

The Adenomatous Polyposis Coli Tumor Suppressor Protein Localizes to Plasma Membrane Sites Involved in Active Cell Migration

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Abstract. Mutations in the adenomatous polyposis coli (APC) gene are linked to polyp formation in familial and sporadic colon cancer, but the functions of the protein are not known. We show that APC protein localizes mainly to clusters of puncta near the ends of microtubules that extend into actively migrating regions of epithelial cell membranes. This subcellular distribution of APC protein requires microtubules, but not actin filaments. APC protein-containing membranes are actively involved in cell migration in response to wound-

ing epithelial monolayers, addition of the motogen hepatocyte growth factor, and during the formation of cell-cell contacts. In the intestine, APC protein levels increase at the crypt/villus boundary, where cell migration is crucial for enterocyte exit from the crypt and where cells accumulate during polyp formation that is linked to mutations in the microtubule-binding domain of APC protein. Together, these data indicate that APC protein has a role in directed cell migration.

THE adenomatous polyposis coli (APC)¹ tumor suppressor gene encodes a 310-kD cytoplasmic protein (6, 16). Mutations in APC constitute the genetic defect in the inherited colon cancer syndrome, familial polyposis (FAP), and represent an early event in a high percentage of sporadic colon cancers (reviewed in reference 26). These mutations also correlate with abnormalities in bone and retinal pigment epithelium development (Gardner's syndrome) (2), as well as the formation of brain tumors, most commonly of medulloblastoma (Turcot's syndrome) (8). To date, the function of APC protein is not known.

Mutations in APC protein lead to the accumulation of enterocytes near the crypt-villus transition in the intestine (26), indicating a possible defect in cell migration. Normally, enterocytes, which are derived from stem cells in the crypt, migrate up to the villus as a tight layer of epithelial cells. Thus, during normal enterocyte differentiation, there is a balance between cell-cell adhesion, cell-substratum adhesion, and cell migration. This balance may be affected as a consequence of mutations in APC protein.

Recently, a role for APC protein in cell adhesion was suggested by the discovery that APC protein forms com-

plexes with a family of cytosolic proteins termed catenins (29, 33). Catenins also bind to the cytoplasmic domain of cadherin cell adhesion molecules (25) and may be associated with other cellular processes, since they exist in cadherin-independent protein pools (11, 24). The protein complex between APC protein and catenins is similar to the cadherin-catenin complex. APC protein binds directly to either β -catenin or plakoglobin, and this interaction provides the link between APC protein and α -catenin (14, 30). α -Catenin is thought to interact directly with actin filaments and thus provides the link between catenin-protein complexes and the actin cytoskeleton (28). β -Catenin is a target of intracellular signaling pathways (7, 13, 17), and an increase in the phosphorylation of β -Catenin correlates with a decrease in cadherin-mediated cell adhesion (1). Significantly, when dominant negative forms of cadherin are introduced into normal, differentiating enterocytes, defects in cell adhesion and migration occur, and the normal structure and function of intestinal villi are disrupted (9, 10).

APC protein has also been shown to associate with microtubules. Transiently overexpressed APC protein colocalizes with microtubules in tissue culture cells (23, 32). This colocalization requires the COOH-terminal third of APC protein. In addition, APC protein fragments containing the COOH-terminal third of the molecule induce assembly and bundling of microtubules in vitro (23). Significantly, the COOH-terminal portion of APC protein is deleted in most of the mutations that are commonly found in FAP patients (26). This suggests that the ability of APC

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1. *Abbreviations used in this paper:* APC, adenomatous polyposis coli; FAP, familial polyposis; HGF, hepatocyte growth factor.

protein to interact with microtubules is crucial for its normal function.

We have determined the subcellular localization of endogenous APC protein in epithelial cells. APC protein was detected near the ends of microtubules that protrude into actively migrating membrane structures. APC protein localization was dependent on intact microtubules but not on the actin cytoskeleton. The localization of APC protein correlated with sites of active migration of epithelial cells into wounded monolayers, and the leading membrane of scattering cells treated with hepatocyte growth factor (HGF). In intestinal tissue, expression of APC protein increased at the crypt-villus boundary and was higher in differentiated enterocytes in the villus compared to cells in the crypt. Together, our results indicate that APC protein plays a role in microtubule-dependent cell migration in epithelial cells. In addition, the fact that catenins bind both APC protein and cadherins suggests that these protein complexes may be involved in regulating the balance between cell migration and cell-cell adhesion that is critical for normal enterocyte differentiation and function.

Materials and Methods

Antibodies and Reagents

Five antibodies against APC protein were generated in rabbits with the APC.2 protein fragment, which contains amino acid residue 1034-2130 of human APC protein, as described previously (29). Immune sera were affinity purified using the APC.2 fragment coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's instructions. A mouse mAb against APC protein, CC1, was the kind gift from Dr. David Hill (Oncogene Science, Manhasset, NY). Rabbit polyclonal antibodies against α -catenin, β -catenin, and plakoglobin were raised against the COOH-terminal 14 amino acids of either α -catenin, β -catenin, or plakoglobin. Polyclonal antibodies against α -catenin and plakoglobin were affinity-purified using immobilized cognate peptide. The characterization of these antibodies has been described previously (12). In addition, mouse mAbs were obtained against β -catenin (Transduction Laboratories, Lexington, KY) and plakoglobin (a kind gift from Pamela Cowin, New York University, New York). The DM1- α mAb against α -tubulin was provided by Arshad Desai (University of California, San Francisco, CA). mAb 3G8 against E-cadherin was kindly provided by Dr. Warren Gallin (University of Alberta, Alberta, Canada), and the monoclonal E-cadherin antibody DECMA-1 was obtained from Sigma Immunochemicals (St. Louis, MO). A polyclonal antibody against the cytoplasmic domain of E-cadherin was described previously (19). HGF was provided by Genentech (San Francisco, CA). Pefabloc was from Boehringer Mannheim (Indianapolis, IN), and all other protease inhibitors were from U.S. Biochemical Corp. (Cleveland, OH).

Tissue Culture and Cell Treatment

MDCK and IEC-6 cells were maintained in DME medium supplemented with 10% FCS (Gemini, Calabasas, CA). Cells were passaged by mild trypsinization. To disrupt microtubules, cells were incubated at 4°C for 30 min. The media was supplemented with 33 μ M nocodazole (Sigma), and cells were incubated at 4°C for an additional 30 min. Cells were then warmed and maintained at 37°C for 1 h in the continued presence of nocodazole. These conditions are sufficient to depolymerize >99% of microtubules in MDCK cells, as determined by Western blotting (data not shown). To stabilize microtubules, MDCK cells were incubated in DME/10% FCS supplemented with 12 μ M taxol (Molecular Probes, Inc., Eugene, OR) for 2 h. To disrupt the actin cytoskeleton, cells were treated with 2 μ M cytochalasin D (Sigma) in DME/10% FCS for 2 h. Cells treated with HGF were seeded at a density of 100,000 cells per tissue culture dish with a diameter of 10 cm and grown for 2 d to allow the formation of isolated colonies. To stimulate cell scattering with HGF, normal media was replaced with DME/5% FCS overnight, and then incubated in DMEM without serum for 1 h. To initiate cell scattering, medium was replaced

with DME containing 20 ng/ml HGF, 5% FCS. In all cases, control cells underwent the same treatment without the addition of drugs or growth factors. To wound epithelial monolayers, confluent monolayers of MDCK cells were scratched with either a 25-G syringe needle or a razor blade.

Immunofluorescence

MDCK cell were grown on collagen-covered coverslips in DME medium supplemented with 10% FCS. Cells stained with antibodies to tubulin were washed once quickly with TBS, 1 mM EGTA at room temperature, and then fixed in 100% methanol at -20°C for 10 min. Fixed cells were washed three times with TBS before incubation in blocking buffer containing TBS, 1% BSA, 5% donkey serum. Alternatively, cells were fixed with 2% *p*-formaldehyde, 0.01 M *m*-sodium periodate, 0.075 M lysine, 0.037 M Na-phosphate, pH 7.4 paraformaldehyde lysine periodate (PLP) (20) for 20 min at room temperature. These cells were permeabilized with 50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100 (CSK buffer) for 5 min and washed three times before incubation in blocking buffer containing TBS, 1% BSA, 5% donkey serum, 50 mM NH₄Cl for either 1 h at room temperature or overnight at 4°C. After removal of the blocking buffer, cells were incubated with primary antibodies in TBS, 5% donkey serum, 0.2% BSA for 1 h at room temperature or overnight at 4°C. After removal of the blocking buffer, cells were incubated with primary antibodies in TBS, 5% donkey serum, 0.2% BSA for 1 h at room temperature. The antibodies were used at the following dilutions: E-cadherin, 1:100; α -catenin, 1:100; β -catenin polyclonal, 1:100; β -catenin monoclonal, 1:400; APC, 1:100; and tubulin, 1:5,000. Primary antibody was removed by aspiration and cells were washed with TBS, 0.2% BSA three times for at least 5 min each time. Binding of primary antibodies was detected with fluorescein or rhodamine-conjugated donkey secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Secondary antibody was diluted 1:75 in TBS, 0.2% BSA, 5% donkey serum, and was allowed to bind for 30–60 min at room temperature. Actin filaments were visualized with rhodamine-conjugated phalloidin (Molecular Probes). After removing the secondary antibody, coverslips were washed three times in buffer containing TBS, 0.2% BSA and once with TBS. Coverslips were mounted in a mixture of 16.7% (wt/vol) Mowiol (Calbiochem, CA), 33% glycerol, 0.1% phenylenediamine (Sigma), or in Vectashield (Vector Laboratories, Burlingame, CA), and then viewed in an Axiophot inverted fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) using either a 40 \times or 63 \times oil immersion objective. Alternatively, cells were viewed using a Delta Vision™ full-spectrum optical sectioning microscope system (Applied Precision, Inc., Seattle, WA).

It has recently been suggested that members of the *armadillo* protein family, including β -catenin, plakoglobin, and APC protein, are present in the nucleus (5, 38). Some nuclear staining was observed with APC antibodies in MDCK cells (see Figs. 2 c and 4 A, d–f). However, the degree of staining varied with the fixation method. APC antibodies did not stain the nucleus in IEC-6 cells and an mAb against APC protein did not stain the nucleus of MDCK cells (data not shown). In light of these data, we regard the nuclear staining in our images as background reactivity.

