

# Apical Membrane Localization of the Adenomatous Polyposis Coli Tumor Suppressor Protein and Subcellular Distribution of the $\beta$ -Catenin Destruction Complex in Polarized Epithelial Cells

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**Abstract.** The adenomatous polyposis coli (APC) protein is implicated in the majority of hereditary and sporadic colon cancers. APC is known to function as a tumor suppressor through downregulation of  $\beta$ -catenin as part of a high molecular weight complex known as the  $\beta$ -catenin destruction complex. The molecular composition of the intact complex and its site of action in the cell are still not well understood. Reports on the subcellular localization of APC in various cell systems have differed significantly and have been consistent with an association with a cytosolic complex, with microtubules, with the nucleus, or with the cortical actin cytoskeleton. To better understand the role of APC and the destruction complex in colorectal cancer, we have begun to characterize and isolate these complexes from confluent polarized human colon epithelial cell monolayers and other epithelial cell types. Subcellular fractionation and immunofluorescence microscopy reveal that a predominant fraction of APC associates tightly with the apical plasma membrane in a variety of epithelial cell types.

This apical membrane association is not dependent on the mutational status of either APC or  $\beta$ -catenin. An additional pool of APC is cytosolic and fractionates into two distinct high molecular weight complexes, 20S and 60S in size. Only the 20S fraction contains an appreciable portion of the cellular axin and small but detectable amounts of glycogen synthase kinase  $3\beta$  and  $\beta$ -catenin. Therefore, it is likely to correspond to the previously characterized  $\beta$ -catenin destruction complex. Dishevelled is almost entirely cytosolic, but does not significantly cofractionate with the 20S complex. The disproportionate amount of APC in the apical membrane and the lack of other destruction complex components in the 60S fraction of APC raise questions about whether these pools of APC take part in the degradation of  $\beta$ -catenin, or alternatively, whether they could be involved in other functions of the protein that still must be determined.

**Key words:** adenomatous polyposis coli • destruction complex • epithelial cells • localization • fractionation

## Introduction

The tumor suppressor protein adenomatous polyposis coli (APC)<sup>1</sup> is implicated in the development of hereditary and sporadic colon carcinoma (Joslyn et al., 1991; Kinzler et al., 1991; Miyoshi et al., 1992). Mutations in the APC gene occur early during tumor formation (Powell et al., 1992), indicating the important role of APC as the “gatekeeper” gene in colon carcinogenesis. APC mutations generally cause chain termination and expression of truncated proteins that lack a COOH-terminal portion of the protein (Polakis, 1995).

An important insight into understanding the biological function of APC has emerged through the finding that

APC binds to and regulates  $\beta$ -catenin, a protein that functions in cell adhesion as well as in signal transduction in the Wnt signaling pathway (Rubinfeld et al., 1993; Su et al., 1993). The best documented function of wild-type APC is the inhibition of  $\beta$ -catenin-mediated signaling by stimulating the degradation of cytosolic  $\beta$ -catenin protein (Munemitsu et al., 1995). This function is lost in cells harboring APC mutations. The posttranslational stabilization and accumulation of cytoplasmic  $\beta$ -catenin is now considered a key mechanism in colon carcinogenesis because it leads to the formation of  $\beta$ -catenin/TCF transcription complexes and the induction of target genes such as the oncogene *c-myc* (Korinek et al., 1997; Morin et al., 1997; He et al., 1998). However, downregulation of  $\beta$ -catenin may not be the only biological role of APC. Studies in *Xenopus* and *Caenorhabditis elegans* point to additional functions of APC or APC-related protein potentially as an activator of the Wnt signaling pathway (Rocheleau et al., 1997; Vleminckx et al., 1997). Also, recent studies provide

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<sup>1</sup>Abbreviations used in this paper: APC, adenomatous polyposis coli; Dlg, *Drosophila* discs large; GBP/FRAT1, GSK-3-binding protein/frequently rearranged in advanced T cell lymphomas 1; GSK, glycogen synthase kinase.

evidence for a role of APC in the nuclear export of  $\beta$ -catenin (Henderson, 2000; Rosin-Arbesfeld et al., 2000).

The regulation of  $\beta$ -catenin turnover and signaling by APC occurs in conjunction with other proteins in a high molecular weight complex in which  $\beta$ -catenin is phosphorylated and subsequently targeted for destruction by the proteasome. Other key members of this destruction complex are glycogen synthase kinase (GSK)-3 $\beta$ , a kinase that presumably phosphorylates  $\beta$ -catenin, and axin, a protein which acts as a scaffold bringing the components of the complex into close proximity, thereby facilitating  $\beta$ -catenin phosphorylation (Zeng et al., 1997; Polakis, 1999; Peifer and Polakis, 2000). The protein dishevelled (dsh in flies, dvl in mammals) mediates signaling from frizzled, the Wnt receptor, and interacts with the destruction complex to inhibit GSK-3 $\beta$  indirectly (Kishida et al., 1999b; Li et al., 1999; Smalley et al., 1999; Salic et al., 2000). Additional components of the complex are the protein phosphatase PP2A (Seeling et al., 1999) and the GSK-3 $\beta$  inhibitor GBP/FRAT1 (Li et al., 1999; Salic et al., 2000). Apart from the destruction complex, APC has been found by yeast two-hybrid assay to interact with the human homologue of the *Drosophila* discs large tumor suppressor protein Dlg (Matsumine et al., 1996) and the microtubule binding protein EB-1 (Su et al., 1995) and has been found to associate with microtubules (Munemitsu et al., 1994; Smith et al., 1994). However, the physiological relevance of these latter interactions is not known.

Although the functional roles for APC and other components of the destruction complex are well established, the dynamics of their interactions and the biochemical nature of the complex in the cell remain poorly understood. For example, the properties, composition, and structure of the intact isolated complex have not yet been determined. Moreover, it is not yet entirely clear where in the cell APC and the destruction complex function. A cell-free analysis of the ability of the complex to stimulate  $\beta$ -catenin degradation indicates that it functions in the cytosol (Salic et al., 2000), and many biochemical analyses of interactions between components are consistent with this view (Rubinfeld et al., 1993, 1996; Hart et al., 1998; Itoh et al., 1998). In epithelial cells of *Drosophila* embryos, APC has been found to colocalize with cortical actin and perhaps adherens junctions (McCartney et al., 1999; Yu and Bienz, 1999; Yu et al., 1999), whereas certain loss of function APC mutants become more cytosolic (McCartney et al., 1999). In several studies, APC has also been observed to associate with microtubules (Munemitsu et al., 1994; Smith et al., 1994; Nathke et al., 1996; Mimori-Kiyosue et al., 2000). However, the relationship of this interaction to its known function in  $\beta$ -catenin degradation is not known. Other components of the destruction complex including GSK-3 $\beta$  and dishevelled have often been described as soluble cytosolic proteins (Yanagawa et al., 1995; Diehl et al., 1998). Recently however, dishevelled has been reported to localize along actin filaments and at focal adhesions in isolated embryonic kidney cells (Torres and Nelson, 2000) or to be associated with cytoplasmic transport vesicles in *Xenopus* eggs (Miller et al., 1999). In some experiments, dishevelled has been observed to associate with the plasma membrane in response to frizzled or soluble wingless (Yanagawa et al., 1995; Axelrod et al., 1998; Rothbacher et al., 2000). Much less is known about axin, but when overexpressed, it has been localized to

the plasma membrane as well as the cytoplasm (Fagotto et al., 1999). These studies have been performed in a variety of cell types, in some cases in the context of Wnt signaling and/or regulation of  $\beta$ -catenin signaling, but often without much regard to the physiological state of the cell. Thus, to attempt to understand the function and dynamics of the complex, it would be very informative to examine the association and subcellular localizations of all the endogenous components of the complex in a well-characterized cell system in which APC and the destruction complex are known to function.

