEF-mycAPC or pEF and neomycin-resistant clones selected. Reverse-transcribed APC cDNA was amplified over the region containing the mutation in SW480 cells and digested with *Pst*I, which cleaves only wild-type APC (Hargest and Williamson, 1995). LIM1215 colon cancer cells contain wild-type APC. RT-PCR products from untransfected SW480 cells and control SW480 clones (.2, .7 and .9) are not cleaved by *Pst*I. SW480APC cells (pEF-mycAPC-transfected, independent clones .23, .15, .34, .24 and .38) contain both endogenous, mutated APC mRNA that cannot be digested by *Pst*I (436 bp RT-PCR fragment) and APC mRNA that is cleaved by *Pst*I (294 and 142 bp RT-PCR products), demonstrating the presence of ectopically expressed APC. Some SW480APC cells (clones .14, .18 and .5) that were transfected with pEF-mycAPC and selected in neomycin did not show expression of ectopically expressed APC by RT-PCR analysis. (B) Expression of APC results in translocation of β-catenin to the cell periphery. SW480 (parental), SW480 control (pEF vector transfected, independent clones .2, .7 and .9) and SW480APC cells (pEF-mycAPC-transfected, independent clones .23, .15, .34, .24, .14, .18, .38 and .5) were stained immunochemically with antibodies to β-catenin and Alexa488-conjugated anti-mouse IgG. Fluorescent staining was imaged by laser-scanning confocal microscopy. β-catenin is predominantly nuclear in SW480 cells, SW480 control clones and SW480APC clones negative for APC by RT-PCR analysis, but translocates to the cell periphery in SW480APC clones containing ectopically expressed APC (.23, .15, .34, .24 and .38). Bar, 10 μm. The cellular distribution of β-catenin was scored as nuclear and cytoplasmic (N), nuclear, cytoplasmic and peripheral (N/P), or peripheral and no nuclear (P), and graphed. Shown is a representative of at least two independent experiments; n, total number of cells counted. (C) β-catenin/Tcf/LEF reporter activity is downregulated in SW480APC cells. SW480 and SW480APC (independent clones .15, .34 and .24) cells were transfected in triplicate with Tcf/LEF luciferase (TOPflash) reporter gene constructs (van de Wetering et al., 1997). Shown is the ratio of TOPflash to FOPflash luciferase activity from a representative of at least three independent experiments. Error bars indicate s.e.m. from triplicate measurements. (D) Western blot analysis demonstrating expression of full-length mycAPC in SW480APC cells. SW480 and SW480APC cells were lysed, proteins separated by 4% SDS-PAGE and immunoblotted using an anti-myc (9E10) mAb. (E) Western blot analysis of total cellular levels of β-catenin in SW480APC cells. SW480, SW480 control and SW480APC cells were lysed, 5 μg protein separated by 4-20% SDS-PAGE and immunoblotted using an anti-β-catenin mAb and an anti-actin mAb to control for protein loading (lower panel). The level of β-catenin in each clone was quantitated by densitometry, normalized to actin, and is represented as a ratio to β-catenin in SW480 cells. Shown is a representative of at least three independent experiments.

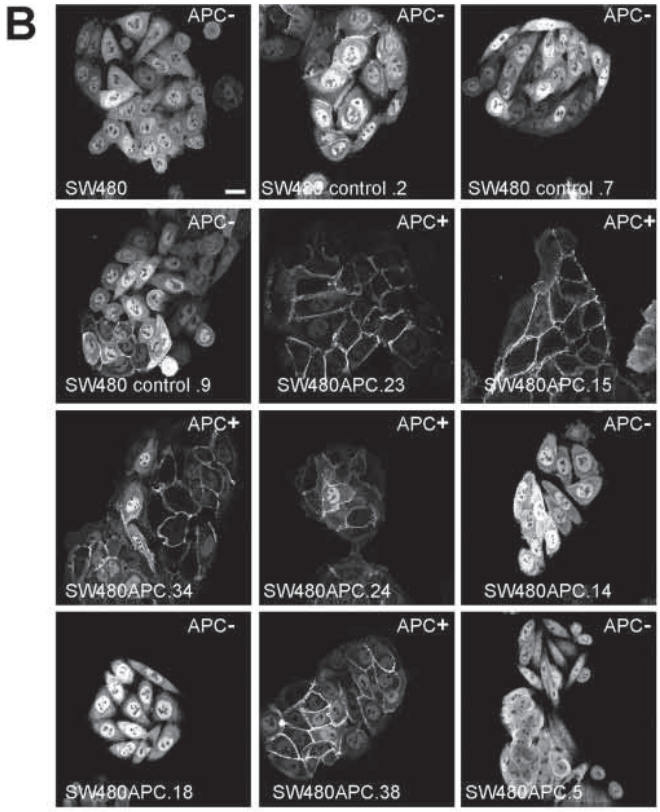
Expression of full-length APC in SW480 cells alters morphology

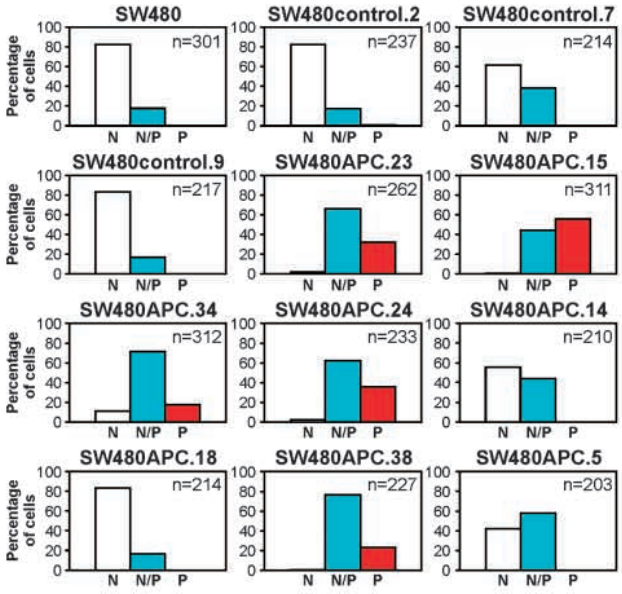
Stable expression of full-length APC in SW480 cells induces changes in cell shape and morphology. SW480 cells are typically heterogeneous and exhibit two cell populations with distinct morphologies: one adherent population of triangular, elongated cells and one population of rounded, refractile and less-adherent cells (Fig. 2A and B, arrows indicate adherent cells) (Palmer et al., 2001). By contrast, SW480APC clones are homogeneous and exhibit a more epithelial phenotype. The SW480APC cells are more flattened and adherent, and form compact colonies that do not contain rounded cells (Fig. 2C and D, arrowheads). The boundaries between adjacent SW480APC cells are less refractile and therefore less visible by phase contrast microscopy, suggesting that cell-cell contacts are tighter. Differences in morphology do not appear to be affected by cell density (Fig. 2, compare panels A versus B and C versus D).