Time Lapse Recording of Migrating Epithelial Cells and Retrospective Immunofluorescence

MDCK cells were seeded onto collagen-coated coverslips at a density of 200,000–400,000 cells per 3-cm dish. After cells had attached for 1–2 h, the coverslip was mounted in the stage of a custom-built laser scanning confocal microscope (4, 21). After equilibrating the temperature of the stage to 37°C for 15 min, 12 sites were selected and digital images of the selected sites were recorded every 2 min for a total of 1.5–3 h using a custom programmed x, y motorized stage controller. An autofocus mechanism was applied at each site before the image was collected. For nocodazole treatment, cells were recorded for 1 h, then medium was replaced with medium containing 33 μ M nocodazole and recording was resumed immediately. At the end of the recording, the coverslip was washed quickly in TBS, 1 mM EGTA at room temperature, fixed in 100% methanol at -20°C for 10 min, and then processed for immunofluorescence as described above, except that the secondary antibody used for the detection of tubulin was coupled to Cy-5. Stained cells were imaged with the same laser scanning confocal microscope used to record living cells. The final images were prepared by combining maximum intensity pixels from a series of confocal stacks 0.5 μ m apart. Stacked images were created using Metamorph Imaging System software (Universal Imaging Corporation, West Chester, PA).

Immunoprecipitation and Immunoblotting

MDCK cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 0.5% (vol/vol) NP-40 (Sigma), aprotinin (2 μ g/ml), leupeptin (2 μ g/ml), Pefabloc (1 μ g/ml), pepstatin (5 μ g/ml), TPCK (20 μ g/ml), α_2 -macroglobulin (12 U/liter) (MEBC buffer) for 10 min at 4°C. Insoluble proteins were removed by centrifugation at 12,000 g for 10 min. Lysates were precleared by incubation with 5 μ l of nonimmune rabbit serum plus 100 μ l Pansorbin (Calbiochem-Novabiochem Corp., La Jolla, CA) for 1 h at 4°C. After removing the Pansorbin by centrifugation, lysates were incubated for 2–3 h at 4°C with 60 μ l protein A-Sepharose (Pharmacia) to which immune serum had been prebound. Immunoprecipitates were washed four times with lysis buffer (MEBC) and boiled in 60 μ l of SDS-reducing sample buffer.

Total cell lysates were prepared by adding hot SDS-reducing sample buffer to MDCK cells on tissue culture dishes. Cells were collected by scraping and the lysate was boiled for 10 min. Insoluble material was removed by centrifugation at 12,000 g for 5 min.

Immunoprecipitates and cell lysates were separated by PAGE in 6% or 10–5% gradient polyacrylamide gels as indicated. Gels were transferred to nitrocellulose membranes with a pore size of 0.1 μ m (Schleicher & Schuell, Inc., Keene, NH) in a buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS, and 10% methanol. Nitrocellulose membranes were blocked for at least 2 h at room temperature in buffer containing TBS, 0.05% Tween-20 (T-TBS), 10% powdered milk, and 1% serum from the animal species that was used to generate the secondary antibody. Primary antibodies were incubated with the blots for 1 h at room temperature in T-TBS containing 0.2% BSA and 1% serum. Antibodies were used at the following dilutions: 3G8, 1:500; β -catenin monoclonal, 1:500; α -catenin polyclonal, 1:500; plakoglobin monoclonal supernatant, 1:5; and APC, 1:1000. After washing the membranes at least three times with T-TBS (10 mins each wash), appropriate secondary antibodies coupled to HRP (Amersham) were added at a dilution of 1:3,000 for 1 h. The membranes were washed for at least 2 h (more than five changes), and antibody reactivity was visualized with the ECL reagent (Amersham, Arlington Heights, IL).

Tissue Isolation and Preparation

C57BL/6J mice were killed and their entire intestinal tracts were removed. The intestinal tract was divided into small intestine, cecum, and colon. The tissue was washed briefly in cold PBS and fixed in PLP (see Immunofluorescence Methods) for 1 h. Tissue was then rinsed in PBS and cryoprotected in 2.5 M sucrose in PBS overnight at 4°C. Tissue was embedded in OCT (VWR) and flash frozen in liquid nitrogen. Sections (5- μ m) of small intestine and colon were prepared using a Reichart-Jung Frigocut 2800 N cryostat (Cambridge Instruments GmbH, Nussloch, Germany). After treating tissue sections with CSK buffer (see Immunofluorescence Methods) for 10 min, they were blocked in buffer containing TBS, 20% donkey serum, 0.2 mg/ml of each of donkey anti-rabbit, donkey anti-mouse, and donkey anti-rat antibodies, 1% BSA, 50 mM NH_4Cl for 2 h. The blocking solution was removed, and the sections were washed extensively in TBS buffer containing 0.2% BSA. Primary antibodies were added in TBS buffer containing 0.2% BSA, 20% donkey serum overnight at 4°C; 1 mM CaCl_2 was included in all solutions for staining with the DECMA-1 antibody. The tissue sections were washed extensively (three to four changes, 10 min each), and then incubated in buffer containing either donkey anti-rabbit, donkey anti-mouse, or donkey anti-rat antibodies conjugated to either FITC or rhodamine (Jackson ImmunoResearch Laboratories) for 2 h. Sections were then washed in TBS containing 0.2% BSA (three to four times, 5 min each) and mounted in Vectashield (Vector Laboratories).

Results

APC-Catenin Protein Complexes in MDCK Cells

To identify protein complexes containing endogenous APC protein and to analyze their subcellular distribution, we used MDCK cells and the intestinal epithelial cell line IEC-6. Cell adhesion and migration have been extensively characterized, and the dynamics of cadherin/catenin complex formation are well understood in MDCK cells (11, 24). Full-length APC protein was detected biochemically

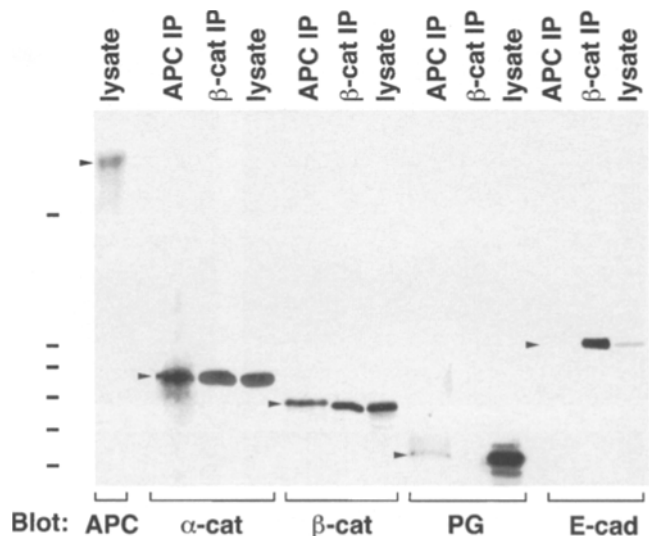


Figure 1. APC protein binds to catenins in MDCK cells. MDCK cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies to APC protein, α -catenin, β -catenin, plakoglobin, and E-cadherin as indicated. In addition, β -catenin and APC protein were immunoprecipitated from MDCK cell lysates (β -cat IP and APC IP) and probed with the same panel of antibodies as used in the whole-cell lysate. For the APC immunoprecipitation that was immunoblotted with α -catenin antibodies, six times as much lysate was used as that for the β -catenin immunoprecipitation. The migration of marker proteins with molecular masses of 210, 120, 110, 100, 90, and 80 kD is indicated on the left.

by immunoblotting MDCK cell lysate (Fig. 1). Association of catenins with APC protein was demonstrated by probing immunoprecipitates of APC protein with antibodies against either α -catenin, β -catenin, plakoglobin, or E-cadherin (Fig. 1). All catenins coimmunoprecipitated in a complex with APC protein. E-cadherin was not detected in APC immunoprecipitates (29, 33). For comparison, we also showed that E-cadherin and α -catenin, but not plakoglobin, coimmunoprecipitated with β -catenin. As a control, APC protein, catenins, and E-cadherin were also detected in whole-cell lysates (Fig. 1). These findings demonstrate that the APC-catenin protein complex is present in MDCK epithelial cells.

Localization of APC Protein in Epithelial Cells

MDCK and IEC-6 cells were immunostained with antibodies against E-cadherin, α -catenin, β -catenin, and APC protein (Fig. 2). α -Catenin (Fig. 2 a), E-cadherin (Fig. 2 b), and β -catenin (Fig. 2 d) were detected predominantly at plasma membrane sites involved in cell–cell contacts. In addition, there were substantial cytoplasmic pools of both α -catenin and β -catenin (see also reference 24). APC protein distribution was detected with affinity-purified antibodies. Two distributions of APC protein were found in MDCK cells. APC protein was distributed throughout the cytoplasm, and in clusters of puncta near the basal plasma membrane, often near the margin of protruding membrane structures (Fig. 2 c, arrowheads). The specific staining pattern of APC protein in clusters near the margin of protruding membranes was observed with five indepen-