A great deal of attention has been given to the association of APC with microtubules. Overexpressed APC co-distributed with or moved along microtubules in cultured cells (Munemitsu et al., 1994; Smith et al., 1994; Mimori-Kiyosue et al., 2000), whereas endogenous APC has sometimes been observed by immunofluorescence to localize to the tips of protrusions of subconfluent epithelial cells where microtubules seem to project (Nathke et al., 1996). From these findings, it has been proposed that APC regulates epithelial cell motility by virtue of its control of microtubule behavior (McCartney and Peifer, 2000). However, most of these studies were carried out on very well spread, subconfluent epithelial cells in order to facilitate immunofluorescence imaging and, therefore, it is not clear how well the findings reflect the state of cells in the real epithelia in which APC is known to function.

We chose to undertake a biochemical and cellular analysis of APC and the destruction complex in human colon epithelial tumor cells, for which the role of APC as a tumor suppressor and regulator of  $\beta$ -catenin signaling is well established. Such cultured cells are homogenous and very accessible to a variety of biochemical and microscopic approaches. We focused our efforts on fully confluent, polarized cultures of these cells because we believe it is the most physiologically relevant state for comparison to normal human colon epithelia and adenomas derived from them. Moreover, we decided to employ a subcellular fractionation approach in combination with immunolocalization analysis in order to begin to isolate and characterize complexes containing the APC protein and other components of the destruction complex in polarized human colon cancer cells. Indeed, our findings indicate that the steady state distribution of APC differs significantly from observations on subconfluent cells, and that it exists in multiple complexes rather than as a single unique protein complex as had been inferred previously (Miyahiro et al., 1995; Hart et al., 1998; Itoh et al., 1998; Polakis et al., 1999; Peifer and Polakis, 2000; Salic et al., 2000).

## Materials and Methods

### Cell Culture

Colon carcinoma cell lines HCT116, DLD-1, LS411, and LoVo were obtained from American Type Culture Collection and maintained in McCoy's 5a medium/10% FBS (HCT116), RPMI 1640/10% FBS (DLD-1 and LS411), or Ham's F12 medium/20% FBS (LoVo), respectively. The breast cancer cell line MCF-7 was kindly provided by Dr. N. Rosen (Memorial Sloan-Kettering Cancer Center) and maintained in DME high glucose supplemented with 12 nonessential amino acids and 10% FBS. MDCK II cells were cultured in DME/10% FBS medium.

### Cell Fractionation

Cell fractionation procedures were carried out at 4°C unless otherwise noted. Protease inhibitors were added to solutions at the following final

concentrations: PMSF, 0.017 mg/ml; leupeptin, 0.002 mg/ml; aprotinin, 0.004 mg/ml; antipain, 0.01 mg/ml; benzamidin, 0.05 mg/ml; ST inhibitor, 0.01 mg/ml; iodoacetamide, 0.1 mg/ml. HCT116 and MCF-7 cells were grown to confluency in 14-cm tissue culture dishes, washed once with PBS, and harvested by scraping. Cells were pelleted at 200 g for 5 min and resuspended in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.8, 10 mM KCl) and lysed by Dounce homogenization after a 20-min (HCT116) or 10-min (MCF-7) incubation on ice. Lysis was assessed by light microscopy after staining with trypan blue. The sample was centrifuged at 5,000 rpm for 30 min to pellet unlysed cells and nuclei. Isotonic salt concentrations were restored (140 mM KCl) and the supernatant was further fractionated by centrifugation at 100,000 g for 1 h at 4°C. The resulting fractions were labeled Pellet 100 (P100) and Supernatant 100 (S100).

### Sucrose Equilibrium Density Gradient Centrifugation: Flotation

To evaluate a potential membrane association of APC in HCT116 and MCF-7 cells, the P100 fraction was resuspended in a 55% sucrose solution (in buffer A: 100 mM NaCl, 20 mM Tris-HCl, pH 7.8, 5 mM EDTA) and loaded onto 0.5 ml of a 66% sucrose solution (cushion). 5 ml of a continuous 20–50% sucrose gradient was poured on top (in buffer A). The samples were spun in a Beckman Coulter SW 55Ti rotor at 45,000 rpm for 16 h at 4°C. 0.55-ml fractions were collected and analyzed by Bradford protein assay and Western blotting.

To characterize biochemical properties of APC after flotation, gradient fractions containing plasma membrane material (sucrose concentrations of ~30–37%, density 1.12–1.16 g/cm<sup>3</sup>) were pooled. A portion of the membrane pool (two thirds) was treated with detergents (1% NP-40 or 1% octyl glucoside) or high salt (1 M NaCl) and spun at 100,000 g for 1 h. Samples were analyzed by Western blotting.

### Velocity Sizing of the S100 Fraction

To further fractionate the S100 sample according to size, velocity gradient centrifugation was performed. 4.5 ml of a continuous 10–40% sucrose gradient was layered over 0.5 ml of a 66% sucrose solution (cushion), and an aliquot of the S100 fraction was loaded on top of the gradient. The sample was spun at 55,000 rpm in a Beckman Coulter SW55 rotor for 4.5 h. The marker proteins catalase (4–5S pool as monomer derived from 11.5S pool as tetramer) and thyroglobulin (19S) were spun in parallel gradients. 625- $\mu$ l fractions were collected and analyzed for protein content by Bradford protein assay and Western blotting. To estimate S values >19S, a software program for simulation calculation of velocity gradients was used based on a method developed by McEwen (1967).

### Western Blotting

To detect APC, samples were separated in 3% agarose gels and transferred by capillary transfer to nitrocellulose overnight as described (Smith et al., 1993). For the detection of E-cadherin,  $\beta$ -catenin, dishevelled, and axin samples were loaded on 7% SDS polyacrylamide gels (for GSK-3 $\beta$ ), on 10% SDS polyacrylamide gels, separated, and transferred to nitrocellulose by semidry transfer. Western blotting was performed as described (Vlemingcx et al., 1997). The following primary antibodies were used: APC, anti-APC NH<sub>2</sub> terminus (Ab-1, mouse IgG1), anti-APC COOH terminus (diluted 1:1,000) (Ab-2, mouse IgG1; Oncogene Research Products), or anti-APC COOH terminus (diluted 1:500) (C-20, rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.);  $\beta$ -catenin, anti- $\beta$ -catenin NH<sub>2</sub> terminus (polyclonal rabbit antiserum, diluted 1:5,000); E-cadherin, anti-E-cadherin (diluted 1:7,500) (mouse IgG; Zymed Laboratories); and GSK-3 $\beta$ , anti-GSK-3 $\beta$  (diluted 1:2,500) (mouse IgG1; BD Transduction Laboratories). Polyclonal rabbit antisera directed against murine dishevelled and murine axin (diluted 1:2,000) were kindly provided by R. Nusse (Stanford University, Stanford, CA).

### Immunofluorescence Microscopy: Cultured Cells

To analyze confluent epithelial cells, glass coverslips were coated with poly-L-lysine (100  $\mu$ g/ml) for 1 h at room temperature, and cells were allowed to adhere to the coverslips for at least 72 h in appropriate culture medium (see above). To examine cells at subconfluency, glass coverslips were coated with collagen (50  $\mu$ g/ml), and cells were allowed to adhere for 24–48 h. State of confluency was assessed by light microscopy before fixation. Cells were fixed (100% methanol, 5 m at –20°C), washed three times in PBS, blocked with BSA (1% in PBS, 30 min at room temperature), and

incubated with primary antibody (diluted in 1% BSA/PBS 1 h at room temperature). Samples were then incubated in appropriate secondary antibodies conjugated to Cy3 (Jackson ImmunoResearch Laboratories) or FITC (Molecular Probes) (diluted 1:750 or 1:200, respectively, in 1% BSA/PBS, 30 min at room temperature). Specimens were examined by confocal microscopy equipped with a 25 $\times$  or 63 $\times$  objective.

