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The adenomatous polyposis coli protein unambiguously localizes to microtubule plus ends and is involved in establishing parallel arrays of microtubule bundles in highly polarized epithelial cells

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Loss of full-length adenomatous polyposis coli (APC) protein correlates with the development of colon cancers in familial and sporadic cases. In addition to its role in regulating β-catenin levels in the Wnt signaling pathway, the APC protein is implicated in regulating cytoskeletal organization. APC stabilizes microtubules in vivo and in vitro, and this may play a role in cell migration (Näthke, I.S., C.L. Adams, P. Polakis, J.H. Sellin, and W.J. Nelson. 1996. *J. Cell Biol.* 134:165–179; Mimori-Kiyosue, Y., N. Shiina, and S. Tsukita. 2000. *J. Cell Biol.* 148:505–517; Zumbrunn, J., K. Inoshita, A.A. Hyman, and I.S. Näthke. 2001. *Curr. Biol.* 11:44–49) and in the attachment of microtubules to kinetochores during mitosis (Fodde, R., J. Kuipers, C. Rosenberg, R. Smits, M. Kielman, C. Gaspar, J.H. van Es, C. Breukel, J. Wiegant, R.H. Giles, and H. Clevers. 2001. *Nat. Cell Biol.* 3:433–438; Kaplan, K.B., A. Burds, J.R. Swedlow, S.S. Bekir, P.K. Sorger, and I.S. Näthke. 2001. *Nat. Cell Biol.* 3:429–432). The localization of endogenous APC protein is complex: actin- and microtubule-dependent pools of APC have been identified in cultured cells (Näthke et al., 1996; Mimori-Kiyosue et al., 2000; Reinacher-Schick, A., and B.M. Gumbiner. 2001. *J. Cell Biol.* 152:491–502; Rosin-Arbesfeld, R., G. Ihrke, and M. Bienz. 2001. *EMBO J.* 20: 5929–5939). However, the localization of APC in tissues has not been identified at high resolution. Here, we show that in fully polarized epithelial cells from the inner ear, endogenous APC protein associates with the plus ends of microtubules located at the basal plasma membrane. Consistent with a role for APC in supporting the cytoskeletal organization of epithelial cells in vivo, the number of microtubules is significantly reduced in apico-basal arrays of microtubule bundles isolated from mice heterozygous for APC.

Introduction

Loss of functional adenomatous polyposis coli (APC)* protein occurs early in the progression of colon cancer (Powell et al., 1992). APC has been characterized most extensively in the context of the Wnt signaling pathway, where it is a crucial component of a protein complex that regulates the degradation of β-catenin (Peifer and Polakis, 2000). Recently, APC has emerged as an important cytoskeletal regulator. It binds to microtubules directly (Munemitsu et al., 1994; Näthke et al., 1996; Zumbrunn et al., 2001) and indirectly via EB1 (Su et al., 1995; Askham et al., 2000) and may also be involved in regulating actin dynamics via its interaction with Asef, a Rac-specific nucleotide exchange factor (Kawasaki et al., 2000). Consistent with the idea that APC has links to both F-actin and microtubules, a number of intracellular locations have been described for APC. In subconfluent cells, APC concentrates in clusters at the dynamic ends of microtubules (Näthke et al., 1996; Mimori-Kiyosue et al., 2000; Rosin-Arbesfeld et al., 2001). In highly confluent cultured cells, APC has been described in two major locations: in clusters near the basal surface and near the lateral plasma membrane (Näthke et al., 1996; Reinacher-Schick and Gumbiner, 2001; Rosin-Arbesfeld et al., 2001). The basal clusters require an intact microtubule network, whereas the localization to the lateral membrane is dependent on a stable actin network (Näthke et al., 1996; Rosin-Arbesfeld et al., 2001). Truncation mutations in APC found in sporadic and familial colon

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*Abbreviation used in this paper: APC, adenomatous polyposis coli.