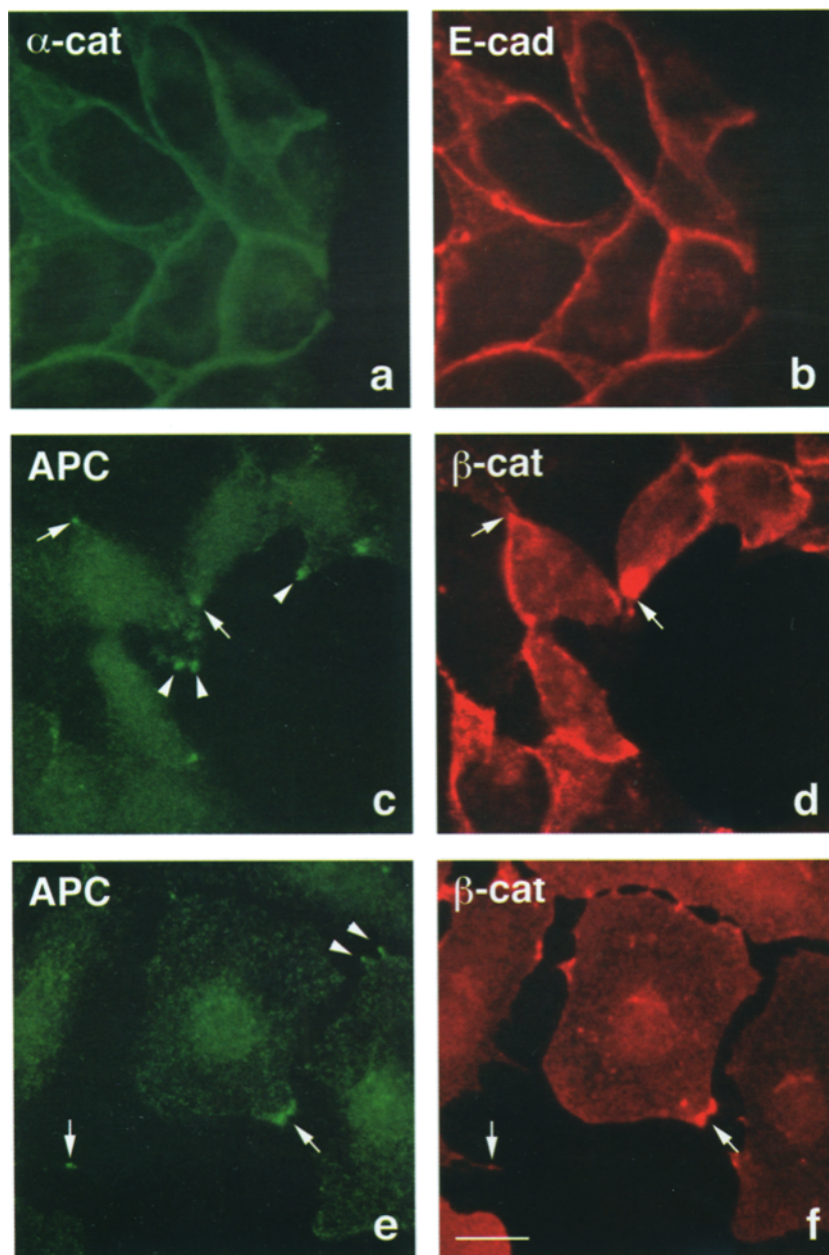


Figure 2. APC protein localizes to clusters of puncta near the plasma membrane in extending membranes of MDCK and IEC-6 cells. MDCK (*a-d*) cells were double stained with antibodies against α -catenin (*a*) and E-cadherin (*b*), APC protein (*c*) and β -catenin (*d*). IEC-6 cells were double stained with antibodies against APC protein (*e*) and β -catenin (*f*). Arrowheads in *c* and *e* point to clusters of puncta of APC protein, and arrows in *c-f* indicate areas of colocalization between β -catenin and APC protein. Bar, 23 μ m.

dently raised polyclonal antibodies and one mAb against APC protein (data not shown). In IEC-6 cells, which are derived from small intestine epithelium (27), APC protein was also present diffusely in the cytoplasm and in clusters of puncta near the cell margin (Fig. 2 *e*). The overall staining intensity for APC protein in IEC-6 cells was less than that in MDCK cells.

The localization of endogenous APC protein is different from that of transiently overexpressed APC protein, which decorated microtubules along their entire length (23, 32). This difference is likely caused by the large excess of the exogenously expressed APC protein in transiently transfected cells compared to the endogenous levels in these cells.

To determine whether the protruding membrane structures containing APC protein were altered by our fixation procedure, DIC images of cells before fixation were com-

pared to those after fixation and staining. Fig. 3 shows DIC images of cells before (Fig. 3, *a* and *e*) and after fixation (Fig. 3, *b* and *f*). The general morphology of the membrane structures containing APC protein was not altered by fixation. When an additional 30 MDCK cells were examined in this fashion, >85% of protruding membrane structures were found to be preserved. Superimposing the localization of APC protein (shown in Fig. 3, *c* and *g*) onto the DIC image of fixed cells revealed that APC protein localized close to the edge of membrane structures (Fig. 3, *e* and *h*).

Colocalization of β -catenin and APC protein was observed in MDCK cells and in IEC-6 cells. (Fig. 2, *c-f*, arrows). However, not every APC protein cluster was enriched with β -catenin. This indicates that the association between APC protein and β -catenin may be transient (see

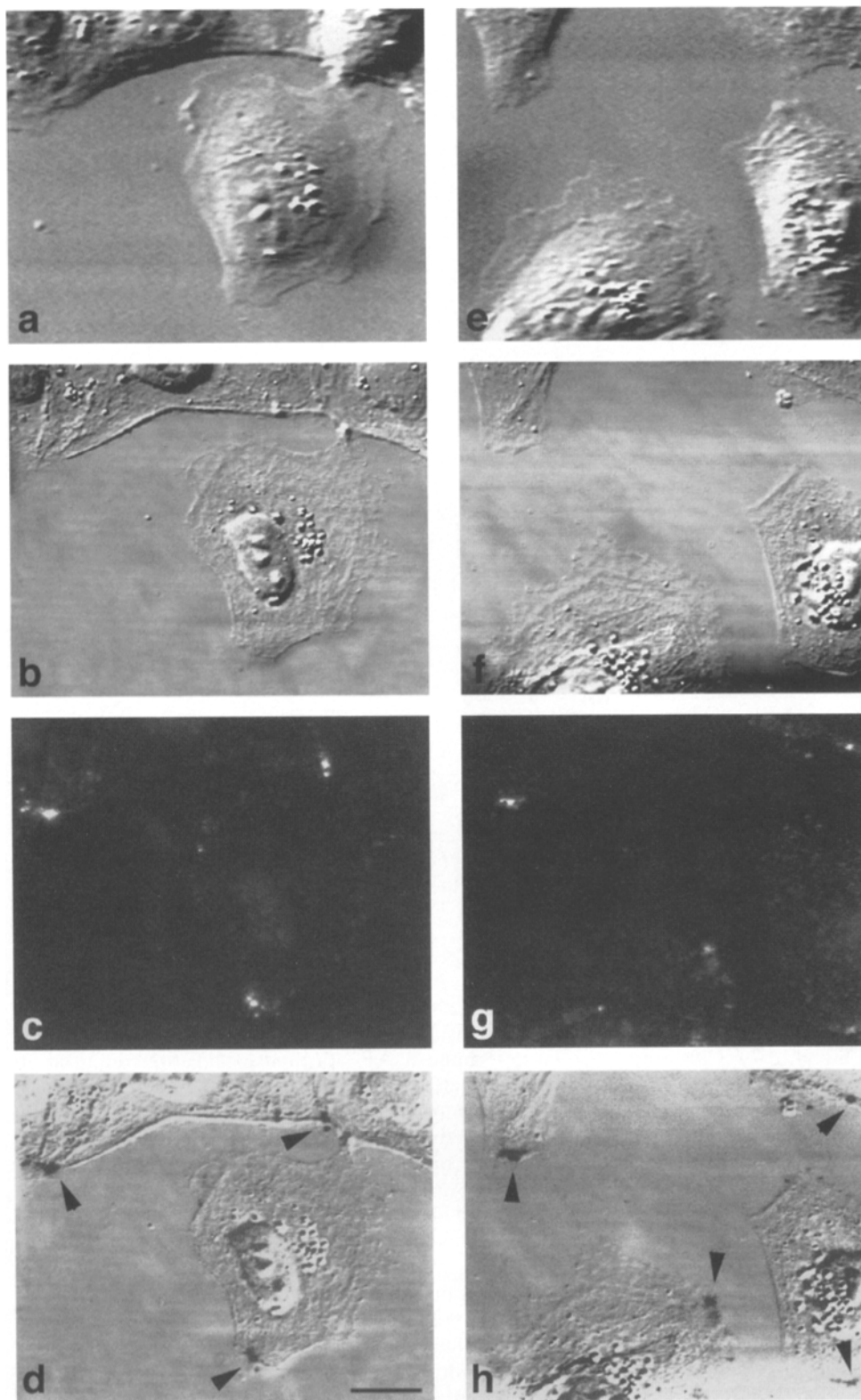


Figure 3. Membrane structures are preserved after fixation and staining, and APC protein is localized close to the plasma membrane. DIC images of MDCK cells were recorded using a scanning confocal microscope before (*a* and *e*), and after fixation and staining (*b* and *f*). Localization of APC protein was detected with affinity-purified antibody against APC protein and fluorescein-coupled secondary antibody (*c* and *g*). An overlap between the fixed DIC images and the APC protein localization is shown in black (also indicated by the arrowheads) in *d* and *h*.

Discussion). Alternatively, enrichment of β -catenin in APC protein clusters could be optically masked because of the abundance of β -catenin in the plasma membrane. It is also possible that the complexes between β -catenin and APC protein that can be detected biochemically are present in the cytoplasm.