Expression of full-length APC in SW480 cells suppresses cell growth and tumourigenicity

SW480APC clones grow more slowly than parental SW480 cells (Fig. 3A). Parental SW480 cells proliferate rapidly when they reach a density of approximately 2.5×10^5 cells/cm^2 (Fig. 3Ai, day 5, open squares, and day 7, open diamonds), whereas SW480APC cells do not. As SW480APC cells do not reach the density at which the SW480 cells proliferate rapidly until the end of the assay period (day 8) (Fig. 3Ai, filled squares), growth assays were performed at a higher cell density (Fig. 3Aii). SW480 cells again proliferate rapidly until reaching a plateau, whereas SW480APC cells plateau at a lower cell density, which is suggestive of contact inhibition of growth (Orford et al., 1999). Enhanced contact inhibition in







SW480APC cells relative to SW480 cells is consistent with the observed alterations in cell morphology.

Truncation of APC correlates with tumour formation (Shibata et al., 1997). Restoration of functional, full-length APC in a cell line containing only truncated APC would therefore be predicted to decrease its tumourigenic properties. Anchorage-independent growth is often correlated with the tumourigenic phenotype (Orford et al., 1999), therefore we used colony formation in soft agar as a measure of the degree of transformation of SW480 versus SW480APC cells. Crystal Violet-stained cells were photographed (Fig. 3Bi-iv) and counted (Table 1). SW480 control cells form typical refractile soft agar colonies, whereas the SW480APC colonies are smaller and less compact, with fewer cells in each colony. Both the size and number of colonies grown from SW480APC cells are markedly reduced compared with SW480 cells (Fig. 3B, Table 1). At both 1×10^4 and 5×10^4 cells/ml there are significantly fewer large colonies (Fig. 3B, arrows) in the

Table 1. Soft agar colony formation by SW480 and SW480APC cells†

		Number of colonies			
		SW480		SW480APC	
Cell density	Experiment number	Large	Small	Large	Small
1×10^4	1	12.2±1.1	36.5±1.6	0.5±0.1	13.6±1.0
	2	18.4±1.5	66.3±2.2	2.9±0.4	44.9±1.2
	3	28.8±3.6	76.6±3.9	6.5±0.4	60.2±3.5
5×10^3	1	7.1±0.3	32.9±0.9	0.7±0.2	24.8±0.3
	2	11.8±1.0	42.3±2.4	2.3±0.4	37.1±2.0

†Cells from each cell line were seeded in triplicate into 0.3% agar overlayed on 0.6% agar at the densities indicated. Colonies were stained with Crystal Violet and counted (3 fields for cells seeded at 1×10^4 cells/ml and >4 fields for cells seeded at 5×10^3 cells/ml). Shown are mean number of colonies ± s.e.m. from triplicate samples.

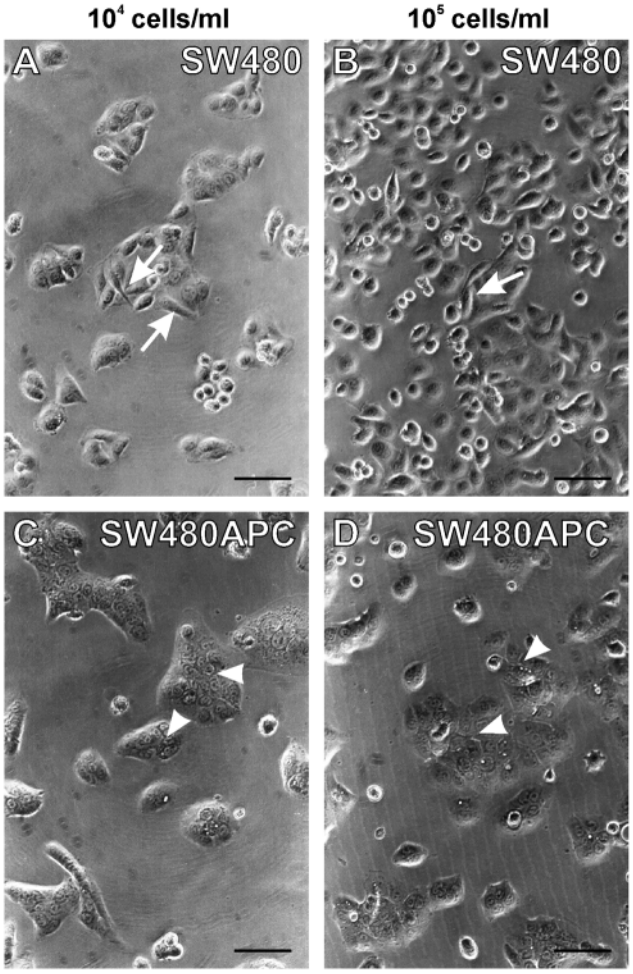


Fig. 2. Expression of full-length APC in SW480 cells alters cell morphology. Phase contrast micrographs of SW480 cells (A and B) and SW480APC.15 cells (C and D) plated at 10^4 cells/ml (A and C) or 10^5 cells/ml (B and D). Elongated SW480 cells (arrows) and tightly packed SW480APC colonies (arrowheads) are indicated. Bars, 20 μm.