Primary antibodies used for immunofluorescence microscopy were as follows: APC, anti-human APC NH<sub>2</sub> terminus (diluted 1:75) (N-15, rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.);  $\beta$ -catenin, anti- $\beta$ -catenin COOH terminus (diluted 1:200) (clone 14, mouse IgG; BD Transduction Laboratories);  $\beta$ -tubulin, anti- $\beta$ -tubulin (diluted 1:200) (clone Tub 2.1, mouse IgG; Sigma-Aldrich). As controls for APC staining, either normal rabbit IgG was substituted for the primary anti-APC antibody or an excess of the neutralizing peptide against which the anti-APC antibody was raised, was incubated with the anti-APC antibody before immunofluorescence analysis (five times excess by weight, overnight at 4°C in 1% BSA/PBS).

### Immunofluorescence Microscopy: Tissue

To determine the subcellular localization of APC in mouse colon, specimens were briefly washed in ice cold PBS, embedded in OCT compound (Miles, Inc.) for 1 h on ice, and frozen in liquid nitrogen. Frozen samples were then cut into 12- $\mu$ m sections. Sections were air dried and fixed in 100% acetone for 10 min on ice, rehydrated in PBS, and blocked with 10% donkey serum in 2% BSA/PBS for 30 min at 37°C. Samples were then incubated with primary antibody in 2% BSA/PBS for 1 h at 37°C (anti-APC antibody N-15, diluted 1:50; anti- $\beta$ -catenin antibody clone 14, diluted 1:100). Slides were washed in 0.1% BSA/PBS for 5 min, washed in 0.05% Triton X-100/PBS for 5 min, and additionally washed in 0.1% BSA/PBS twice for 5 min. Samples were then incubated in appropriate secondary antibodies conjugated to Cy3 (Jackson ImmunoResearch Laboratories) or FITC (Molecular Probes) (diluted 1:750 or 1:200, respectively, in 2% BSA/PBS, 30 min at 37°C). After additional washing, sections were mounted and examined by confocal microscopy equipped with a 63 $\times$  objective. Normal rabbit IgG and preincubation of the anti-APC antibody with the competing peptide against which the antibody was raised were used as controls.

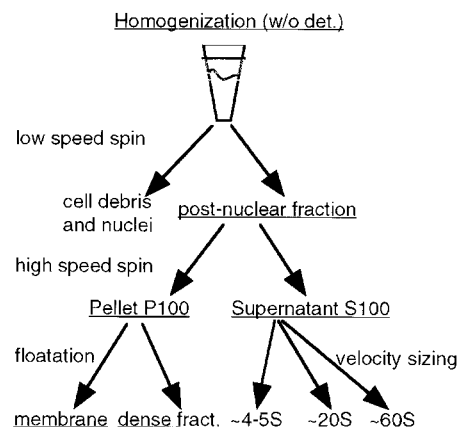
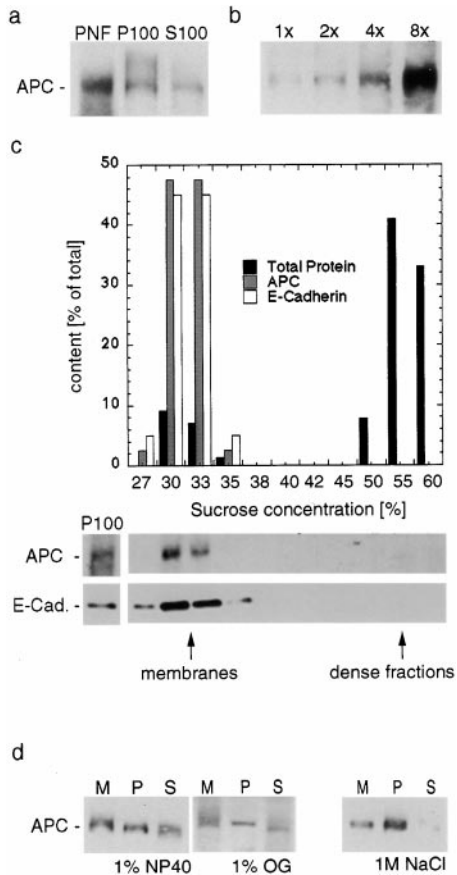


Figure 1. Schematic representation of fractionation protocol used to analyze subcellular distribution of APC and components of the  $\beta$ -catenin destruction complex in HCT116 and MCF-7 cells. Cells were lysed in hypotonic lysis buffer without detergents (w/o det.) and homogenized using a Dounce homogenizer. Unbroken cells and nuclei were removed by low speed centrifugation (5,000 rpm for 30 min). Postnuclear supernatants were further fractionated by high speed centrifugation (100,000 g for 1 h) into a pellet fraction (P100, >90S) and a supernatant fraction (S100, <90S). Membrane association of proteins was analyzed by sucrose density equilibrium flotation of P100 fractions (density of membranes ~1.13 g/cm<sup>3</sup>; density of dense fractions ~1.3 g/cm<sup>3</sup>). The S100 sample was further fractionated by velocity sedimentation. Size fractions of ~4–5S, ~20S, and ~60S were distinguished based on distribution of total protein, APC, and axin.

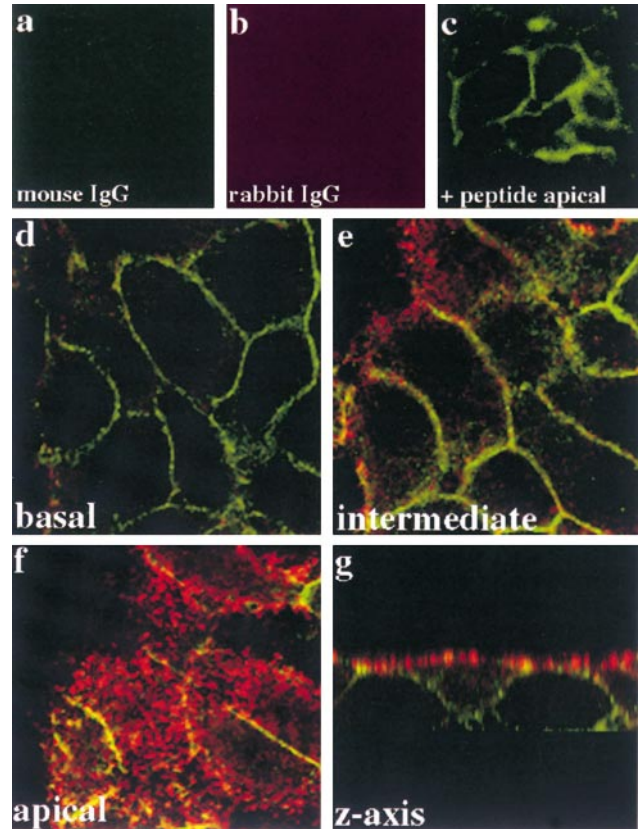


**Figure 2.** Membrane association of APC in HCT116 colon carcinoma cells. (a) Western blot showing distribution of APC after high speed centrifugation. APC present in the postnuclear fraction (PNF) distributes into both the high speed pellet fraction (P100) and the high speed supernatant fraction (S100). Equal proportions of P100 and S100 samples were loaded. (b) Dilution series for measurement of APC by Western blotting. (c) APC floats with membranes in equilibrium density gradients. Graph and corresponding Western blot illustrating distribution of APC, E-cadherin (marker for membranes), and total protein in each fraction after equilibrium density flotation of P100 fractions. (d) Solubility of membrane-bound APC after treatment with detergents or high salt. Membrane-containing fractions after P100 density flotation (fractions 30–33% in panel c) were pooled, treated with 1% NP-40, 1% octyl glucoside (OG) or 1 M NaCl, and subjected to a high speed spin. One third of starting material was loaded in lane M (membrane) and resulting pellet and supernatant fractions were loaded in lanes P and S, respectively. Membrane-bound APC is only partially solubilized by solubilization of membranes with detergents, whereas high salt treatment does not result in the release of APC into the soluble fraction. Moreover, after high salt treatment, APC continues to float with membranes in density gradients (data not shown).