Localization of APC Protein with Respect to the Cytoskeleton

APC protein colocalizes with microtubules when transiently overexpressed in tissue culture cells, and bundles microtubules in vitro (23, 32). To characterize the spatial

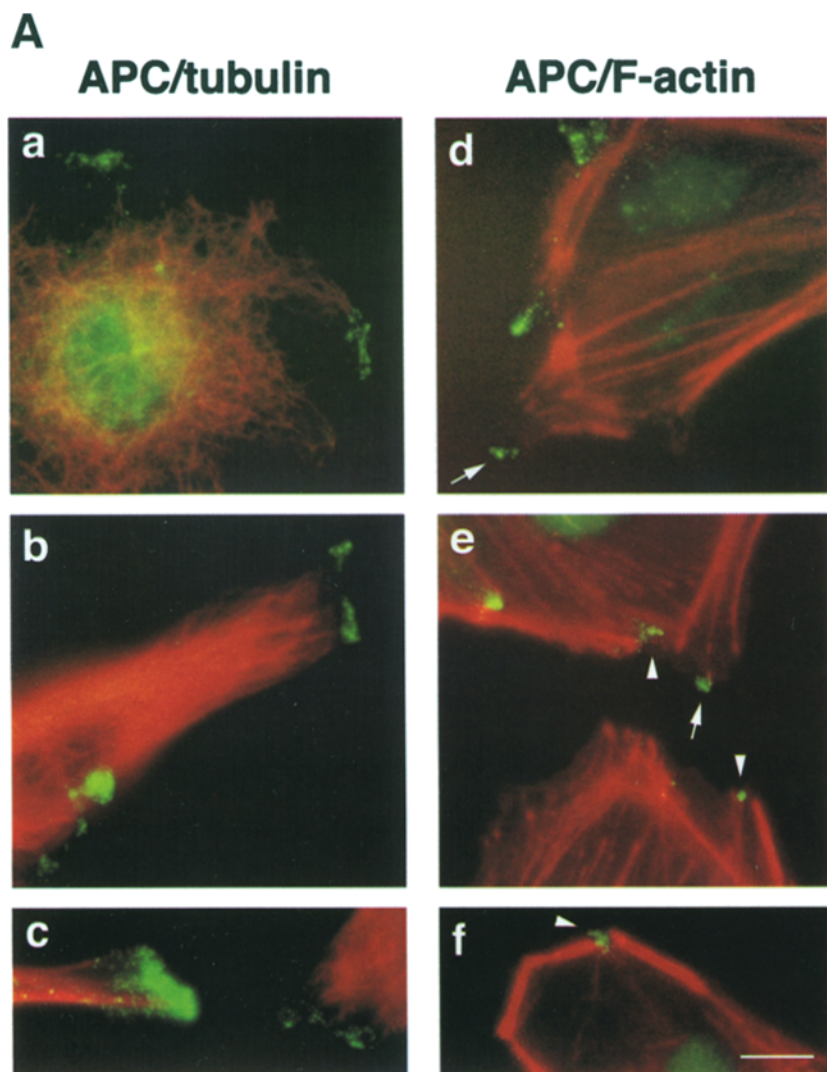


Figure 4. APC protein localizes near the ends of microtubules invading extending membrane structures. (A) MDCK cells were double stained with antibodies against APC protein (fluorescein) and tubulin (rhodamine) (a–c), or APC protein antibodies (fluorescein) and phalloidin (rhodamine) (d–f). Note the clusters of puncta of APC protein at the ends of microtubules that invade prominent membrane protrusions. Arrows in d–f indicate APC protein near the ends of actin filaments. Arrowheads in d–f indicate the localization of APC protein to sites near the cell membrane where the actin belt is interrupted. Bar, 23 μ m.

relationship between endogenous APC protein and microtubules, MDCK cells were double stained with antibodies against APC protein and tubulin (Fig. 4, a–c). Clustered puncta of APC protein localized specifically near the ends of microtubules at the margins of membranes not involved in cell–cell contact (Fig. 4 A, a–c). APC protein clusters also localized near the ends of some actin filaments (Fig. 4 A, d–f, arrows). In a few cases, APC protein was concentrated in areas where the prominent actin belt at the cell periphery was interrupted and short actin filaments were more abundant (Fig. 4 A, d–f, arrowheads). High resolution images of MDCK cells stained with phalloidin or antibodies against tubulin and antibodies against APC protein (Fig. 4 B, a–c) confirmed that APC protein localized near the ends of microtubules and, in general, not to the ends of actin filaments in lamellipodia (Fig. 4 B, d–f). In addition, actin was detected in many types of flatter membrane protrusions (e.g., ruffled edges, filopodia) that did not contain APC protein (see also Fig. 6 A). However, we cannot exclude the possibility that APC protein is also transiently associated with actin filaments when microtubules are also present. As shown in Fig. 4 B, d–f, APC protein localized to an area surrounded by a circular actin filament. It cannot be distinguished from these images whether the end of

a microtubule bundle extends into this area or if APC protein is actually associated with this actin filament.

To determine if APC protein localization required an intact cytoskeleton, we used nocodazole and cytochalasin D to specifically depolymerize microtubules and actin filaments, respectively. In cells treated with nocodazole, APC protein was diffusely distributed throughout the cytoplasm, and APC protein clusters were not present. This was observed in individual cells (data not shown) and cells growing in small colonies (Fig. 5, a and b). When cells were treated with the microtubule-stabilizing drug taxol, microtubules became bundled and appeared much shorter (Fig. 5 e). In the presence of taxol, clusters of APC protein were not detected, but staining for APC protein at the plasma membrane was more prominent (Fig. 5 e, arrowheads). Whether this plasma membrane staining is increased as a result of redistribution of APC protein clusters to the entire plasma membrane, or is simply more visible in the absence of the bright clusters containing APC protein in untreated cells, is not clear. In contrast to the effects of nocodazole and taxol, APC protein still localized to clusters at the ends of microtubules in retraction fibers that remained in cells treated with cytochalasin D (Fig. 5, c and d). Since APC protein staining appeared sim-

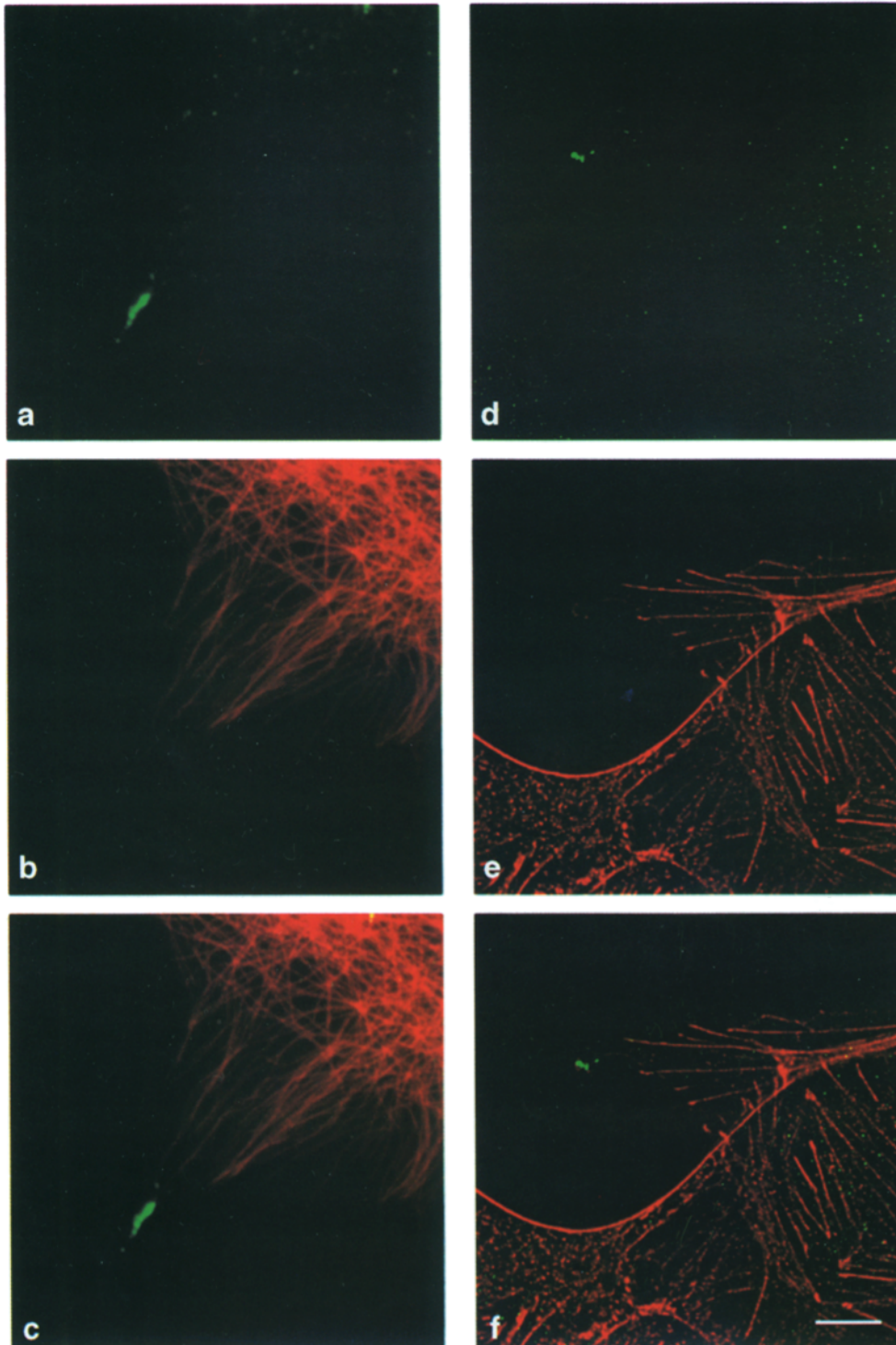
B**APC/tubulin****APC/F-actin**

Figure 4. (B) MDCK cells were double stained with phalloidin (*d*) and antibodies against APC protein (*a* and *d*) and tubulin (*b*). They were imaged with a Delta Vision™ full-spectrum optical sectioning microscope system. Overlay of the two staining patterns are shown in *c* (tubulin and APC) and *f* (phalloidin and APC). Bar, 5 μ m.

ilar in control cells and cells treated with cytochalasin D, we consider that the staining pattern in the latter cells is not an artifact of changes in either actin or cell organization. Thus, the localization of APC to clusters near the edges of membranes invaded by microtubules is maintained independently of actin filaments.