Key words: microtubule organization; microtubule hook decoration; cytoskeleton; cochlea; min mouse

cancer lead to loss of its cytoskeletal association (Polakis, 1995; Polakis, 1997; Rosin-Arbesfeld et al., 2001), suggesting that this function of APC is important for the maintenance of normal epithelial function. Mutations in APC manifest themselves most prominently in polarized epithelial cells of the gut, making the localization of APC in polarized cells important to determine. The detailed analysis of APC localization with respect to cytoskeletal organization has been restricted to cultured cells so far. Because the correct three dimensional organization of epithelial tissues is of particular importance for their function, the information obtained from cultured cells may be incomplete. Therefore, we determined the distribution of APC protein in highly polarized epithelial cells in vivo using supporting cells from the organ of Corti in the inner ear (see Fig. 1 A for schematic). The organ of Corti consists mainly of a strip of neuroepithelial tissue. Sensory hair cells and the adjacent supporting cells are arranged in rows that extend along the length of this strip (Lim, 1986; see Fig. 1 A). This organization together with the cytoskeletal organization of the supporting cells is crucial for efficient transmission of vibrations to the sensory hair cells and thus for auditory perception (Patuzzi, 1996). Supporting cells contain an extremely high number of microtubules organized in an apico-basal array that facilitates the detection of low abundance proteins that specifically associate with microtubule ends (Henderson et al., 1994; Tucker et al., 1992, 1995; Mogensen et al., 1997). In comparison, epithelial cells in the gut contain an order of magnitude fewer microtubules, making their preservation and the detection of microtubule ends significantly more difficult.

We found that APC concentrates near the basal plasma membrane of supporting cells where microtubule plus ends terminate in a dense matrix. Hook decoration was used to confirm that the microtubules in these highly polarized cells are oriented with their plus ends near the base and their minus ends near the apex. Ninein, a microtubule minus end binding and anchoring protein is found near the apex of the cell, further supporting this organization (Bouckson-Castaing et al., 1996; Mogensen et al., 2000; Piel et al., 2000). During the development and assembly of this highly structured, polarized microtubule array, APC associates with microtubules as they extend toward the cellular base. These data suggest that APC may play an important role in the stabilization of the microtubule arrays during their formation. This was further supported by our finding that in the cochlea of *Min* mice, which are heterozygous for APC and express reduced levels of full-length APC protein, these apico-basal microtubule arrays showed a significant reduction in the number of microtubules present in the parallel bundles when compared with wild-type litter mates.