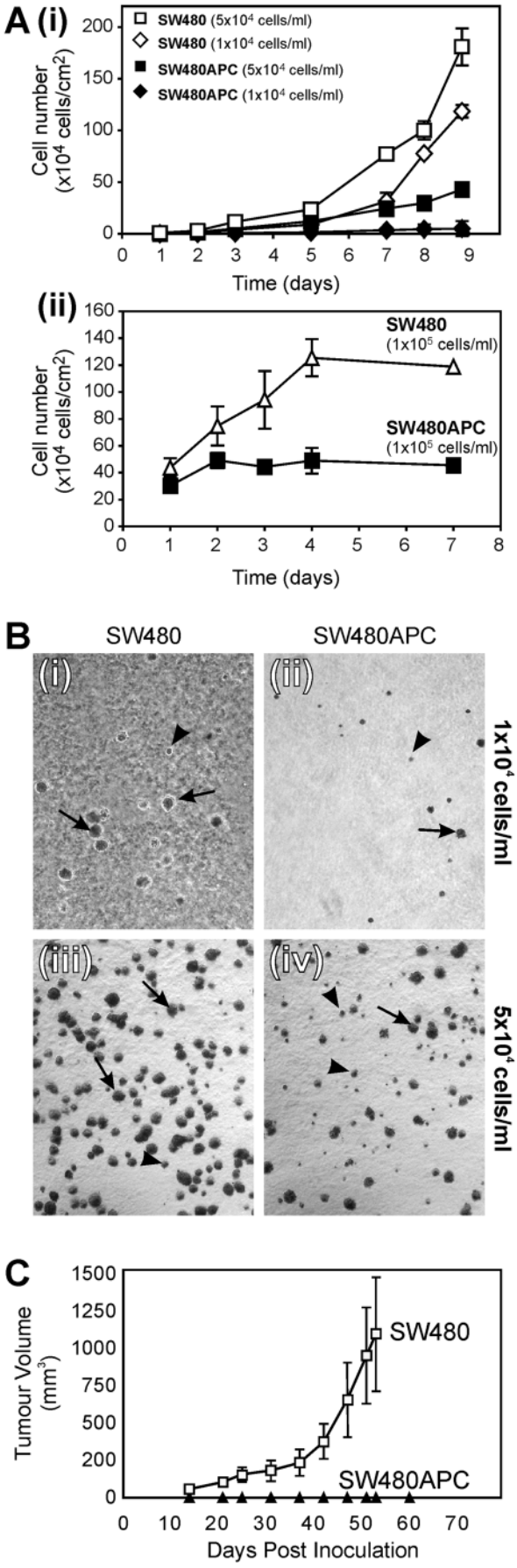


Fig. 3. Expression of full-length APC in SW480 cells inhibits cell growth, soft agar colony formation and tumour growth. (A) Cell growth. SW480 and SW480APC.15 cells, seeded in triplicate, were harvested at the time points indicated and counted using a haemocytometer (four fields counted in duplicate). Shown is the mean (±s.d.) number of cells counted from a representative of at least three independent experiments. (i) SW480APC cells (filled symbols) or parental SW480 cells (open symbols) were seeded at either 5×10^4 cells/ml (squares) or 1×10^4 cells/ml (diamonds). (ii) SW480 (triangles) and SW480APC (squares) cells were seeded in triplicate at 10^5 cells/ml. (B) Soft agar colony assay. SW480 or SW480APC.15 cells were seeded in triplicate into 0.3% agar overlayed on 0.6% agar in 35 mm dishes at the densities indicated. Phase contrast micrographs were taken of colonies stained with Crystal Violet formed by SW480 [(i) and (iii)] or SW480APC [(ii) and (iv)] cells after 10 days growth in soft agar. Arrows and arrowheads indicate colonies classified as large and small, respectively. (C) Xenograft tumour growth rate. 5×10^6 SW480 control cells (clone .7) and 5×10^6 SW480APC (clone .15) cells were injected into nude Balb/c mice (n=8/group) and tumour volumes of xenografts measured. Shown is the mean (±s.d.) tumour volume (n=8) for SW480 control cells (squares) and SW480APC cells (triangles).

SW480APC cultures than the SW480 culture. The difference in number of smaller colonies (Fig. 3B, arrowheads) is significant but not as marked (Table 1). Expression of intact APC in SW480 cells therefore inhibits anchorage-independent growth in soft agar, suggesting that expression of the ectopic APC gene is maintained in these cell lines and that stably expressed mycAPC is functional and sufficient to reverse the transformation of SW480 cells.

Suppression of tumourigenicity following the restoration of intact APC in SW480APC cells was confirmed in tumour xenografts (Fig. 3C). The tumour growth rate for SW480 control cells injected into nude mice is shown, whereas SW480APC cells did not grow tumours. Thus, stable expression of APC in SW480APC cells is sufficient to eradicate the tumourigenic growth of SW480 colon cancer cells.

Expression of full-length APC in SW480 cells results in redistribution of E-cadherin

SW480 cells have been reported to express little or no E-cadherin (Gottardi et al., 2001; Palmer et al., 2001). However, northern blot analysis with an E-cadherin-specific probe demonstrates expression of E-cadherin mRNA in SW480 cells (data not shown). Furthermore, mass spectrometric sequence analysis of a 120 kDa Coomassie Brilliant Blue-stained protein from an anti-E-cadherin immunoprecipitate unequivocally demonstrates the presence of E-cadherin protein in SW480 cells (data not shown). An E-cadherin-immunospecific protein is also detected in SW480 cells by both immunofluorescence and western blotting (migrating on SDS-PAGE at approximately 120 kDa) (Fig. 4).

Immunofluorescent staining of MDCK epithelial cells using this E-cadherin mAb localizes E-cadherin to the cell periphery, at sites of cell-cell contact, which is consistent with the known distribution of E-cadherin in epithelial cells (Nathke et al., 1996). By contrast, immunofluorescent staining of fixed, permeabilized SW480 and SW480 control cells reveals a punctate, perinuclear distribution for the E-cadherin protein (Fig. 4A; SW480, SW480 control clones .7 and .9, arrowheads). The punctate nature of the staining is unusual: the E-cadherin is