## Results

### APC Associates with the Apical Plasma Membrane in Human Epithelial Cells

To localize APC at the subcellular level, we initially used the human colon carcinoma cell line HCT116, because this cell line expresses wild-type APC. It does express a mutant  $\beta$ -catenin protein that carries a deletion of ser45 (Rubinfeld et al., 1997). The fractionation protocol described in



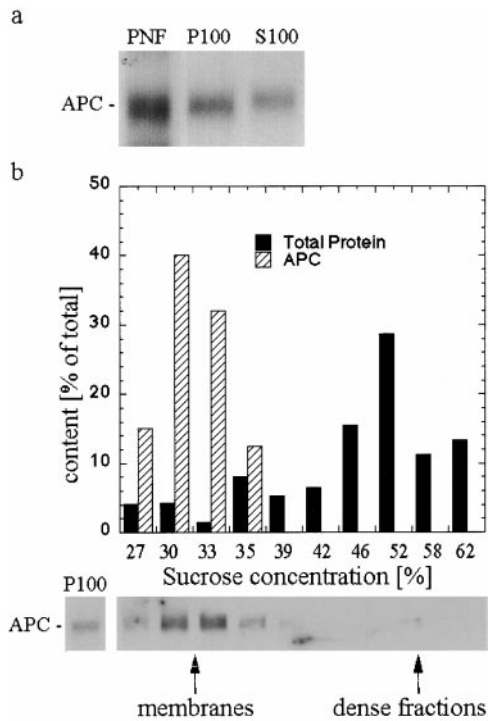
**Figure 3.** Apical localization of APC in the HCT116 colon carcinoma cell line. Immunolocalization of endogenous APC (red) and  $\beta$ -catenin (green) in HCT116 cells. (a and b) Negative control for APC and  $\beta$ -catenin staining, respectively. Normal mouse (a) or normal rabbit IgG (b) was substituted for primary antibodies before incubation with respective secondary antibodies. (c) Peptide competition (+ peptide apical). Anti- $\beta$ -catenin and anti-APC antibodies were preincubated with an excess of the neutralizing peptide against which the anti-APC antibody was raised. A transverse apical section is shown. (d–f) Images of successive sections of fully confluent, polarized HCT116 cells using confocal microscopy: basal (d), intermediate (e), and apical (f) sections. (g) Corresponding perpendicular section (z-axis).

Fig. 1 was used. As shown in Fig. 2 a, APC distributed approximately equally into pelletable and soluble material after the initial 100,000-g spin. This distribution pattern was consistently observed when fully confluent monolayers were used.

To determine whether the pool of APC that sedimented at high speed (P100) was associated with membranes equilibrium, density flotation centrifugation was performed. With this method, APC mostly fractionated at light densities ( $\sim 1.13 \text{ g/cm}^3$ ) and comigrated with vesicles containing the integral membrane protein E-cadherin, suggesting that APC associated with membranes in HCT116 cells (Fig. 2 c). Although APC was consistently found to float with membranes, in some experiments APC could also be detected in dense fractions of the gradient ( $1.3 \text{ g/cm}^3$ ) where most of the total protein of the P100 fraction sedimented (data not shown).

To determine the nature of the association of APC with the membrane, this fraction was treated with mild non-ionic detergents or high salt concentrations (Fig. 2 d).

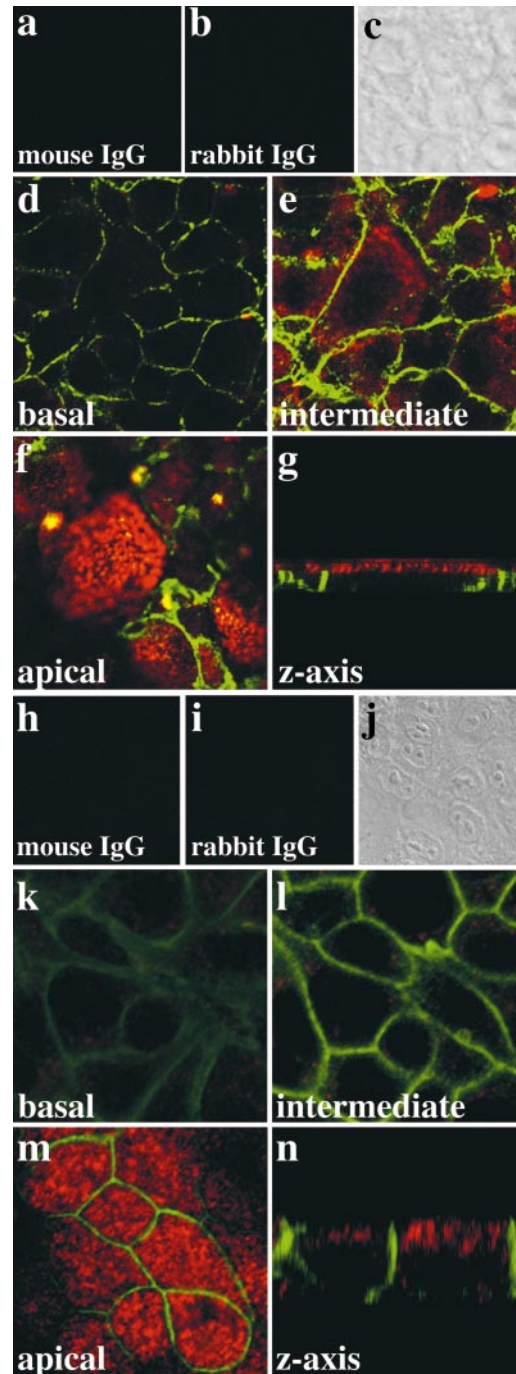




**Figure 4.** Membrane association of APC in MCF-7 breast epithelial cancer cells. (a) Western blot for APC illustrating distribution of APC after fractionation of postnuclear fractions (PNF) into a high speed pellet fraction (P100) and a high speed supernatant fraction (S100). Equal proportions of P100 and S100 were loaded. (b) APC in MCF-7 cells fractionates with membranes in equilibrium density gradients. Graph illustrating relative distribution of total protein and APC in each fraction after density flotation of P100 samples. Corresponding Western blot for APC is shown below.

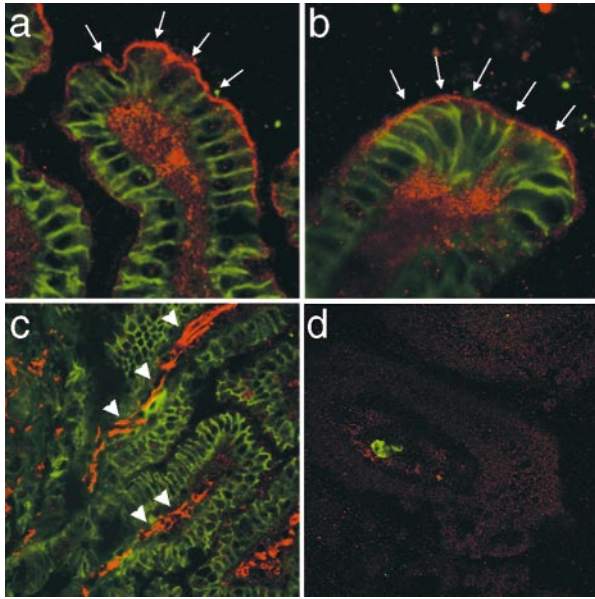
High salt did not release APC from the membrane fraction, suggesting that APC is tightly associated with the membrane. Neither NP-40 nor octyl glucoside efficiently solubilized APC. The fraction that pelleted after detergent solubilization of membranes did not refloat during subsequent density equilibrium centrifugation (data not shown), indicating that this APC subfraction is not associated with classic lipid rafts (Simons and Ikonen, 1997), but rather is present in a very large submembranous protein complex.