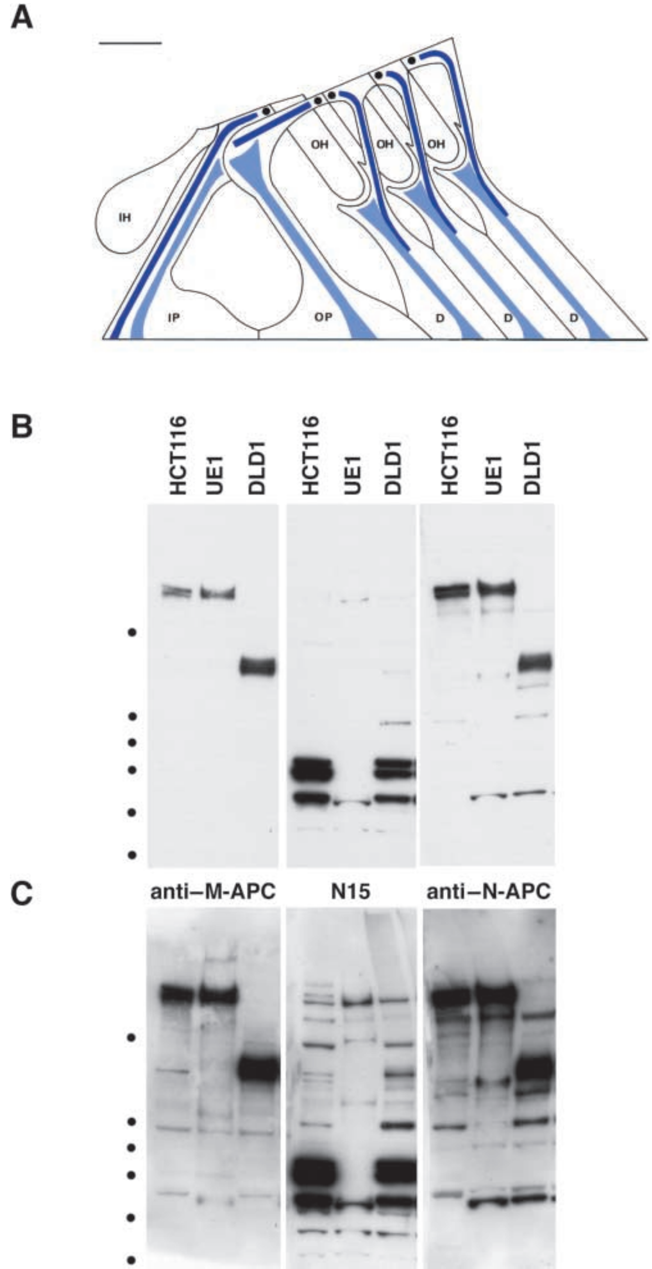

Figure 1. **Endogenous APC protein in cell lysates.** (A) Schematic of the organ of Corti. Inner and outer hair cells (IH and OH) are embedded into rows of highly polarized epithelial supporting cells, of which there are three types: inner pillar cells (IP), outer pillar cells (OP), and Deiters cells (D). The centrosomal microtubule bundles are shown in dark blue, whereas the noncentrosomal bundles are indicated in light blue. Centrosomes are represented by black dots. Bar, 10 μm. (B) Immunoblot of lysates from HCT116, UE1, and DLD1 cells using affinity-purified anti–M-APC polyclonal antibody, commercially obtained, affinity-purified anti–N-APC antibody N15, and a different crude anti–N-APC antiserum. (C) Overexposure of the blots shown in B shows many cross-reacting bands in cell lysates when the N15 antibody is used. Migration of 40-, 60-, 80-, 100-, 120-, and 200-kD standards is indicated by the bullets on the left.

Results and discussion

Specificity of APC antibodies

To confirm the specificity of available antibodies against APC, cell lysates from human colonic tumor cells with wild-type (HCT116) and truncated APC (DLD1) (Rowan et al., 2000) and UE1 cultured mouse inner ear cells expressing full-length APC (Lawlor et al., 1999) were probed with a panel of three different polyclonal APC antibodies (Fig. 1, B and C). Affinity-purified antiserum raised against the middle domain of APC, and crude serum raised against the NH_2-terminal domain (Midgley et al., 1997), detected APC as the major protein in all lysates (Fig. 1, B and C). A commercially available, affinity-purified anti-APC antiserum,

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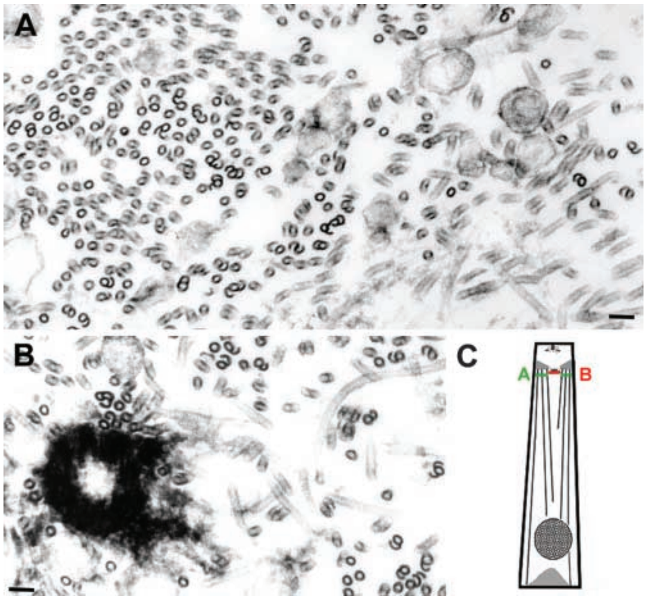

Figure 2. **Hook decoration in 6-d inner pillar cells.** (A) Electron micrograph of hook decoration in a cross section through the microtubule bundle below the apical sites showing predominantly counterclockwise hooks when viewed from the apex, indicating that microtubule plus ends point toward the basilar membrane. (B) Cross section through a centriole surrounded by microtubules showing clockwise and counterclockwise hooks indicating mixed microtubule polarity. (C) Schematic indicating the position of the cross sections in A and B. Bars, 50 nm.