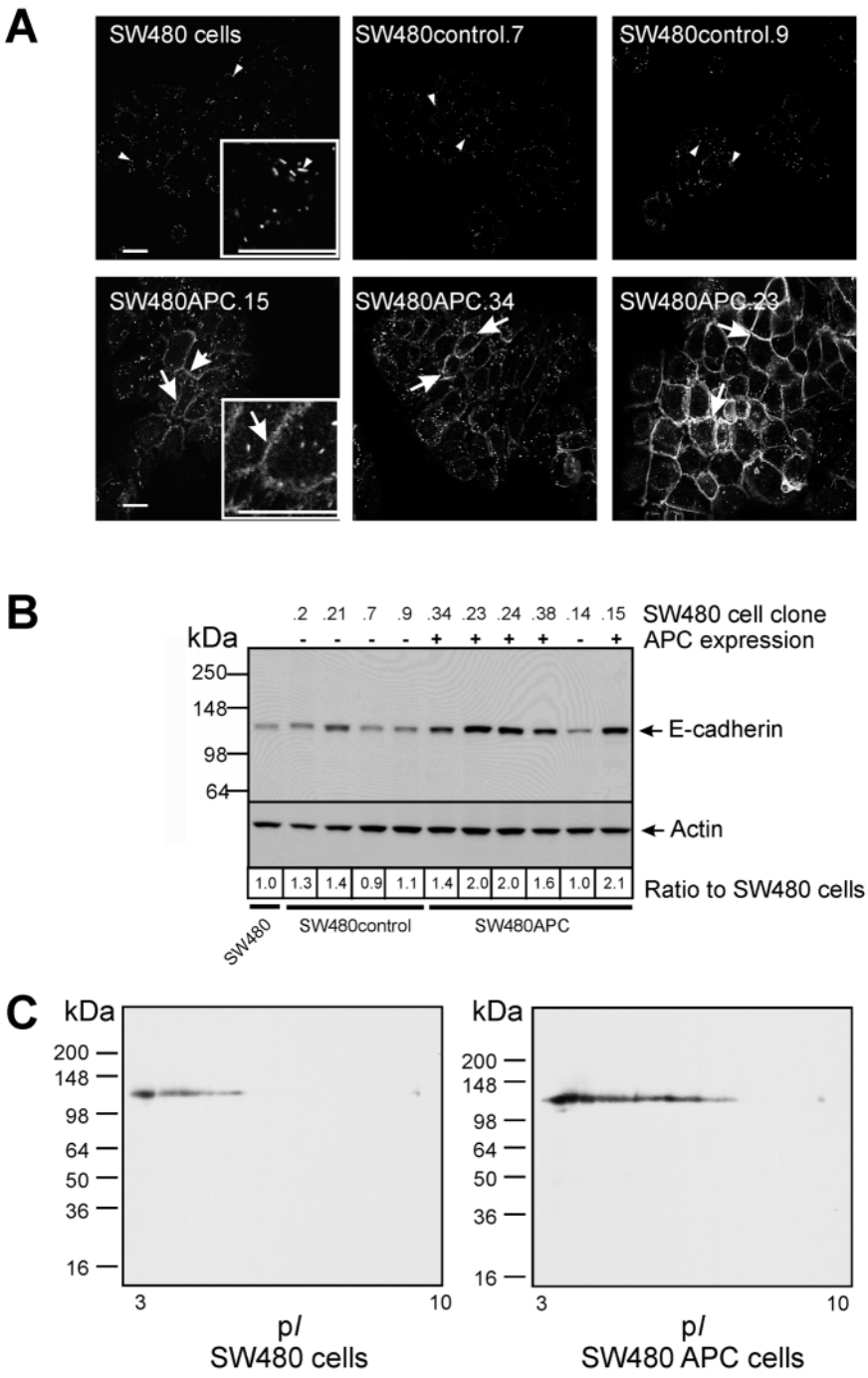


Fig. 4. Alterations in the localization, expression levels and charge of E-cadherin in SW480APC cells. (A) Redistrubution of E-cadherin in SW480APC cells. Cells were immunostained using an antibody against E-cadherin and Alexa488-conjugated anti-mouse IgG. Confocal microscopy reveals a punctate distribution for E-cadherin in SW480 and SW480 control cells (indicated by arrowheads) that is redistributed to sites of cell-cell contact in SW480APC cells (arrows). Bars, 10 μm. (B) Increased expression of E-cadherin in SW480APC cells by western blot analysis. Cells were lysed, 20 μg protein separated by 4-20% SDS-PAGE and immunoblotted using anti-E-cadherin and anti-actin (lower panel) mAbs. The level of E-cadherin in each clone was quantitated by densitometry, normalized to actin, and is represented as a ratio to E-cadherin in SW480 cells. Shown is a representative of at least three independent experiments. (C) 2DE gel analysis. SW480 or SW480APC.15 cells were lysed in 2DE gel extraction buffer containing 9 M urea, 4% (v/v) Chaps, 50 mM DTT, separated in two dimensions (IEF and SDS-PAGE), and analysed by immunoblotting with an anti-E-cadherin antibody. A paired sample of a representative of at least three independent experiments is shown.

concentrated in distinct, short, linear structures (Fig. 4A, inset). In the SW480APC cells, E-cadherin is localized predominantly to sites of cell-cell contact at the periphery of the cell (Fig. 4A; SW480APC clones .15, .34 and .23, arrows). In cells at the edges of SW480APC colonies, where there are no cell-cell contacts, there is very little membrane-associated E-cadherin staining. Not all E-cadherin translocates to the cell periphery in SW480APC cells, as there is still some punctate staining in all SW480APC clones.

Immunofluorescent staining of E-cadherin in SW480APC cells reveals that E-cadherin levels appear to be enhanced in cells expressing APC. Indeed, western blot analysis demonstrates an increase in total cellular protein levels of E-cadherin in SW480 cells expressing APC compared with SW480 control cells (Fig. 4B). Two-dimensional SDS-PAGE (2DE) was used to assess changes in E-cadherin at the molecular level. Western blot analysis of 2DE separations of whole cell lysates from SW480 and SW480APC cells shows the difference in the pI range for E-cadherin from the two cell lines (Fig. 4C). There is no detectable change in the apparent molecular weight of E-cadherin in the two cell lines, suggesting that changes in post-translational modifications of the protein were occurring. The calculated pI for unmodified E-cadherin is 4.55; however, the extracellular domain of E-cadherin is modified by variable carbohydrate moieties (Shore and Nelson, 1991), which will contribute to the spread of apparent pIs. Translocation of E-cadherin to the periphery of SW480APC cells correlated with a wider spread of pIs for E-cadherin, with an increased proportion of E-cadherin immunoreactivity towards the basic end of the gel (Fig. 4C). 2DE analysis also demonstrates that total cellular levels of E-cadherin are increased in SW480APC cells. However, the altered charge distribution of E-cadherin in SW480APC cells is unlikely to be due to increased protein levels. Even when western blots of 2DE gels of SW480 cells were exposed for longer time periods, the spread of E-cadherin in SW480 cells did not match that of SW480APC cells. Stable expression of full-length APC in SW480 cells results in redistribution of E-cadherin to the plasma membrane, increases in the levels of E-cadherin and changes in the post-translational modifications of E-cadherin. APC may therefore regulate E-cadherin at both the transcriptional or translational level and the post-translational level. It is also possible that APC regulation of E-cadherin is indirect and alterations in E-cadherin result from increased processing that occurs during trafficking of E-cadherin to the cell surface.