Localization of APC Protein in Migrating Cells

Colocalization of APC protein clusters and microtubule ends to margins of protruding membrane structures suggests that APC protein localization and cell migration may be connected. To test this hypothesis, APC protein localization was determined by retrospective immunofluores-

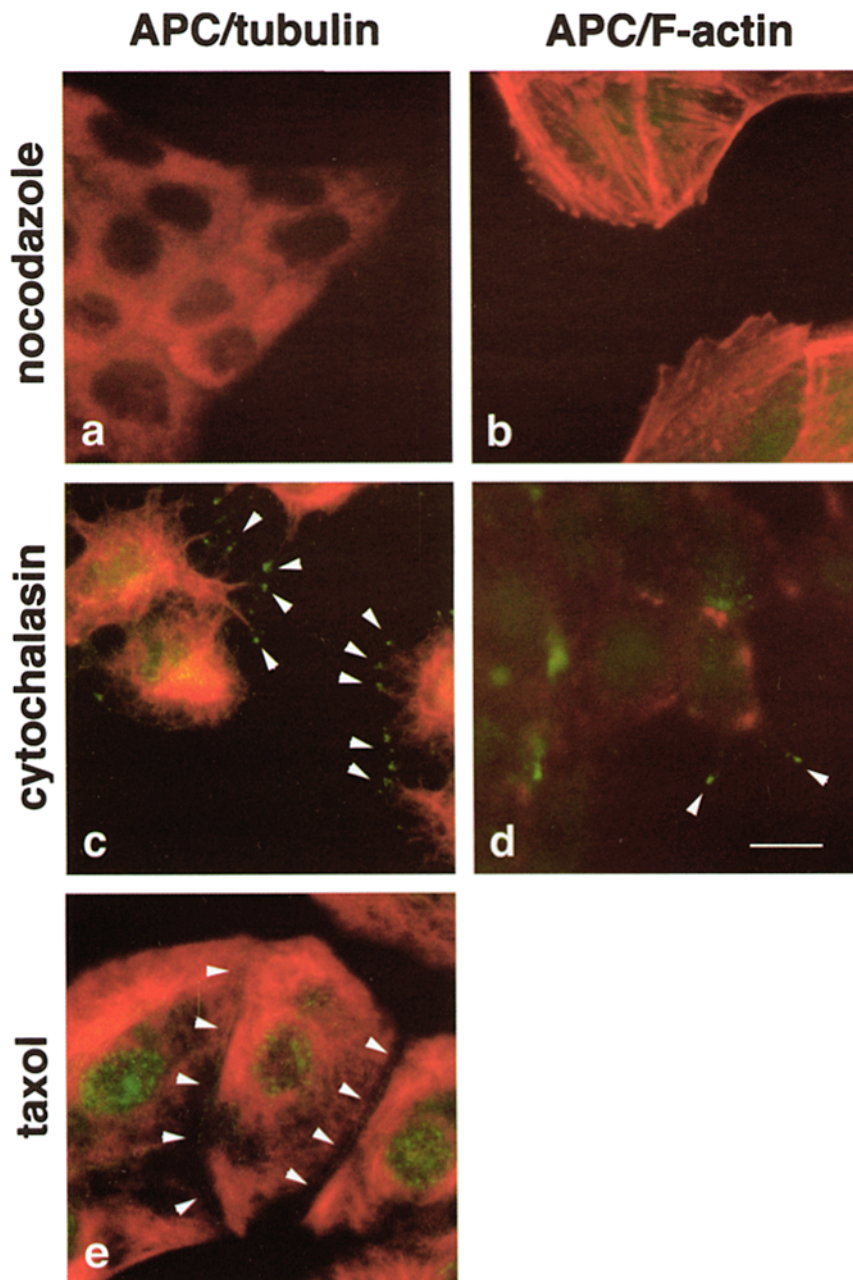
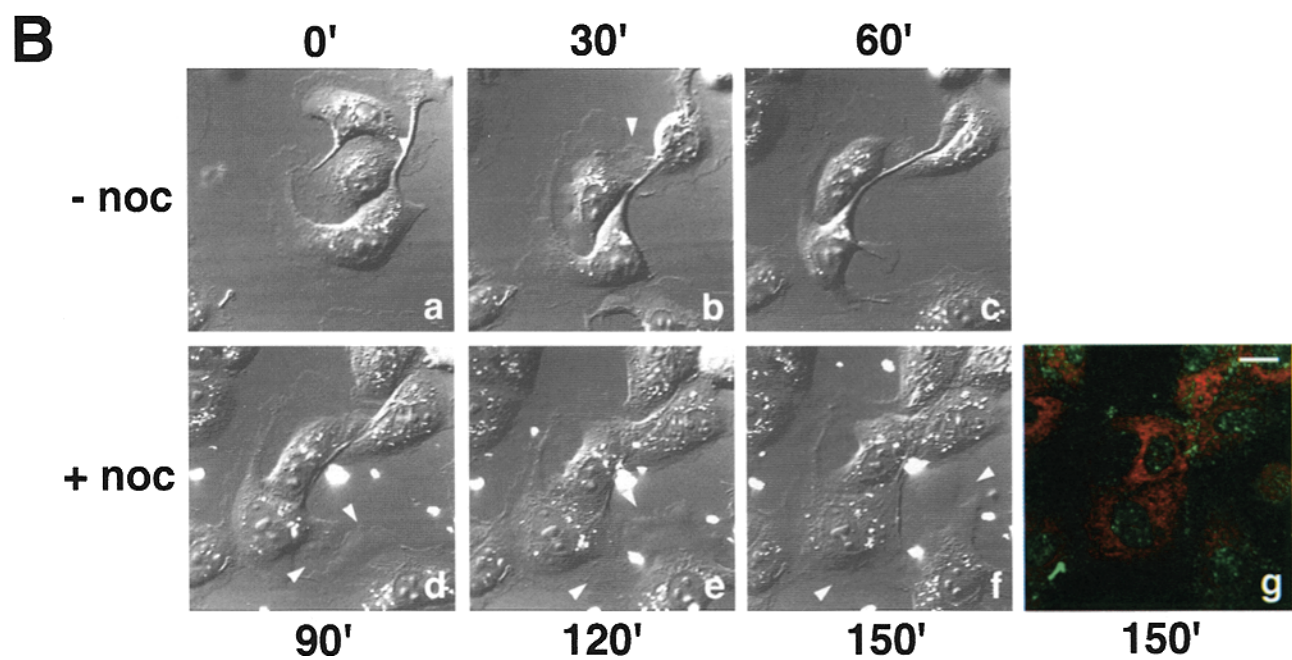
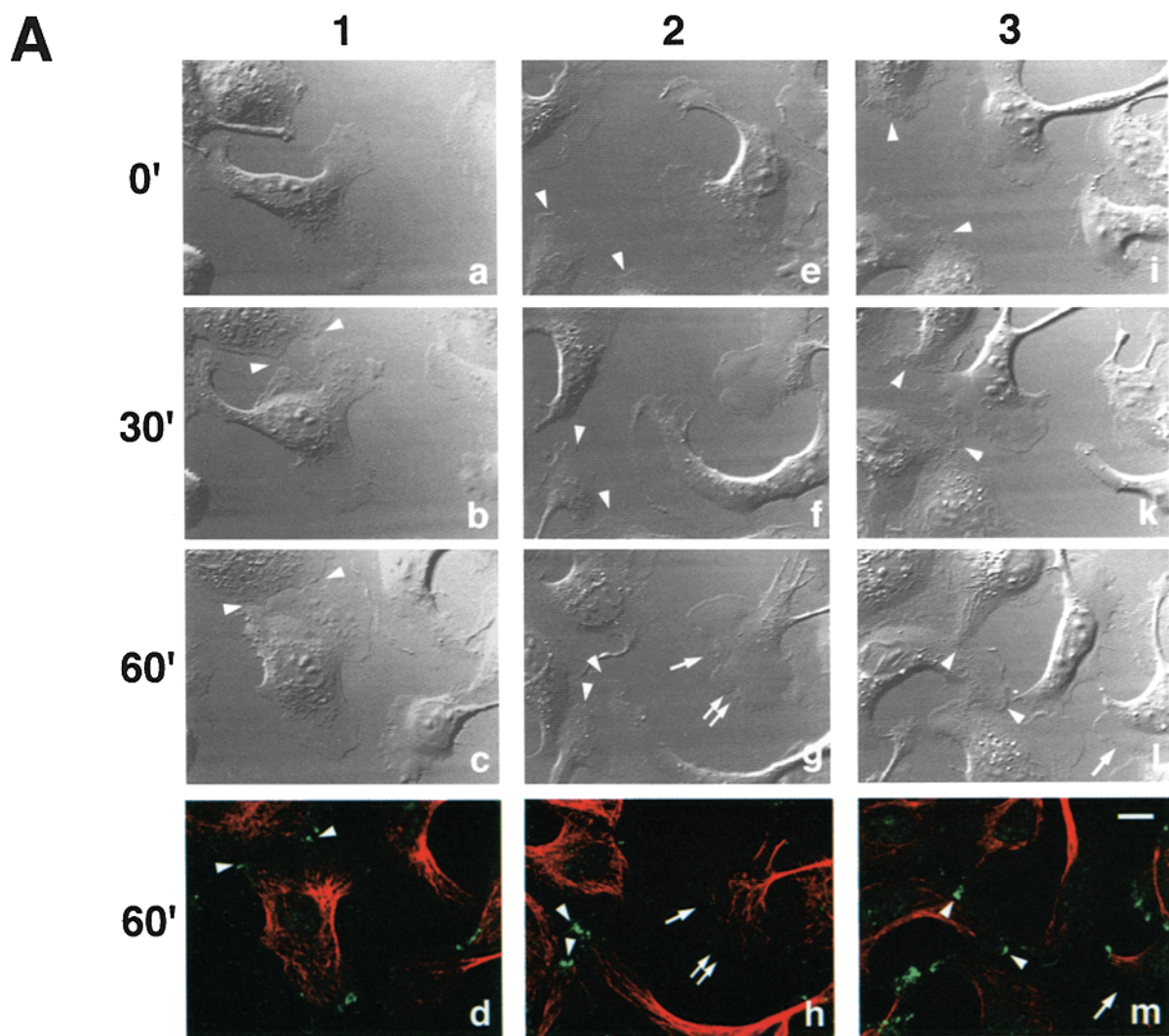


Figure 5. The distribution of APC protein is dependent on assembled microtubules but not assembled actin filaments. MDCK cells were treated with nocodazole (*a* and *b*), cytochalasin D (*c* and *d*), or taxol (*e*), and double stained with antibodies against APC protein (fluorescein) and tubulin (rhodamine) (*a*, *c*, and *e*), or APC protein antibody (fluorescein) and phalloidin (rhodamine) (*b* and *d*). Arrowheads in *c* and *d* indicate where APC protein remains in clusters near the ends of microtubules extending into the remaining retraction fibers after cytochalasin D treatment. Arrowheads in *e* indicate the localization of APC protein in the plasma membrane at sites of cell contact. Bar, 23 μm .