To determine where the membrane-associated fraction of APC localizes in HCT116 cells, we used indirect immunofluorescence microscopy with the polyclonal rabbit IgG antibody N-15 raised against an NH<sub>2</sub>-terminal peptide of human APC. Immunolocalization was carried out on fully confluent, polarized epithelial cell monolayers. Confocal microscopic analysis of immunostained HCT116 colon carcinoma cells showed that APC predominantly localized to the apical plasma membrane and to a very small degree to the apicolateral cell border (Fig. 3, d–g). APC and β-catenin, for the most part, did not colocalize. β-Catenin staining was mostly seen laterally with very little overlap of the two proteins detectable at the apicolateral cell borders (Fig. 3 g). Essentially no APC was seen in more basal sections of the cell (Fig. 3 d). APC staining was most pronounced in apical sections of the cells (Fig. 3 f). The apical localization of APC is best observed in the z-section of analyzed monolayers (Fig. 3 g). For controls using normal mouse or rabbit IgG, no specific



**Figure 5.** Apical localization of APC in the MCF-7 breast tumor cell line and the MDCK cell line. Immunolocalization of APC (red) and β-catenin (green) in MCF-7 (a–g) and MDCK cells (h–n). Images of successive sections of fully confluent MCF-7 and MDCK cells using confocal microscopy. (d and k) Basal; (e and l) intermediate; and (f and m) apical sections as well as (g and n) corresponding perpendicular section (z-axis) for MCF-7 and MDCK cells. Negative controls (substitution of normal mouse or rabbit IgG for primary antibodies) are shown for MCF-7 cells in a and b and for MDCK cells in h and i, respectively. (c and j) Phase-contrast image of controls.

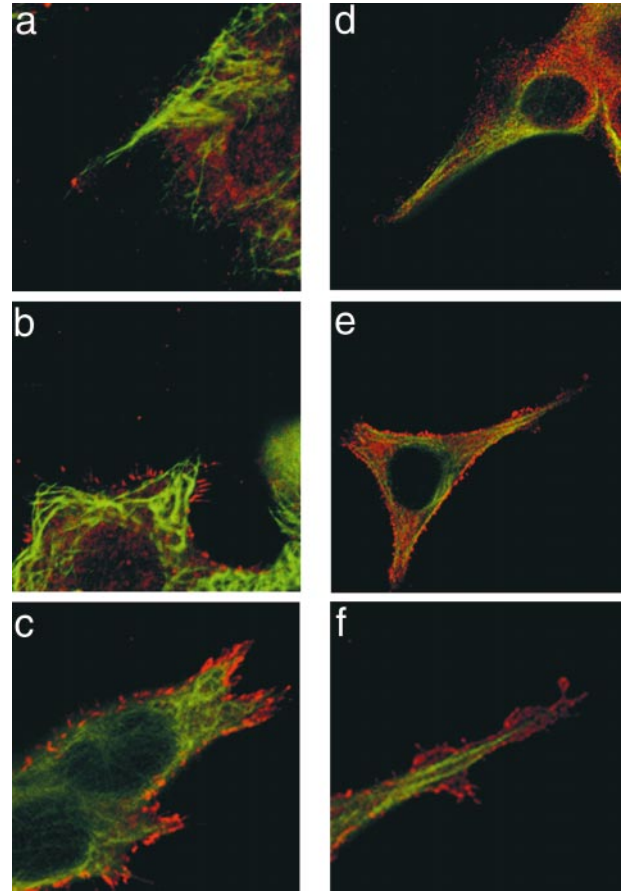
staining was detected (Fig. 3, a and b). Furthermore, apical staining of APC was effectively blocked by preincubation of the antibodies with the neutralizing peptide against which the anti-APC antibody was raised (Fig. 3 c).



**Figure 6.** Apical membrane localization of APC in normal mouse colon. Immunolocalization of APC (red) and  $\beta$ -catenin (green) in tissue sections of normal mouse colon. (a and b) Apical membrane staining of epithelial cells in the upper portion of colonic crypts which face the lumen of the digestive tract in normal mouse colon (arrows). (c) Low APC immunoreactivity in epithelial cells towards the base of the crypts. Additional APC staining in nonepithelial cells in the lamina propria (arrowheads) and throughout the submucosa and muscularis layers. (d) APC staining is effectively blocked with the specific neutralizing peptide against which the antibody was raised. There is no  $\beta$ -catenin staining, because normal mouse IgG was substituted for  $\beta$ -catenin primary antibody in control sections. Note residual immunoreactivity of secondary anti-mouse IgG antibody with cells in the lamina propria (green).

### Apical Membrane Localization of APC in Other Epithelial Cell Types and Mouse Colon

To determine whether the apical membrane localization of APC is unique to the HCT116 colon carcinoma cell line or due to the presence of a mutant  $\beta$ -catenin found in HCT116 cells, we analyzed the breast cancer cell line MCF-7, which expresses wild-type  $\beta$ -catenin and wild-type APC. The distribution of APC during the initial 100,000-g spin was similar in MCF-7 cells compared with HCT116 cells. APC fractionated to about equal proportions into pellet and supernatant (Fig. 4 a). Furthermore, as found in HCT116 cells, APC in MCF-7 cells also sedimented at low densities characteristic of plasma membranes during equilibrium centrifugation, suggesting that APC similarly associates with the plasma membrane in this cell line (Fig. 4 b). To assess whether APC also localizes specifically to the apical plasma membrane in MCF-7 cells, we analyzed this cell line by confocal immunofluorescence microscopy (Fig. 5, a–g). APC localized predominantly to the apical membrane and in general did not overlap with  $\beta$ -catenin, which mainly localized to lateral cell borders (Fig. 5, d–g; negative controls for MCF-7 cells shown in a–c). Also, to evaluate APC localization in a nontransformed epithelial cell line, we examined fully confluent, well-polarized MDCK cells by immunofluorescence microscopy (Fig. 5, k–n; neg-



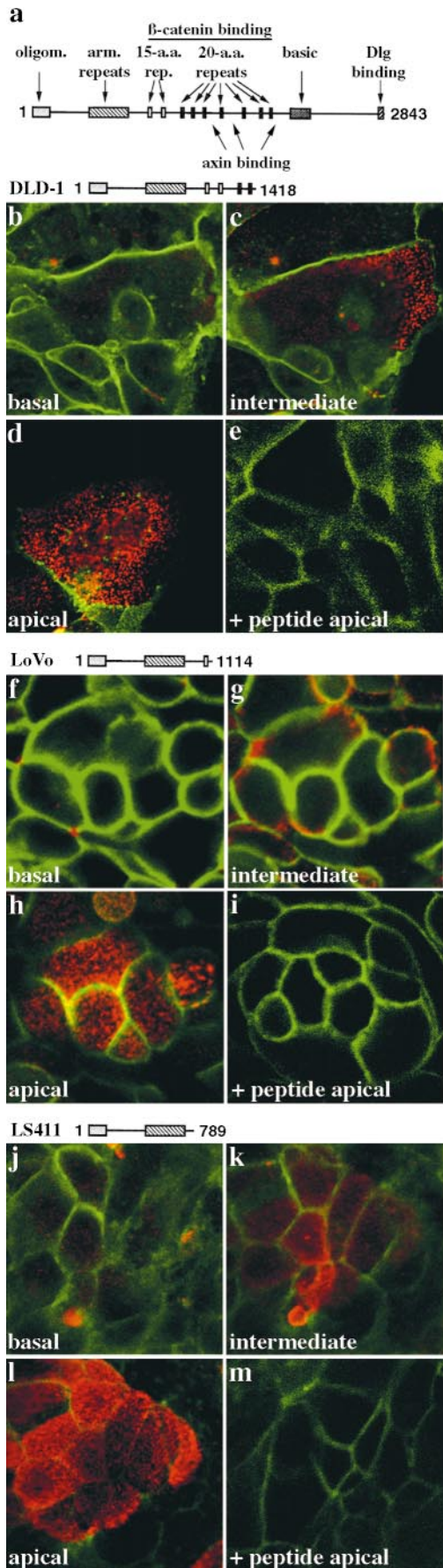
**Figure 7.** Localization of APC and microtubules in subconfluent epithelial cells. Immunolocalization of APC (red) and  $\beta$ -tubulin (green) in subconfluent HCT116 (a–c) and MDCK cells (d–e). (a) Localization of APC at the tips of cell processes containing microtubules is detected, albeit infrequently (<5% of all microtubule-containing protrusions). (b–f) APC is present over much of the cell surface and enriched all along the edges of the cell body and cell protrusions. (f) Higher magnification view of e.

ative controls for MDCK cells shown in h–j). APC also localized apically in MDCK cells, whereas  $\beta$ -catenin was found laterally. In general, no overlap between the two proteins was observed.