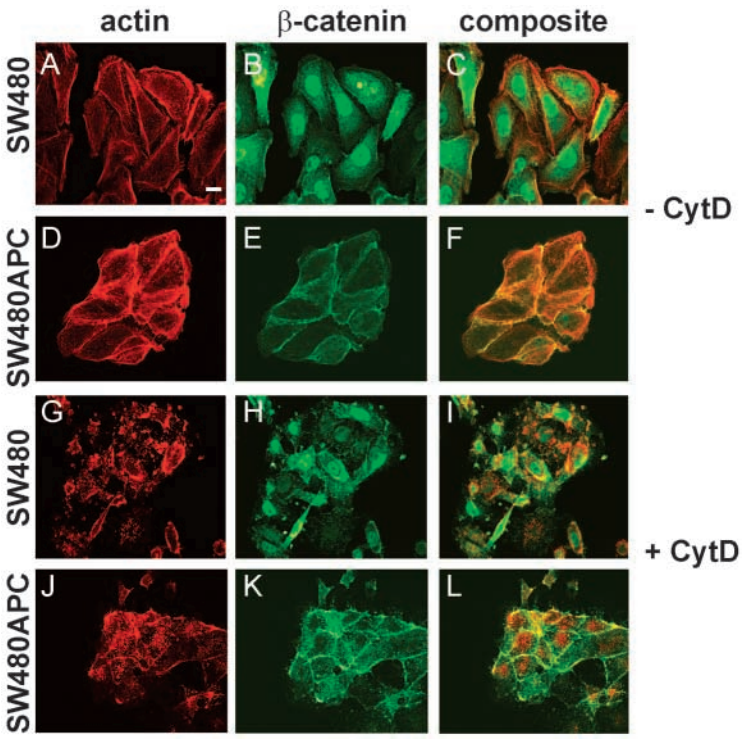


Fig. 5. Reorganization of the actin cytoskeleton in SW480APC cells. Vehicle control-treated SW480 (A-C) or SW480APC.15 (panels D-F), and cytochalasin D-treated SW480 (G-I) or SW480APC.15 (J-L) cells, were co-stained with Rhodamine-Phalloidin to visualize F-actin (A,D,G,J) and antibodies to β-catenin and Alexa488-conjugated anti-mouse IgG to visualize β-catenin (B,E,H,K). Areas with overlapping actin (Rhodamine, red) and β-catenin (Alexa488, green) staining are seen as yellow in C, F, I and L (composite). Fluorescent staining was imaged by laser-scanning confocal microscopy. The images shown are single-representative focal planes. Bar, 10 μm.

Effect of APC expression on the actin cytoskeleton

Given the increased cell-cell contact and the redistribution of β-catenin and E-cadherin in SW480APC cells (Figs 2, 1A and 4B, respectively), and the connection between E-cadherin and β-catenin in adherens junctions with the actin cytoskeleton (Jamora and Fuchs, 2002), the distribution of actin was determined by staining with Rhodamine-Phalloidin (Fig. 5). In both SW480 and SW480APC cells, actin is concentrated at the periphery of the cell. In SW480 cells, most staining is close to the outer edges of the cells, but there is clear definition between individual cells (Fig. 5A). By contrast, peripheral actin in SW480APC cells is associated with the close cell-cell contacts (Fig. 5D). The distribution of β-catenin overlaps with that of the actin cytoskeleton in SW480APC cells, but not SW480 cells (Fig. 5F and C, respectively). Disruption of the actin cytoskeleton by treatment of cells with cytochalasin D has little effect on the localization of β-catenin (Fig. 5G-L). Although cytochalasin D treatment causes collapse of cortical actin, β-catenin remains at the cell periphery in SW480APC cells (Fig. 5K), thus it seems unlikely that peripheral localization of β-catenin in SW480APC cells is mediated through a direct association with actin filaments. Treatment with latrunculin A also showed that peripheral β-catenin was maintained in SW480APC cells despite disruption to the actin cytoskeleton (data not shown). Interestingly, cytochalasin D disrupts the actin cytoskeleton even more severely in SW480 cells than in SW480APC cells (Fig. 5G,J); however, β-catenin remains in the nucleus of SW480 cells (Fig. 5H). The expression of full-length APC therefore results in reorganization of the actin cytoskeleton, which is consistent with the reported association of APC and actin (Rosin-Arbesfeld et al., 2001). However, the exact role of APC in regulating actin dynamics is not clear. Changes in actin may