Figure 6. APC protein localizes to actively migrating, microtubule-dependent membrane protrusions in MDCK cells. (A) Living MDCK cells were recorded for 60 min using a scanning confocal microscope. (*a*, *e*, and *i*) DIC images of cells at the beginning of the recording. (*b*, *f*, and *k*) The corresponding cells 30 min later. (*c*, *g*, and *l*) The same cells after 60 min, at the end of the recording period. (*d*, *h*, and *m*) The same cells stained with antibodies against APC protein (fluorescein) and tubulin (rhodamine). Arrowheads in *e–m* indicate the migrating edges of cells where APC protein is concentrated. The arrowheads in *b–d* mark a cell contact that is formed during the recording. Note that APC protein is localized to membranes that are involved in “zippering up” the contact between adjacent cells, but is absent from the membranes that have made stable contact. Arrows in *g*, *h*, *l*, and *m* point to ruffling edges or filopodia that are devoid of APC protein. Bar, 10 μm . (B) Migration of living MDCK cells was recorded for 60 min and DIC images of one field are shown at 0, 30, and 60 min after the beginning of the recording. Arrowheads indicate migratory edges of cells. At this time the medium was replaced with medium containing 33 μM nocodazole. The cells were recorded for an additional 90 min, and DIC images of the same field of cells are shown 30 (*d*), 60 (*e*) and 90 (*f*) min after nocodazole was added. At the end of the recording, cells were fixed and double stained with antibodies against APC protein (fluorescein) and tubulin (rhodamine) (*g*). Cell motility is greatly reduced after nocodazole treatment and is limited to membrane ruffling and the extension and retraction of filopodia (e.g., arrowheads in *d–f*). Well-defined microtubules are not detected, and APC protein is present in a punctate pattern throughout the cytoplasm, but it is not detected in prominent membrane protrusions (*g*). The confocal slices of APC protein staining were restricted to the basal surface and superimposed on a stacked set of tubulin images. Bar, 10 μm .



cence (21) of migrating MDCK cells. Fig. 6 *A* shows three examples (1–3) of living cells at the beginning, during, and end of a time lapse recording of their migration, as well as the corresponding immunostaining of APC protein and tubulin in fixed cells at the end of the recording. In migrating cells, APC protein appeared to concentrate near the end of microtubules invading migrating membrane structures. We examined 72 APC protein-containing membrane structures, and found that 49 (79%) of these were involved in migration. Of the remaining APC protein-containing membrane structures, 2 (3%) localized to sites that were stationary and 11 (18%) were obscured so that their migration could not be distinguished. Note that regions of membranes that appeared flatter than the APC-containing membrane structures and exhibited dynamic, transient ruffling typical of purely actin-based motility contained few microtubules and little or no staining of APC protein (Fig. 6 *A*, *g*, *h*, *l*, and *m*, arrows).

Fig. 6 *A*, *b–d*, show cells that had established a cell–cell contact during the recording. This contact broadened between the cells during the time of the recording (Fig. 6 *A*, *b–d*, arrowheads). APC protein staining was not detectable within the boundary of the contact site. However, prominently stained clusters of APC protein were detected at the edges of contact, where the membranes of opposing cells were moving toward each other to extend the contact (Fig. 6 *A*, *d*, arrowheads).

To examine whether microtubules are important for MDCK cell migration, we treated living cells with nocodazole during the time lapse recording (Fig. 6 *B*). Although cells migrated rapidly before the addition of nocodazole (Fig. 6 *B*, *a–c*), they became almost stationary after the addition of nocodazole. However, membrane ruffling, filopodia, and pseudopodia extensions, which were likely generated by actin polymerization, were still observed (Fig. 6 *B*, *d–f*, arrowheads). These data are consistent with a recent report describing the requirement of intact microtubule cytoskeleton for cell migration (18). At the end of the recording, immunofluorescence microscopy of these nocodazole-treated cells showed that APC protein was localized throughout the cytoplasm as fine puncta and diffuse staining, but not in clusters at specific sites near cell margins (Fig. 6 *B*, *g*) of the remaining actin-based membrane protrusions. Note that APC protein staining is more punctate in these nocodazole-treated cells than those shown in Fig. 5. This probably results from the fact that the living cells were only subjected to nocodazole-containing media for a total of 1 h, and their microtubules were not completely depolymerized by pretreatment at 4°C. Nevertheless, nocodazole treatment of living cells decreased migration, disrupted APC protein localization, and inhibited formation of APC and microtubule-containing membrane structures.

APC Protein Localization to Migrating Edges of Cells Closing an Epithelial Wound

To further investigate the correlation between APC protein localization and cell migration, we examined the distribution of APC protein in cells adjacent to a wound in an epithelial monolayer. MDCK cells were grown to confluence on coverslips, and lines were cut in the monolayer

with either a syringe needle or razor blade to create a wound. Immediately after wound formation, APC protein staining was diffuse in cells within the intact monolayer and in cells directly adjacent to the wound (Fig. 7 *A*, *a*). After 2 h, cells had begun to migrate from the edges of the monolayer into the wound (Fig. 7 *A*, *b*). In these cells, clusters of APC protein localized to distinctive membrane protrusions containing microtubules. In addition, the staining intensity of APC protein was stronger in cells near the edge of the wound compared to that of APC protein staining in cells three to five cell widths from the edge of the wound (Fig. 7 *A*, *b*). This suggests that APC protein expression was upregulated or that APC protein redistributed from a diffusely localized cytoplasmic pool to clusters near the basal membrane in response to increased cell migration (see below).

APC Protein Expression and Localization in MDCK Cells Treated with HGF

HGF induces epithelial cells to flatten and scatter, resulting in the dispersal of tightly associated colonies of cells (37, 39). We examined whether the distribution of APC protein changes in response to MDCK cell migration induced by HGF. Within 1 h of HGF treatment, cells at the membrane edges of most colonies became ruffled and some contained APC protein at their margins (Fig. 7 *B*, *a* and *b*, arrowheads). These membranes contained interrupted actin structures, but a correlation between the distribution of actin filaments in these structures and the localization of APC protein clusters was not detected. As illustrated in Fig. 7 *B*, *c* and *d*, APC protein localized near the ends of microtubules extending into the leading margin of cells that were actively migrating away from the colony.

Unlike the epithelial wounding experiment in which only a few cells close to the site of the wound respond, HGF treatment results in a synchronous migratory response in all cells. Thus, we could examine whether induction of cell migration affected APC protein levels. When equal amounts of protein from lysates of control or HGF-treated cells were immunoblotted with APC antibodies, there was approximately twice as much APC protein in the HGF-treated cells compared to the untreated cells (Fig. 8). The amount of β -catenin that could be coimmunoprecipitated with APC protein from HGF-treated cells also increased approximately twofold (Fig. 8, indicating that the additional APC protein associates with β -catenin, at least transiently (see Discussion). The overall expression levels of β -catenin or tubulin did not change significantly after HGF treatment (Fig. 8).

Localization of APC Protein and Catenins in Intestinal Epithelia In Situ

In intestinal epithelia, proliferative stem cells in the crypts give rise to differentiating enterocytes that migrate out of the crypt and up adjacent villi. Mutations in APC protein lead to the formation of numerous intestinal polyps that represent an extension of the proliferative zone of the crypt (15); a schematic diagram of a normal intestinal villus and the adjacent crypt is shown in Fig. 9 *a*. Tissue sections of normal mouse small intestine were immunostained with antibodies against either APC protein (Fig. 9, *o–q*),

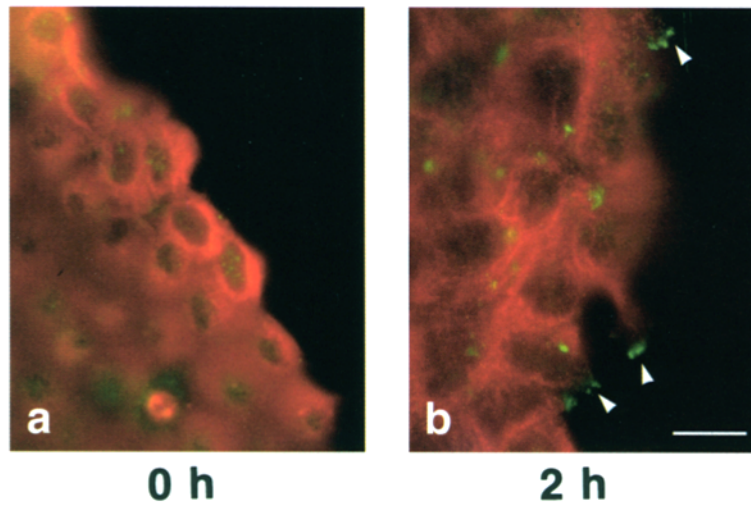
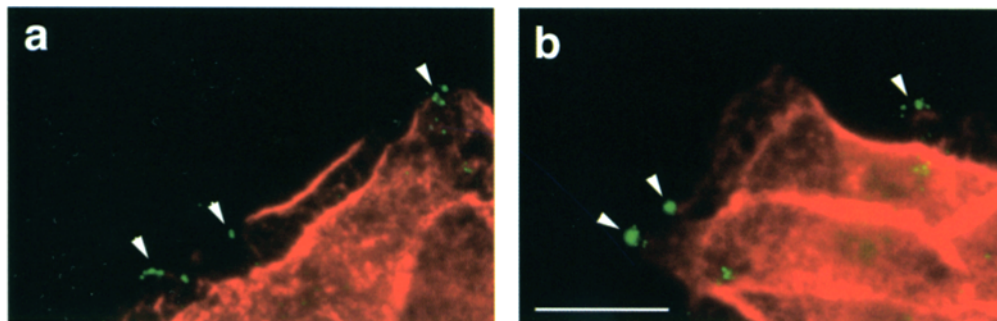
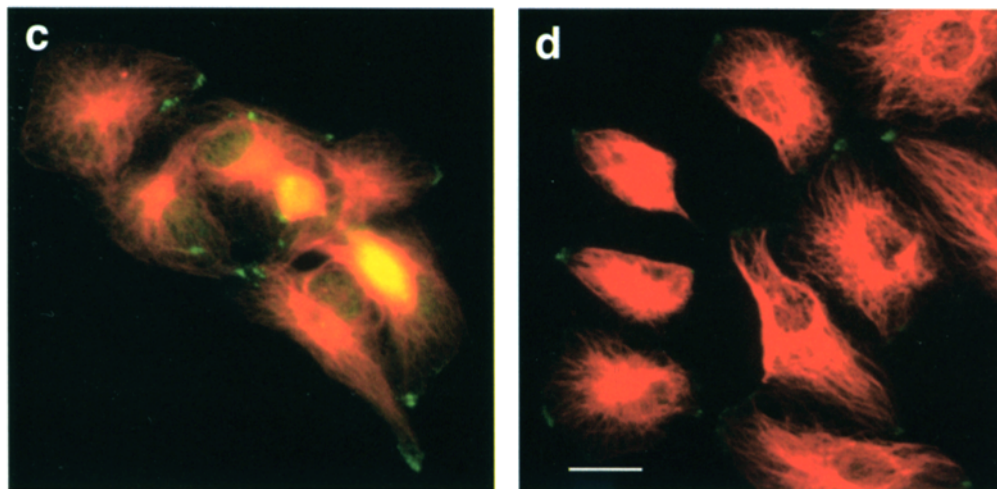
A**B****APC/
F-actin****APC/
tubulin**