To determine where APC localizes in situ, we examined samples of normal mouse colon by immunofluorescence microscopy. APC was enriched at the apical membrane of epithelial cells predominantly in upper regions of colonic crypts towards the lumen of the digestive tract (Fig. 6, a and b). In addition, some diffuse cytoplasmic staining was detected. In contrast,  $\beta$ -catenin was seen mainly at the lateral borders of colonocytes and no obvious colocalization with APC was found. Immunoreactivity towards the APC protein in epithelial cells decreased substantially towards the base of the crypts (Fig. 6 c). Additionally, APC was detected in nonepithelial cells in the lamina propria and throughout the submucosa and muscularis layers. APC staining was effectively blocked with the competing peptide against which the anti-APC antibody was raised (Fig. 6 d).

Since a lot of attention has been given to the association of APC with microtubules, we also performed double immunostaining for APC and  $\beta$ -tubulin. In fully confluent



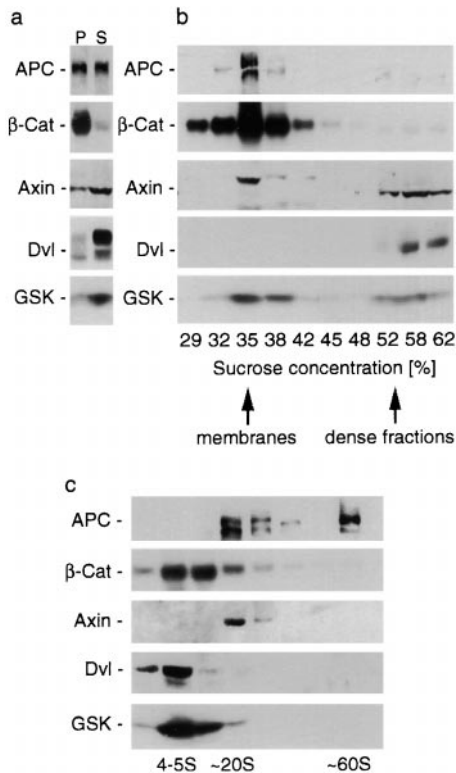


cell monolayers, no apparent colocalization of APC and microtubules was observed (data not shown). In subconfluent HCT116 cells (Fig. 7, a–c) and subconfluent MDCK cells (Fig. 7, d–f), APC was found over large regions of the cell surface and perhaps in the cytoplasm. We did occasionally detect APC at the tips of cell protrusions where microtubules project, as reported previously (Nathke et al., 1996); however, these were very rare (<5% of identified microtubule-containing protrusions) (Fig. 7 a). Much more commonly observed was APC distributed all along the surface of the cell protrusions (Fig. 7, b–f). Staining patterns for HCT116 and MDCK cells were very similar.

### Mutant Forms of APC also Localize to the Apical Membrane in Colon Carcinoma Cells

Truncations of APC are loss of function mutations that are responsible for loss of tumor suppressor activity. Therefore, we asked whether truncation mutations in APC affect its apical membrane localization. The distributions of APC in several colon carcinoma cell lines with known truncations of the APC protein were examined by confocal immunofluorescence microscopy. The linear representation of the full-length human APC protein is shown in Fig. 8 a. The DLD-1 cell line (Fig. 8, b–e) harbors a truncation mutation within the central region of the APC protein, a region which is known to be crucial for the tumor suppressor activity of APC through downregulating  $\beta$ -catenin (Munemitsu et al., 1995). The cell line LoVo (Fig. 8, f–i) carries a mutation at codon 1114 of APC, retaining the NH<sub>2</sub> terminus of APC including one of the three 15-amino acid repeats. The cell line LS411 (Fig. 8, j–m) carries a mutation at codon 789 which causes chain termination shortly after the seven armadillo repeats (Homfray et al., 1998). A polyclonal antibody directed against the NH<sub>2</sub> terminus of the APC protein was used to detect APC. In all cell lines, the mutant APC protein predominantly localized to the apical plasma membrane as observed in serial transverse sections through confluent monolayers. Again, most APC protein did not colocalize with most of the lateral membrane-associated  $\beta$ -catenin in DLD-1, LoVo, and LS411 cells. APC staining was effectively abolished by competition with the specific neutralizing peptide

**Figure 8.** Localization of mutant forms of APC to the apical membrane in colon cancer cell lines. (a) Linear representation of the full-length human APC protein. Several known motifs are shown on top, including the oligomerization domain (oligom.), armadillo repeats (arm. repeats), the 15- and 20-amino acid repeats (both known to bind  $\beta$ -catenin), basic domain, and Dlg binding site. Regions for axin binding are shown below. Immunolocalization of APC (red) and  $\beta$ -catenin (green) in DLD-1 (b–e), LoVo (f–i), and LS411 (j–m) cells. Linear representation of truncated mutant forms of the APC protein expressed by the respective cell line is also shown. Images of successive sections of fully confluent DLD-1, LoVo, and LS411 cells using confocal microscopy. (b, f, and j) Basal; (c, g, and k) intermediate; and (d, h, and l) apical sections are shown. (e, i, and m) Peptide competition (+ peptide apical). Apical section through DLD-1 (e), LoVo (i), and LS411 cells (m) after preincubation of primary anti- $\beta$ -catenin and anti-APC antibodies with the neutralizing peptide against which the anti-APC antibody was raised.

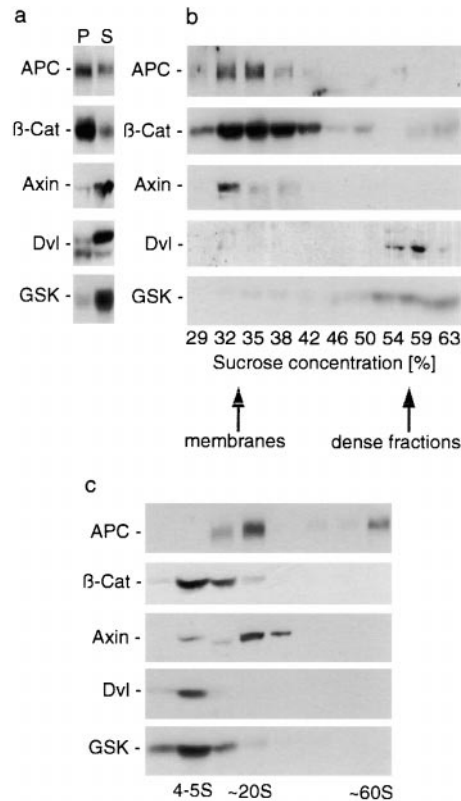


**Figure 9.** Distinct fractionation pattern of components of the  $\beta$ -catenin destruction complex in HCT116 cells. Cells were fractionated according to the scheme in Fig. 1 and analyzed by Western blotting for APC,  $\beta$ -catenin ( $\beta$ -Cat), axin, dishevelled (Dvl), and GSK-3 $\beta$  (GSK). (a) Distribution of proteins into pellet (P100) and supernatant (S100) after high speed centrifugation. Equal proportions of P100 and S100 were loaded. Note that axin, dishevelled, and GSK are mostly cytosolic. (b) Distribution of proteins of the destruction complex and  $\beta$ -catenin after density equilibrium flotation of P100 fractions. Note that only a small fraction of total axin, dishevelled, and GSK-3 $\beta$  is analyzed by P100 flotation since these components are mostly cytosolic. (c) Distribution of components of the destruction complex and  $\beta$ -catenin after velocity sizing of S100 fractions. APC distributes into two high molecular weight pools of  $\sim$ 20S and 60S.

against which the anti-APC antibody was raised (Fig. 8, e, i, and m). These data suggest that an  $\text{NH}_2$ -terminal fragment of the APC protein containing the oligomerization domain and the arm repeat region is sufficient to localize APC to the apical membrane.