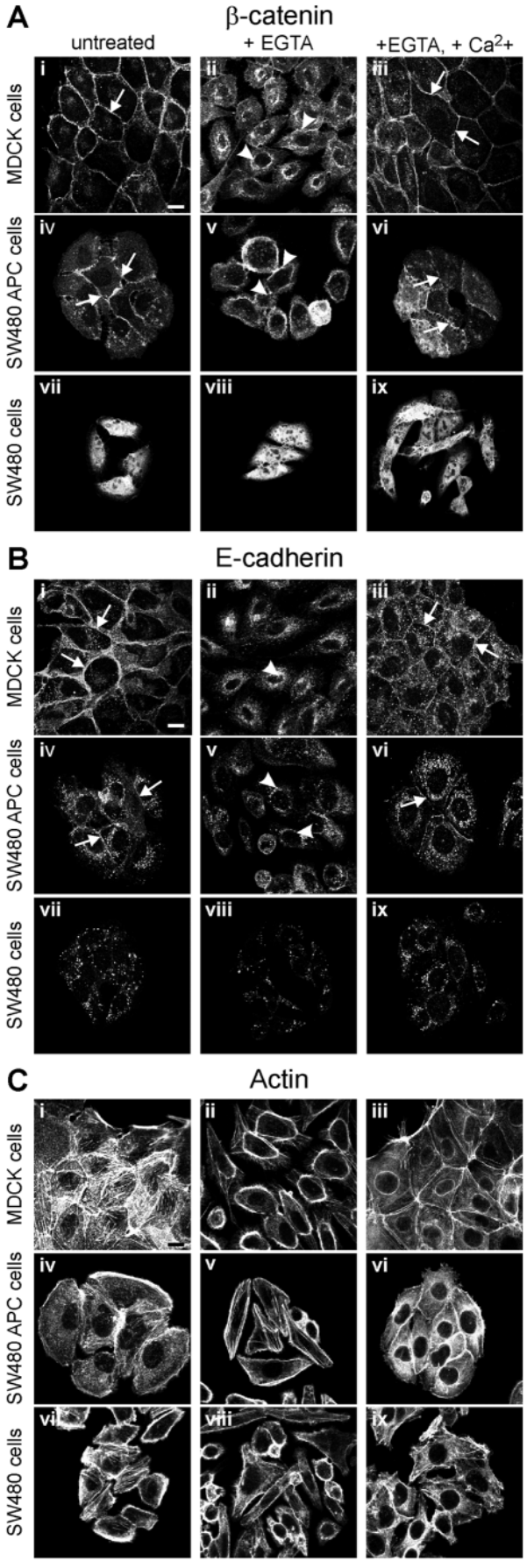


Fig. 6. Junctional β-catenin and E-cadherin staining in SW480APC cells is disrupted by EGTA treatment and restored with excess Ca^{2+}. MDCK (panels i-iii), SW480APC.15 (panels iv-vi) or SW480 (panels vii-ix) cells were stained with antibodies to β-catenin (A), E-cadherin (B) or with Rhodamine-Phalloidin to visualize actin (C). Prior to staining, cells were serum starved overnight and left untreated (i, iv and vii), treated with 4 mM EGTA and 1 mM $MgCl_2$ for 1 our (ii, v, viii) or with 4 mM EGTA and 1 mM $MgCl_2$ for 1 hour followed by treatment with 10 mM Ca^{2+} for 2 hours (iii, vi, ix). Arrows indicate intact adherens junctions, and arrowheads indicate relocalized staining. Fluorescent staining was imaged by laser-scanning confocal microscopy. The images shown are for single-representative focal planes. Bar, 10 μm.

be a consequence of altered cell-cell contacts mediated through the regulation of β-catenin and/or E-cadherin, or there may be a direct interaction between APC and the actin cytoskeleton. In either case, the distribution of β-catenin in full-length APC-expressing cells does not depend on an intact actin cytoskeleton.

SW480APC cells form functional adherens junctions

In order to determine whether translocation of β-catenin and E-cadherin to the cell periphery allows them to associate with the plasma membrane and form functional adherens junctions, disassembly and reassembly of adherens junctions was induced by chelation of calcium, which is required for homotypic E-cadherin binding (Gumbiner et al., 1988), then reintroduction of excess calcium, respectively. The MDCK epithelial cell line is known to form functional adherens junctions, and therefore was used as a positive control. In MDCK cells, both β-catenin and E-cadherin localize to sites of cell-cell contact at the plasma membrane, (Fig. 6Ai,Bi, arrows). Actin staining in these cells also reflects the presence of tight cell-cell contacts (Fig. 6Ci). As described previously, staining patterns for β-catenin, E-cadherin and actin in SW480APC cells (Fig. 6A,B,C, panel iv, arrows in 6A and B indicate junctional staining) are more reminiscent of that in MDCK cells (Fig. 6A,B,C, panel i), than that in SW480 cells (Fig. 6A,B,C, panel vii).

Treatment of MDCK cells with EGTA, to disrupt Ca^{2+}-dependent adherens junctions, results in redistribution of β-catenin and E-cadherin from the cell periphery to a perinuclear location (Fig. 6Aii,Bii, arrowheads) and incubation with excess Ca^{2+} following EGTA treatment restores junctional β-catenin and E-cadherin staining (Fig. 6Aiii,Biii, arrows) as expected. The localization of E-cadherin and β-catenin in SW480APC cells is also altered by treatment with EGTA (Fig. 6Av,Bv, arrowheads indicate redistributed β-catenin and E-cadherin), but calcium chelation does not affect the distribution of either protein in SW480 cells (Fig. 6Aviii,Bviii). Junctional E-cadherin staining is also disrupted using an anti-E-cadherin mAb (HECD1) in SW480APC, but not SW480, cells (data not shown). In addition, treatment with EGTA causes alterations in the actin cytoskeleton in both MDCK and SW480APC, but not SW480, cells (Fig. 6Cii,Cv,Cviii). Interestingly, the actin cytoskeleton in EGTA-treated SW480APC cells more closely resembles actin in untreated SW480 cells (Fig. 6Cv,Cvii). Excess Ca^{2+} restores E-cadherin, β-catenin and actin distributions in both MDCK and SW480APC cells following