Figure 7. APC protein localizes to actively migrating membranes in MDCK cells migrating into a wound or in response to HGF. (A) Confluent monolayers of MDCK cells were scratched with a syringe needle. Cells were fixed immediately after introducing the wound (a) or 2 h later (b), and double stained with antibodies against APC protein (fluorescein) and tubulin (rhodamine). Arrowheads in b indicate the localization of APC protein at tips of microtubules invading membrane protrusions that are migrating into the wound. Bar, 23 μ m. (B) MDCK cells were treated with HGF and fixed 1 h (panels a and b) or 6 h later (c and d), and then double stained with APC protein antibody (fluorescein) and phalloidin (rhodamine) (a and b), or antibodies against APC protein (fluorescein) and tubulin (rhodamine) (c and d). Arrowheads in a and b indicate small puncta of APC protein near the plasma membrane of cells that had started to spread at the edge of the colony. Bar, 23 μ m.

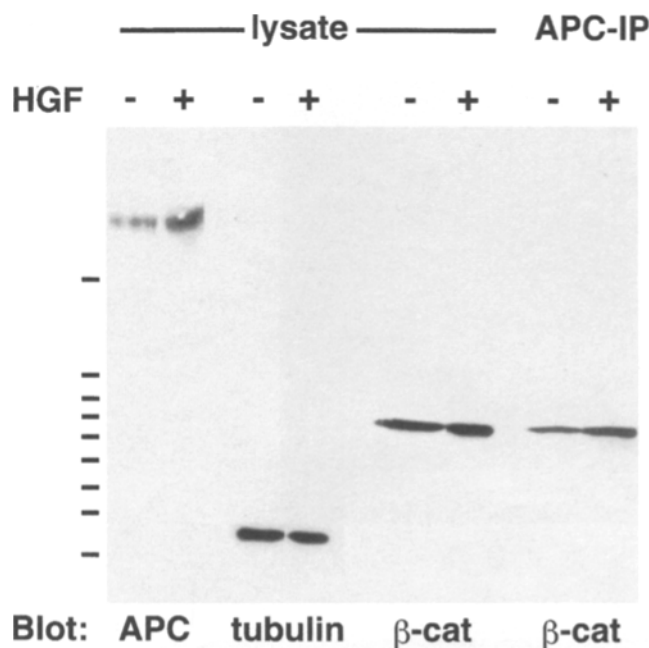


Figure 8. Expression level of APC protein increases in response to HGF treatment. MDCK cell lysates were prepared 24 h after treatment with HGF (*HGF*⁺). As a control, lysates were prepared from cells that had not received HGF (*HGF*⁻). Equal amounts of proteins were separated in 5–10% gradient SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were probed with antibodies against APC protein, tubulin, or β -catenin as indicated. In addition, APC protein was immunoprecipitated from the two different cell lysates containing equal amounts of protein (*APC IP*), and the immunoprecipitate was probed for the presence of β -catenin. The migration of marker proteins with molecular masses of 210, 120, 110, 100, 90, 80, 70, 60, 50, and 40 kD is indicated on the left.

α -catenin (Fig. 9, *b–d*), β -catenin (Fig. 9, *h, i*, and *k*), plakoglobin (Fig. 9, *l–n*), or E-cadherin (Fig. 9, *e–g*). The position of crypts in the stained sections is outlined by arrowheads (Fig. 9, *b, e, h, l*, and *o*). Higher magnification images of villus and crypt regions are shown in Fig. 9, *c, f, i, m*, and *p*, and *d, g, k, n*, and *q*, respectively. Staining with secondary antibody alone gave a faint, uniform, diffuse staining pattern; neither individual epithelial cells nor their plasma membranes could be distinguished (data not shown). E-cadherin and α -catenin localized predominantly to the basal and lateral membranes of intestinal enterocytes in both the crypt and villus; similar staining intensities were detected along the crypt-villus axis (Fig. 9, *b–g*). Some α -catenin was present in the cytoplasm, similar to the distribution of α -catenin in epithelial cells grown in culture (see Fig. 2 *a*).

In contrast to E-cadherin and α -catenin, the staining intensities of APC protein, β -catenin, and plakoglobin were not uniform along the crypt-villus axis. The intensity of staining of these proteins was stronger in enterocytes in the villus than in cells in the crypt (Fig. 9, *h–q*). Although this difference in intensity of APC protein staining between the crypt and the villus is not as striking as that for plakoglobin, direct comparison of the uniform staining of E-cadherin in the crypt-villus axis (Fig. 9, *e–g*) and staining

of APC protein reveals the gradient of APC protein expression. In the crypt, some staining for APC protein was present on the lateral membrane (Fig. 9 *q*). In the villus, however, APC protein was detected throughout the cytoplasm as well as at the plasma membrane (Fig. 9 *p*). The diffuse cytoplasmic distribution of APC protein in villus enterocytes is similar to that observed in MDCK and IEC-6 cells. However, clusters of puncta of APC protein were not detected in the basal membrane of enterocytes. APC protein clusters may be obscured because of the orientation of the tissue in the sections, and the thickness of the basal membrane viewed in the sections compared to the flat projections and clearly identifiable membrane protrusion found in cells in tissue culture. Alternatively, membrane protrusions may not have been maintained during tissue preparation. β -Catenin was detected most strongly at the lateral and basal membranes of enterocytes (Fig. 9, *h–k*); however, some cytoplasmic staining was also observed in enterocytes of the villus (Fig. 9, *h* and *i*). This additional cytoplasmic staining contributed to the increased staining intensity of β -catenin of cells in the villus compared to those in the crypt (Fig. 9 *i*). Plakoglobin was detected almost exclusively in the villus, where it was detected in the cytoplasm of enterocytes and also in the basal and lateral membranes of these cells (Fig. 9, *l* and *m*). In the intestinal crypt, the small amount of plakoglobin that was detected was present in a patchy staining pattern at the basal and lateral membranes (Fig. 9 *n*).

In summary, α -catenin and E-cadherin appear to be present in similar amounts in cells in intestinal crypts and villi, whereas APC protein, β -catenin, and plakoglobin are expressed in higher levels in enterocytes in the villus than cells in the crypt.

Discussion

This is the first study to specifically localize endogenous APC protein at the subcellular level in a distribution consistent with its known interactions with microtubules, catenins, and cell adhesion structures. We used a number of independent experimental approaches to test the significance of APC protein distribution in cell organization, movement, and adhesion. Although the study is at a descriptive stage, our localization of APC protein provides a new foundation for investigating its function in cell adhesion and migration.

In this study, we have established that endogenous APC protein localizes to the margins of membrane structures in epithelial cells that are actively migrating over the substratum. Furthermore, the clusters of puncta of APC protein localize near the ends of microtubules that specifically invade these membranes. Maintenance of this APC protein distribution is dependent on intact microtubules. In contrast, APC protein distribution was not dependent on actin filaments and was not found consistently in actin-enriched membranes (e.g., ruffling edges) and transient filopodia. Based on this data, we suggest that one role for APC may be in regulating directed cell migration.

Our finding that APC protein localizes to the margins of membrane protrusions containing microtubule bundles raises two issues: (*a*) Is the localization of APC protein and morphology of these membranes altered by fixation?