#### ***APC Fractionates into Multiple Subcellular Pools in Human Epithelial Cells but Does Not Completely Cofractionate with Known Components of the $\beta$ -Catenin Destruction Complex***

Although most of the APC localized to the apical membrane in epithelial cells by immunofluorescence, we did identify a soluble pool of APC biochemically upon initial high speed centrifugation (Fig. 2 a). To characterize the soluble fraction of APC further, we determined the size of APC-containing protein complexes by velocity gradient centrifugation. With this method, APC sedimented in two size-fractions corresponding to sedimentation coefficients of  $\sim$ 20S and 60S (Figs. 9 c and 10 c). In particular, the 60S



**Figure 10.** Distinct fractionation pattern of components of the  $\beta$ -catenin destruction complex in MCF-7 breast cancer cells. Cells were fractionated according to the scheme in Fig. 1 and fractions were analyzed by Western blotting for APC,  $\beta$ -catenin, axin, dishevelled, and GSK-3 $\beta$ . (a) Distribution of proteins into high speed pellet (P100) and high speed supernatant (S100) fractions. Equal proportions of the fractions were loaded. Note that axin, dishevelled, and GSK-3 $\beta$  are predominantly cytosolic. (b) Distribution of components of the destruction complex and  $\beta$ -catenin after density flotation of P100 fractions. Note that only a small fraction of total axin, dishevelled, and GSK-3 $\beta$  is analyzed by P100 flotation since these components are mostly cytosolic. (c) Distribution of components of the destruction complex after velocity centrifugation of S100 fractions. Similar to HCT116 cells, APC distributes into two high molecular weight peaks of  $\sim$ 20S and  $\sim$ 60S in MCF-7 cells.

pool of APC fractionated away from most of the total protein in the S100 fraction, most of which sedimented at a smaller size of  $\sim$ 4–5S and some at  $\sim$ 20S (data not shown). This distribution pattern of APC into two distinct pools was repeatedly observed in multiple experiments.

A convergence of biochemical and genetic studies have shown that APC can form complexes with several proteins in the Wnt signaling pathway, including  $\beta$ -catenin, axin, dishevelled, and GSK-3 $\beta$ . APC and its binding partners are thought to form a high molecular weight complex (called the destruction complex) that affects  $\beta$ -catenin signaling by catalyzing  $\beta$ -catenin phosphorylation, which is a prerequisite for proteasomal degradation of  $\beta$ -catenin. To get a better understanding of the localization of the destruction complex in epithelial cells, we determined the subcellular distribution of key components of the complex in confluent polarized epithelial cells. After high speed centrifugation of the postnuclear fraction from HCT116 cells, axin,



**Table I. Summary of Distribution of Components of the  $\beta$ -Catenin Destruction Complex in Subcellular Fractions of HCT116 Colon Carcinoma Cells**

Protein	Membrane association	Soluble fraction	Soluble fraction size
APC	~50% membrane; apical	~50% soluble	20S and 60S
$\beta$ -Catenin	>90% membrane; lateral	<10% soluble	4-5S*
Axin	~5-10% membrane	90-95% soluble	20S
Dishevelled	<5% membrane	>95% soluble	4-5S
GSK-3 $\beta$	~5-10% membrane	90-95% soluble	4-5S*

Data from Fig. 9 resulting from fractionation according to Fig. 1 are summarized. Fraction and percent values are approximate; ranges reflect experiment to experiment variability. \*Small amounts of protein from 4-5S peak also present in 20S fraction.

dishevelled, and GSK-3 $\beta$  remained largely cytosolic, whereas APC distributed to about equal proportions into pellet and supernatant (Fig. 9 a).  $\beta$ -Catenin pelleted almost completely during this fractionation step, presumably because most of the  $\beta$ -catenin in HCT116 cells is bound to the membrane protein E-cadherin. To determine whether the small fractions of axin, dishevelled, and GSK-3 $\beta$  that sedimented at 100,000 g codistributed with APC and membranes, equilibrium density flotation gradients were run (Fig. 9 b). As before, most of the APC present in the P100 floated with membranes (assessed by its codistribution with  $\beta$ -catenin), whereas small amounts of axin and GSK-3 $\beta$  were detected in both the membrane and the dense fractions of the gradient. On the contrary, none of the small fraction of dishevelled protein present in the P100 floated with membranes, but rather sedimented with the bulk of the protein in gradient fractions with high densities.

With the antibodies used, two forms of axin and dishevelled were consistently detected by Western blotting. The higher molecular weight form of dishevelled distributed mostly in the S100 fraction (Fig. 9 a). For axin, small amounts of a higher molecular weight form were seen in the P100 fraction and were enriched in membrane fractions after flotation of the P100 (Fig. 9 b). The significance of these distinct immunoreactive forms of axin and dishevelled is unclear.

To determine whether the large soluble pool of the components of the destruction complex cofractionates with APC, the S100 fraction of HCT116 cells was analyzed by velocity gradient centrifugation (Fig. 9 c). Most of the soluble  $\beta$ -catenin, dishevelled, and GSK-3 $\beta$  sedimented at a size range of ~4-5S. Only minor portions of GSK-3 $\beta$  and  $\beta$ -catenin overlapped with APC in the 20S fraction. Axin, on the other hand, was almost exclusively present in the 20S fraction of the gradient, thereby significantly cofractionating with the 20S pool of APC. None of axin, dishevelled, GSK-3 $\beta$ , or  $\beta$ -catenin codistributed with the 60S pool of APC in HCT116 cells. A summary of the overall approximate distributions of APC and components of the  $\beta$ -catenin destruction complex in HCT116 cells is depicted in Table I.

To assess whether the distributions of these proteins were unique to HCT116 cells or dependent on the NH<sub>2</sub>-terminal mutation of  $\beta$ -catenin expressed by this cell line, we also analyzed their fractionation patterns in the breast cancer cell line MCF-7 (Fig. 10). APC as well as  $\beta$ -catenin, axin, dishevelled, and GSK-3 $\beta$  distributed into subcellular pools in MCF-7 cells very similar to HCT116 cells. APC fractionated equally into P100 and S100 pools, whereas axin, dishevelled, and GSK-3 $\beta$  were largely soluble and  $\beta$ -catenin mostly pelleted (Fig. 10 a). In density flotation gradients, most complex components from MCF-7 cells

behaved comparable to those from HCT116 cells (Fig. 10 b). APC and  $\beta$ -catenin mostly floated with membranes, whereas the small amounts of particulate dishevelled were mainly detected in dense fractions. In contrast to the HCT116 cells where the small P100 pools of axin and GSK-3 $\beta$  distributed in both membrane and dense fractions, the small pool of particulate axin fractionated exclusively in membrane fractions, and most of the pelletable GSK-3 $\beta$  sedimented in the dense fractions. Nonetheless, because the fraction of axin and GSK-3 $\beta$  present in the P100 before flotation is minor compared with the soluble pool of these proteins (see Fig. 10 a), their overall distributions are very similar in HCT116 and MCF-7 cells.

The fractionation pattern of the major soluble pool of the destruction complex in MCF-7 cells was also analyzed by velocity sizing (Fig. 10 c). Largely identical to HCT116 cells, APC fractionated into two peaks, a 20S and a 60S fraction, whereas  $\beta$ -catenin, dishevelled, and GSK-3 $\beta$  again distributed into the 4-5S fraction. Axin was again mainly found in the 20S pool, thus overlapping with the 20S fraction of APC. Again, none of the components of the destruction complex fractionated with the 60S pool of APC.

## Discussion

We have found that the tumor suppressor protein APC is associated with the apical plasma membrane in a variety of polarized epithelial cells. In contrast, only small amounts of axin and GSK-3 $\beta$ , but no dishevelled, are associated with membranes. In addition to the apical membrane pool, APC is present in a cytosolic pool which fractionates into two distinct high molecular weight complexes of ~20S and 60S. Most of the cellular axin codistributes with APC in the 20S fraction, whereas most of the dishevelled and GSK-3 $\beta$  are found uncomplexed (4-5S). Interestingly, none of the components of the destruction complex copurify with APC in the 60S fraction (for a summary see Table I).