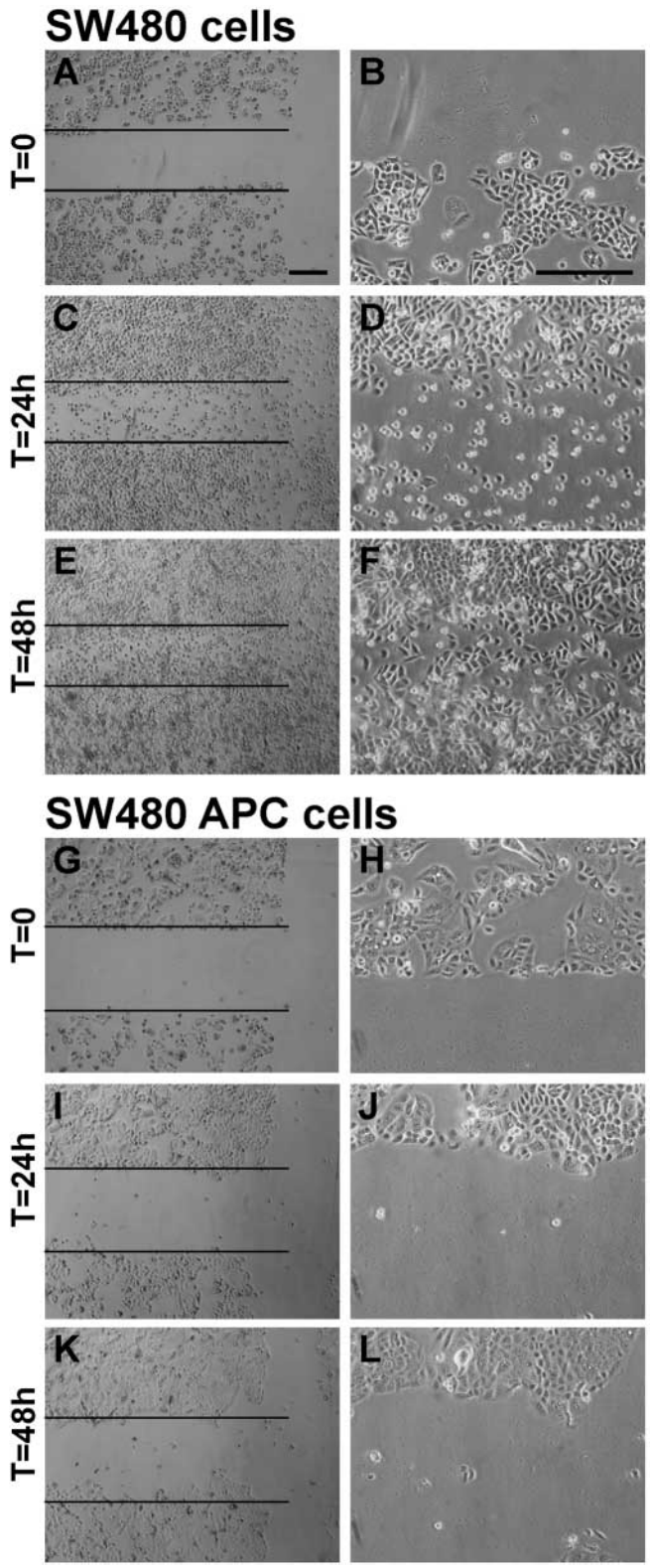


Fig. 7. SW480APC cells migrate differently to SW480 cells in a wound assay. SW480 (A-F) or SW480APC.15 (G-L) cells were grown as confluent monolayers on a plastic surface and a wound applied with a plastic pipette tip. Cells were photographed at 0, 24 and 48 hours after wounding at 4× (A,C,E,G,I,K) and 10× (B,D,F,H,J,L) magnification. The width of the wound at time (T)=0 is indicated by black lines on the 4× magnification micrographs. The data shown are representative of at least three independent experiments performed in triplicate. Bars, 50 μm.

treatment with EGTA (Fig. 6A,B,C, panels iii and vi). Expression of full-length APC in SW480 cells therefore results in translocation of E-cadherin and β-catenin to the plasma membrane, formation of Ca^{2+}-dependent adherens junctions and consequently tighter cell-cell adhesion. Clearly, there is a direct role for APC in the regulation of epithelial cell-cell adhesion.

Effect of full-length APC expression on cell adhesion and migration in wound assays

The changes in SW480APC cell adhesion led us to investigate the effect of full-length APC expression on cell migration. SW480 and SW480APC cells were grown to confluence on a plastic surface and a 'wound' applied with a plastic pipette tip. The ability of cells to migrate into the wound area was assessed over 48 hours. Phase-contrast micrographs of the wound area were taken at various times after formation of the wound at both low (4×) (Fig. 7, left-hand panels) and higher (10×) (Fig. 7, right-hand panels) magnification, to show the whole wound area and more detailed morphology of cells in the wound, respectively. By 24 hours, SW480 cells start to populate the wound and, after 48 hours, the wound is almost entirely occupied (Fig. 7A-F). Indeed, it often proved difficult to find the original wound area in the SW480 cells after 48 hours. At 24 hours, two populations of SW480 cells were evident: a refractile, less-adherent population, and an adherent population. The wound area contains a higher percentage of the refractile, less-adherent SW480 cells (Fig. 7C,D), which may reflect repopulation of the wound by the less-adherent SW480 cells that detach from the culture dish then resettle in the wound area. However, there is also evidence for active cell migration, as the width of the wound narrows significantly over the 48 hour time period (Fig. 7A,E). By contrast, the SW480APC cells do not significantly populate the wound area (Fig. 7G-L). There are consistently very few, if any, single cells in the wound, even after 48 hours. However, by 48 hours, the wound area narrows, suggesting active sheet migration of SW480APC cells at the edges of the wound area (Fig. 7, compare panels G and K). Similar results were observed in wound assays performed in the presence of mitomycin C, demonstrating that the different proliferation of the two cell types did not have an effect in the migration assay (not shown). The observations from the wound assays are consistent with the changes in morphology and adhesion of SW480APC cells, and suggest that the tighter cell-cell adhesion of SW480APC cells inhibits single cell, but not sheet, migration into the wound.

Discussion

Truncation of APC has been reported to be sufficient to initiate colorectal tumourigenesis (Kinzler et al., 1991). Several studies have suggested that this occurs via increases in total cellular levels of β-catenin, causing it to translocate to the nucleus where it acts as a co-transcriptional activator, thus

upregulating several growth-promoting genes (Barker et al., 2000; Mann et al., 1999). However, there is increasing evidence that dysregulation of β-catenin signalling may not be sufficient for initiation of colon tumour formation. Increased levels of β-catenin have been shown to lead to apoptosis unless p53 is also inactivated (Damalas et al., 1999). In addition, it has been shown recently that restoration of wild-type β-catenin in a colon cancer cell line that normally expresses a mutated, constitutively activated form, leads to changes in β-catenin localization rather then levels (Chan et al., 2002). There is also evidence that two of the major growth-promoting genes, c-myc and cyclin D1, reported to be transcriptionally upregulated by the β-catenin/Tcf/LEF complex, do not contribute directly to colon carcinogenesis (Wang et al., 2002; Wilding et al., 2002). In addition, mutations in the APC gene first manifest in vivo as abnormalities in colon crypt architecture rather than changes in the rate of cell proliferation or survival (Oshima et al., 1995). APC may therefore be involved in the regulation of epithelial cell shape, adhesion and/or migration, as well as in cell proliferation by upregulation of β-catenin levels and signalling.

Our success in reintroducing full-length APC in SW480 cells, a colon cancer cell line that normally expresses only truncated APC, provides evidence for a role for APC in the regulation of epithelial cell-cell adhesion. Several independent SW480APC clones were derived and shown to express wild-type epitope-tagged APC mRNA and protein stably by RT-PCR and western blotting, respectively. Ectopically expressed APC was functional as it recapitulated previously reported activities of APC in the regulation of β-catenin signalling. In cells expressing full-length APC, the levels of β-catenin in the nucleus, β-catenin/Tcf/LEF transcriptional signalling and cell proliferation are reduced.

Stably expressed mycAPC is able to influence cell adhesion. Full-length APC has been reported to be a crucial component of the complex that mediates the proteasomal degradation of β-catenin, and thus controls its total cellular levels. In fact, we found that β-catenin levels are not markedly reduced. Instead, β-catenin is translocated from the nucleus and cytoplasm to the plasma membrane. E-cadherin is also translocated to the cell membrane in SW480APC clones, where it forms functional adherens junctions with β-catenin. Junction formation leads to tighter cell-cell interactions and consequential changes in cell morphology, the actin cytoskeleton and cell migration. Previous studies have hinted that APC might modulate epithelial cell adhesion indirectly (Shoemaker et al., 1997), and one form of *Drosophila* APC is clearly involved in cell adhesion (Hamada and Bienz, 2002). However, our results demonstrate for the first time that restoring wild-type APC in a human colon cancer cell line, expressing a truncated form of APC, leads to the re-establishment of epithelial cell-cell contacts.