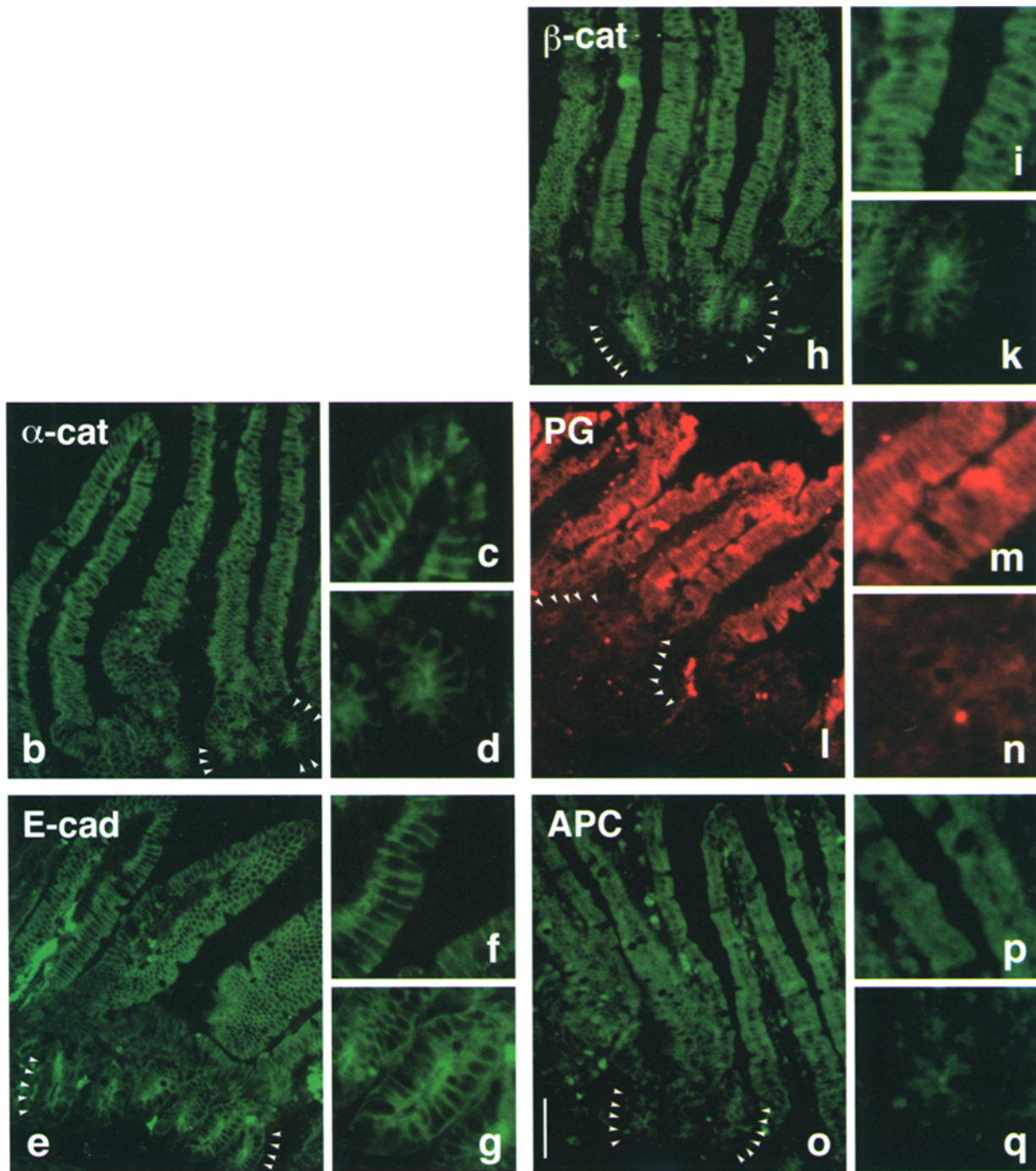


Figure 9. Distribution of catenins, cadherin, and APC protein in intestinal tissue. Tissue sections of mouse small intestine were stained with antibodies against α -catenin (*b–d*), E-cadherin (*e–g*), β -catenin (*h–k*), plakoglobin (*l–n*), or APC protein (*o–q*). Arrowheads in *b*, *e*, *h*, *l*, and *o* outline the right and left margins of the crypts present in the sections. (*a*) A schematic diagram of the crypt–villus axis. Higher magnification images of villus and crypt regions stained with the same panel of antibodies are shown in *c*, *f*, *i*, *m*, and *p*, and *d*, *g*, *k*, *n*, and *q*, respectively. These latter images are magnified approximately twofold over those in *b*, *e*, *h*, *l*, and *o*. Bar in *o*, 40 μ m.

and (*b*) Are these APC/microtubule-containing membrane protrusions related to other well-defined actin-containing membranes structures (i.e., lamellipodia)? Many types of fine membrane structures at the margins of cells are subject to disruption during standard fixation protocols used before immunofluorescence. However, direct comparison of DIC images of living cells and the same

cells after fixation and antibody staining demonstrates that, at the level of the light microscope, these membrane structures are very similar (Figs. 3 and 6). Although we cannot exclude the possibility that there is some disruption of the fine structure of membrane margins after fixation, their overall morphology, dimensions, and distribution remain intact. Therefore, we conclude that the observed dis-

tributions of APC protein and microtubules represents structures that exist in living cells. Our comparative analysis of distributions of APC protein, microtubules, and actin filaments indicates a stronger correlation between APC protein and microtubule distributions than between APC protein and actin filament distributions. Although we cannot exclude the possibility that some fine actin filaments colocalize with APC-protein, the principal actin structures associated with well-defined membrane protrusions (i.e., lamellipodia) do not appear to directly associate with APC protein clusters. Finally, the divergent effects of nocodazole and cytochalasin D on APC protein (Fig. 5) indicate that microtubules, rather than actin integrity, are required for APC protein localization.

The specific subcellular localization of APC protein in extending membrane structures, and the correlation between the presence at these structures and cell migration, raises the question of how APC protein distribution is determined. The localization of APC protein clusters in the basal membrane near the margins of membrane structures indicates that APC protein may interact, directly or indirectly, with the membrane through integral membrane proteins, such as integrins, that are involved in cell binding to the extracellular matrix. By immunofluorescence microscopy, however, we were not able to distinguish between a direct association of integrins with APC protein, or the simple overlap of staining for APC protein and integrins because of their abundance and uniform staining in the basal membrane (data not shown). In addition, the staining pattern of vinculin, which is associated with integrin-mediated "focal adhesions" (3), did not significantly overlap that of APC protein (data not shown). At present time, we cannot exclude the possibility that APC protein associates with a subset of integrins in actively migrating membrane structures that are distinct from stable sites of integrin-mediated focal adhesions that contain actin stress fibers. Potentially, the actin cytoskeleton can bind to APC protein indirectly through catenins. APC protein binds β -catenin, and β -catenin binds α -catenin which, in turn, interacts with actin filaments (Fig. 1; see also references 14, 28, 30). However, we note that colocalization of β -catenin and APC protein was not obligate (see Fig. 2, *c* and *d*), and cytochalasin D did not disrupt clusters of APC protein. Thus, we suggest that any interaction between APC protein, catenins, and the actin cytoskeleton is likely to be transient; however, this interaction may be important in regulating APC protein function in migratory protrusions.

A distinctive feature of the APC protein clusters near the margins of membrane extensions was their proximity to the ends of microtubules (Fig. 4). Formation of these structures (Fig. 6), APC protein localization (Figs. 4–6), and cell migration (Fig. 6) were dependent on an intact microtubule cytoskeleton. It has been shown previously that APC protein induces assembly and bundling of microtubules (23). It is possible that this property of APC protein is important at the migrating edge of a cell where microtubules may be required to stabilize membrane protrusions that are involved in directed cell migration. In neurons, for example, dynamic instability of microtubules is important for exploratory activities of neuronal growth cones, and selective stabilization of microtubules is involved in stabilizing new directions of growth cone migration after a

transient association of the growth cone with guidepost cells (31, 34, 35). In this context, it is significant that preliminary studies show that APC protein specifically localizes to the tips of growth cones in neuronal cells *in vitro* (Näthke, I.S., unpublished observations). We suggest that APC protein might be part of a protein complex involved in sensing extracellular signals that determine the direction of migration, thereby acting as a cellular "nose." Subsequently, APC protein could recruit microtubules to extending membrane structures by nucleating their assembly and bundling, and thus stabilize the direction of cell migration. Alternatively, APC protein might be transported by specific motor proteins to the plus ends of microtubules involved in cell migration. In taxol-treated cells, however, we found that APC protein was not present in clusters near the ends of microtubules or in extending membrane structures, but was detected on the lateral membrane and diffusely distributed in the cytoplasm. Since taxol does not interfere with the association between microtubules and motor proteins (36), motor proteins may not be involved in transporting APC protein to the ends of microtubules. Note that we also detected APC protein staining diffusely throughout the cytoplasm. Although the significance of cytoplasmic APC protein is not clear in the context of cell migration, it is possible that it provides a pool of protein that can be rapidly recruited to the membrane in response to either extracellular or intracellular signals.

In addition to regulating cell migration, APC protein could affect cell–cell adhesion by competing with cadherins for binding to β -catenin (14). The level of cadherin protein expression in all cells examined greatly exceeds that of APC protein. Therefore, a direct competition for β -catenin is not likely to contribute to the overall regulation of cadherin-mediated cell–cell adhesion by APC protein. Overexpression of APC protein leads to increased turnover of β -catenin, however, indicating that APC protein can regulate the level of β -catenin (22). It is possible that in areas near membranes that contain high levels of APC protein, such as the clusters described here, APC protein could locally compete with cadherin for β -catenin and increase β -catenin turnover, thereby decreasing β -catenin availability to cadherin. Locally, this might loosen adhesive cell–cell junctions, thereby allowing cells to migrate past each other. Note that we detected APC protein clusters at the outer boundary of cell–cell contacts, where membranes are moving past each other (Figs. 4 and 6 *A*). The balance between cell migration and cell–cell adhesion has to be particularly dynamic at these sites, and APC protein may contribute to the regulation of this balance.

Dynamic coupling of cell–cell adhesion and cell migration is particularly important in the gut, where a monolayer of epithelial cells constantly maintains a barrier while migrating up the villus. Cadherins and APC protein may play important roles in regulating these events. Expression of a dominant negative form of cadherin specifically in the villus enterocytes of the intestinal epithelium led to an increased rate of migration of enterocytes, decreased cell–cell adhesion, loss of barrier function, and increased apoptosis (9). Expression of the same mutant in the crypt resulted in accumulation of cells in the crypt and formation of polyps (10). Similarly, mutations in APC protein lead to the accumulation of cells near the crypt and formation of polyps.

At the present time, it is not known whether cadherin-mediated adhesion is affected in cells that contain mutant APC protein. However, the localization of APC protein to actively migrating areas of epithelial cells in vitro and to actively migrating cells in the intestine indicates a positive role for APC protein in migration that involves the association of APC protein with microtubules. We suggest that cells expressing APC protein containing a deletion of the microtubule binding domain have a migration defect and accumulate near the crypt-villus transition zone, where further cell proliferation and additional mutations may occur, leading to the induction of a cancerous growth.

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Note added in proof. Matsumine et al. (1996) recently showed that in rat colon epithelium, APC protein is distributed diffusely throughout the cytoplasm of enterocytes and is not enriched at cell boundaries (Fig. 4 A in: Matsumine, A., A. Ogai, T. Senda, N. Okumura, K. Satoh, G.-H. Baeg, T. Kawahara, S. Kobayashi, M. Okada, K. Toyoshima, and T. Akiyama. 1996. *Science (Wash. DC)*. 272:1021–1023). This is similar to the distribution of APC protein in the mouse small intestine shown in the manuscript.

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