The association of APC with the apical plasma membrane of polarized epithelial cells is not unique to colorectal cell lines nor is it due to the activating mutation in  $\beta$ -catenin present in HCT116 cells. APC is also apical in MCF-7 breast cancer cells and MDCK cells, both of which express wild-type  $\beta$ -catenin and APC. Moreover, APC is enriched at the apical membrane of epithelial cells in normal colon tissue in the upper portion of crypts towards the lumen of the digestive tract. Others have also reported some concentration of APC in apical regions of mouse and human intestinal cells (Miyashiro et al., 1995; Senda et al., 1996; Midgley et al., 1997). Furthermore, the reported cortical localization of APC in numerous epithelial cell types

in the *Drosophila* embryo often appears to be concentrated at the apical cell surface (McCartney et al., 1999; Yu and Bienz, 1999; Yu et al., 1999). Thus, we propose that the association of APC with the apical membrane is widespread in a variety of epithelia.

Inactivating truncation mutations in APC do not alter its localization to the apical membrane, indicating that loss of its tumor suppressor function is not attributable to alterations in its localization. Rather, the known tumor suppressor function of APC, that is, the downregulation of  $\beta$ -catenin, resides in the central region of the protein (Munemitsu et al., 1995; Hart et al., 1998), a region that is not present in the truncated APC proteins that still localize apically. These findings also demonstrate that apical localization does not depend on binding to axin,  $\beta$ -catenin, or any other component of the destruction complex that interacts with the central third of the protein. Although our findings do not precisely determine the amino acid sequence of APC required for apical localization, it is worth noting that the NH<sub>2</sub>-terminal quarter fragment sufficient for apical localization in LS411 cells still retains the armadillo repeat domain, a region with high homology to  $\beta$ -catenin and related proteins. The membrane association of a mutant form of *Drosophila* APC2 that carries a deletion of a single amino acid within the arm repeat region (Ser 241) is largely abolished (McCartney et al., 1999), suggesting that an intact arm repeat region is necessary for membrane localization. It will be interesting to determine whether the arm repeat domain interacts, either directly or indirectly, with apical membrane proteins.

It is not yet known whether the apical membrane-associated pool of wild-type APC is functional in the regulation of  $\beta$ -catenin degradation. Cell-free assays and studies on APC expression in cultured cells have implied that it functions in the cytosol (Munemitsu et al., 1995; Salic et al., 2000). In contrast, genetic studies in *Drosophila* have linked the membrane association of APC to its function as an inhibitor of the wingless/ $\beta$ -catenin signaling pathway, because loss of function mutant forms of APC accumulate in the cytoplasmic pool (McCartney et al., 1999). However, we find that most of the other components of the destruction complex, axin, dishevelled, and GSK-3 $\beta$ , are predominately soluble after high speed centrifugation. There are minor portions of cellular axin and GSK-3 $\beta$  in the membrane fraction that could be associated with APC, but the distribution of APC in the membrane fraction is clearly disproportionate relative to other members of the complex. Also very little, if any,  $\beta$ -catenin colocalizes with APC at the apical membrane. It is possible that apical membrane-associated APC interacts with  $\beta$ -catenin and other components of the destruction complex transiently (Salic et al., 2000). Alternatively, apical APC could be a stored and/or inactive pool, with its release into the cytosol regulated physiologically. Finally, apical APC may have additional biological functions not yet elucidated.

A large soluble pool of APC was also detected by cell fractionation. This cytosolic pool was not apparent by immunofluorescence microscopy, presumably because it is extracted during fixation and/or the signal is diluted over the volume of the cell. We also did not detect nuclear APC by immunofluorescence in fully confluent epithelial cells, although as reported by others (Henderson, 2000; Rosin-

Arbesfeld et al., 2000) mutant APC was detected in the nucleus at subconfluency in some cell lines (data not shown). It is unlikely that the soluble pool of APC arises artifactually by dissociation from the membrane during fractionation, because APC is tightly associated with the membrane under the conditions used. The 20S pool of soluble APC cofractionates with some of the other components of the destruction complex, including most of the axin and small amounts of the GSK-3 $\beta$  and  $\beta$ -catenin. This 20S pool is similar in nature to the pool of APC that has been studied in previous studies on the  $\beta$ -catenin destruction complex by coimmunoprecipitation and gel filtration analyses of detergent lysates (Rubinfeld et al., 1993, 1996; Hart et al., 1998; Itoh et al., 1998; Kishida et al., 1999a). However, only minor portions of the total GSK-3 $\beta$  and  $\beta$ -catenin, and very little, if any, dishevelled are present in this fraction. These proteins may interact with the destruction complex dynamically (Salic et al., 2000) or may serve other functions in some cells (i.e., GSK-3 $\beta$  in the insulin and other signaling pathways, dishevelled in the planar cell polarity pathway, and  $\beta$ -catenin in cell-cell adhesion) (Axelrod et al., 1998; Boutros et al., 1998; Srivastava and Pandey, 1998; Wallingford et al., 2000). We propose, therefore, that the APC-associated destruction complex described in previous reports corresponds to the 20S pool of APC that we detect in this study.

If the destruction complex fractionates at 20S, one may ask what is the function of the APC in the 60S fraction? None of the other components of the destruction complex were detected in this fraction. The lack of axin in the 60S fraction indicates that it is not simply a higher order assembly of the 20S complex. This raises the possibility that the different APC pools identified in this study (apical, 20S, and 60S) are independent of each other and that the 60S pool of APC may have novel functions in the cell. Indeed, the known function of APC in the destruction complex is mediated by the central region of this large and complicated protein, yet the NH<sub>2</sub>- and COOH-terminal regions contain interesting domains with less established cellular functions. Furthermore, there is evidence that APC and an APC related protein (APR-1) may have additional, possibly positive acting functions in the Wnt signaling pathway in *C. elegans* and *Xenopus* embryos (Rocheleau et al., 1997; Vleminckx et al., 1997; Nelson and Gumbiner, 1999; Guger and Gumbiner, 2000; Hoier et al., 2000). Also, the relationship of the 60S and/or membrane pool to the recently described nuclear export function of APC is not yet known (Henderson, 2000; Rosin-Arbesfeld et al., 2000).

APC has been reported to be associated with microtubules (Munemitsu et al., 1994; Smith et al., 1994; Mimori-Kiyosue et al., 2000), and therefore has been proposed to regulate microtubule function and cell migration (Nathke et al., 1996; McCartney and Peifer, 2000). We found APC to be largely localized at the apical membrane, with no apparent colocalization with microtubules. Our study was carried out on fully confluent polarized epithelial cells, conditions that correspond to the situation in intact epithelial tissue in vivo. Similar to earlier studies (Nathke et al., 1996), we could detect endogenous APC at the tips of microtubule-containing cell extensions in cells at subconfluency. However, these were infrequent, and in most

cells, APC staining was observed all over the cell surface and all along the extensions. Considering the recent finding that APC moves along microtubules in subconfluent cultured cells (Mimori-Kiyosue et al., 2000), one explanation for all of the reported findings is that APC is transported to the apical membrane via microtubules as cells form polarized epithelia.

The polarization of APC to the apical membrane domain may have implications for a function in epithelial development. For example, localizing the degradation machinery to the apical membrane could serve to keep the destruction activity segregated away from the pool of  $\beta$ -catenin in cadherin adhesion complexes at the lateral membrane. Conversely, active destruction of  $\beta$ -catenin at the apical surface could help keep the apical luminal surface nonadhesive and help maintain epithelial polarity. Another speculation is that apically localized APC could play a role in asymmetric cell division by localizing signaling determinants, similar to other apical proteins that control the localization of cell fate determinants during asymmetric cell division in *Drosophila* (e.g., crumbs, bazooka, inscuteable, and pins) (Knust, 1994; Lu et al., 1998; Schweisguth, 2000). Indeed, the Wnt signaling pathway regulates asymmetric cell division and anterior–posterior cell fate specification in early *C. elegans* embryos (Lu et al., 1998). Regardless of the specific model, the apical localization of APC suggests that APC plays some role in the compartmentalization of signaling functions or in cell polarity.

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