Although full-length APC has previously been inducibly expressed in the HT29 colon cancer cell line, where it was reported to inhibit cell growth owing to increased apoptosis (Morin et al., 1996), previous attempts to restore expression of full-length APC stably in colon cancer cells in which it is truncated, including SW480 cells, have not been successful (Groden et al., 1995). In these cases, over-expression of APC inhibits cell growth to such an extent that the cells undergo apoptosis (Morin et al., 1996). SW480APC cells do not show an increased frequency of apoptosis at any stage of the cell cycle (J.L.R. and M.C.F., unpublished); however, they proliferate substantially more slowly than SW480 cells. It is likely that the relatively low expression levels of mycAPC minimized its growth-suppressive effects and allowed us to isolate these clones. This hypothesis is supported by our difficulty in detecting full-length APC in SW480APC clones by western blot analysis. Furthermore, transient expression of pEF-mycAPC in SW480APC cells is able to decrease Tcf/LEF reporter activity further (M.C.F., unpublished), even in clones with little or no detectable nuclear β-catenin. Several SW480APC clones exhibit both nuclear and peripheral β-catenin staining, suggesting that mycAPC levels in these clones is even lower, and therefore not able to induce complete re-localization of β-catenin. Similarly, Morin et al. showed that growth inhibition of HT29-APC cells was not observed when APC expression was induced in suboptimal conditions, and that morphological changes in these cells correlated with higher levels of wild-type APC (Morin et al., 1996). Clearly, a balance needs to be achieved between the low levels of APC expression required to induce the observed changes in cell adhesion and the concentrations that suppress cell growth to a point where the cells are no longer viable.

Little is known about the regulation of the intracellular transport and subcellular localization of β-catenin and E-cadherin. Transient over-expression of plasma membrane localized E-cadherin (Orsulic et al., 1999; Stockinger et al., 2001) or cytoplasmically localized E-cadherin cytoplasmic domain (Simcha et al., 2001) have been shown to target endogenous β-catenin to the relevant subcellular compartment. Conversely, β-catenin binding to E-cadherin has been reported to promote its delivery from the ER to the basolateral membrane, allowing assembly of adherens junctions (Chen et al., 1999; Hinck et al., 1994; Jamora and Fuchs, 2002). APC has also been proposed to be involved in the regulation of the movement of β-catenin between the nucleus and the cytoplasm (Henderson, 2000; Rosin-Arbesfeld et al., 2000). It has been suggested that the primary role of APC in the cell is as a molecular shuttle, in complex with microtubules (Mimori-Kiyosue et al., 2000) and kinesin superfamily-associated protein 3 (Kap3), a member of a family of proteins that associate with kinesin motors (Jimbo et al., 2002). If this is the case, it is possible that the effect of APC on cell adhesion is due to the regulation of the transport of junctional proteins from intracellular sites to the plasma membrane.

It has been reported that E-cadherin levels are transcriptionally regulated by β-catenin (Novak et al., 1998). Indeed, total cellular levels of E-cadherin are increased in SW480APC cells. The expression of full-length APC also induced changes in the molecular nature of E-cadherin, raising its average pI and suggesting that regulation of E-cadherin localization by APC is at the post-translational level in addition to possible transcriptional regulation by β-catenin. It is possible that APC is involved in regulating the post-translational modification of E-cadherin, which in turn regulates its subcellular distribution and the formation of functional adherens junctions.

Despite the reported interactions of APC and microtubules, it is the actin cytoskeleton rather than the microtubule network (M.C.F., unpublished) that appears to be reorganized as a consequence of full-length APC expression in SW480 cells. The association of APC with the lateral plasma membrane in

polarized MDCK cells has been shown to depend on the actin cytoskeleton (Rosin-Arbesfeld et al., 2001), and this association is lost in cells that only express truncated APC, suggesting that truncated APC cannot maintain junctional β-catenin and cellular adhesion (Rosin-Arbesfeld et al., 2001).

The differences in migration between SW480APC and SW480 cells in the wound assay also seem to be primarily a consequence of changes in cell adhesion. Both SW480 and SW480APC epithelial cells migrate into the wound area at similar rates. However, SW480 cells detach and re-attach to the wound area more easily than SW480APC cells. It is likely that the SW480APC cells are restricted by cell-cell interactions in the epithelial sheet. APC and β-catenin have previously been suggested to play a role in the regulation of cell migration (Barth et al., 1997; Wong et al., 1998), and the subcellular localization of APC near the ends of microtubules at the edges of migrating cells has also implicated it in both cell migration and adhesion (Dikovskaya et al., 2001; Mogensen et al., 2002; Nathke et al., 1996; Pollack et al., 1997). APC has been observed to move continuously along microtubules and to concentrate at their distal ends, suggesting that APC may guide microtubule plus ends in migrating cells (Mimori-Kiyosue et al., 2000).

Reintroduction of full-length, functional APC also reduces the tumourigenic phenotype of SW480APC cells: they have a reduced proliferation rate, reduced ability to form colonies in soft agar and do not grow tumours in nude mice. Over-expression of APC in colon cancer cell lines, such as SW480 and HT29, has been reported to result in growth suppression (Morin et al., 1996; Shih et al., 2000), while over-expression of β-catenin has been shown to promote cell proliferation and colony growth in soft agar (Orford et al., 1999). Likewise, antisense oligonucleotides that downregulate levels of β-catenin inhibit cell proliferation and anchorage-independent growth of SW480 cells (Roh et al., 2001). As well as its effects on cell adhesion, expression of full-length APC therefore also affects cell proliferation and tumourigenicity, probably via β-catenin/Tcf/LEF signalling.

The manipulation of the levels of potent cell regulators, such as APC, is difficult to achieve. Over-expression is likely to lead to significant artefacts that will obscure the physiological role of these proteins. The SW480APC cells express only low levels of APC, but a powerful biological effect is still transmitted. The effects of expression of full-length APC in our study are related to cell-cell adhesion, possibly through the regulation of the intracellular transport of β-catenin and E-cadherin. These effects seem to occur at the post-translational level, and may therefore be independent of β-catenin transcriptional signalling. Nevertheless, SW480APC cells also exhibited changes, such as decreased proliferation rates and anchorage-independent growth, which can be attributed to β-catenin/Tcf/LEF signalling. It is perhaps unsurprising that truncation of APC can initiate tumourigenesis by interfering with more than one function of full-length APC. Defining the functions of APC apart from its ability to regulate total cellular levels of β-catenin, such as its role in protein transport and cell adhesion, will give a clearer picture of why it is such an important tumour suppressor in the colon.

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