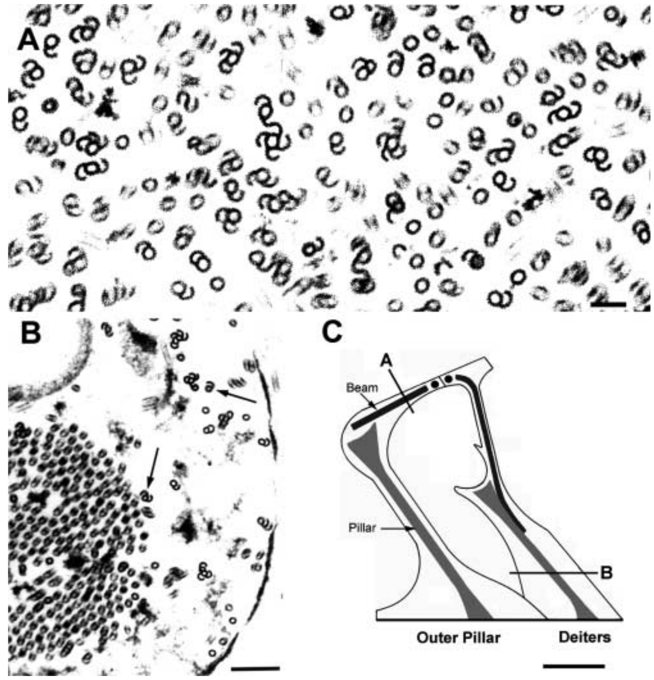

Figure 3. **Hook decoration in mature outer pillar and Deiters cells.** (A) Electron micrograph of cross section of the beam microtubule bundle in an outer pillar cell viewed from the centrosome. All unambiguously decorated microtubules show counterclockwise hooks, confirming that minus ends face the centrosome. Bar, 50 nm. (B) Cross section of the basal portion of a Deiters cell again viewed from the apex of the cell. Most of the bundle microtubules are closely packed together, preventing hook decoration. Hook-decorated microtubules are visible only at the edge of the bundle with counterclockwise hooks (arrows). Bar, 0.2 μm. (C) Schematic indicating the position of the cross sections shown in A and B. Bar, 10 μm.

N15 (raised against the NH_2-terminal domain of APC) did not detect any APC protein in the lysates from human cells, even after prolonged exposure and only very faintly detected APC in mouse UE1 cells. Instead, proteins with molecular masses of 65–85 kD were detected as major bands by this antibody in human cells lysates, and the 65-kD protein was also detected in the mouse cells. After longer exposure (Fig. 1 C), a band that comigrates with full-length APC appeared in blots exposed to N15 in all samples including DLD1 cell lysates, although these cells do not contain full-length APC. It is important to note that the gels shown in Fig. 1 (B and C) were simply scanned and not processed any further. Additionally, the entire gel lanes are depicted showing all proteins above 35 kD. These data confirm that the N15 antibody is not suitable for detecting endogenous APC. For our

Table I. **Hook curvature for apico-basally viewed microtubules in the inner pillar cells of a 6-d-old mouse**

	MT with counterclockwise hooks/hooks per MT[a]				MT with clockwise hooks/hooks per MT[a]				
	1	2	3	Total	1	2	Total	MT with ambiguous hook decoration[b]	Percent decorated MT[c]
Bundle									
A	80	4	0	84	16	1	17	26	50
B	73	1	1	75	7	0	7	43	30
C	60	7	0	67	4	0	4	27	35
D	40	8	0	48	10	0	10	22	34
E	22	4	1	27	2	0	2	6	18
Totals	275	24	2	301	39	1	40	124	Mean = 33

The number of microtubules (classified according to three main categories of hook decoration) for cross sections of bundles in five different cells (arbitrarily designated A–E) is shown. 88% of the unambiguously decorated microtubules bore counterclockwise hooks.

[a]Details of the number of counterclockwise, or clockwise, hooks per microtubule are shown for each bundle. Most microtubule profiles possessed one hook each.

[b]Details of the ambiguous patterns of decoration are given in the text.

[c]The percentage of cross-sectional microtubule profiles in each bundle that had been decorated.

Table II. **Hook curvature for apico-basally viewed pillar microtubules in the outer pillar cells of a 21-d-old mouse**

	MT with counterclockwise hooks/hooks per MT[a]					MT with clockwise hooks/hooks per MT[a]				
	1	2	3	4	Total	1	2	Total	MT with ambiguous hook decoration[b]	Percent decorated MT[c]
Bundle										
A	105	20	13	1	139	6	0	6	114	27
B	35	6	2	0	43	4	0	4	42	12
C	27	11	2	0	40	0	1	1	8	29
D	26	0	2	0	28	2	0	2	25	29
E	12	0	0	0	12	1	0	1	3	6
Totals	205	37	19	1	262	13	1	14	192	Mean = 21

95% of the unambiguously decorated microtubules bore counterclockwise hooks.
[a]Details of the number of counterclockwise, or clockwise, hooks per microtubule are shown for each bundle. Most microtubule profiles possessed one hook each.
[b]Details of the ambiguous patterns of decoration are given in the text.
[c]The percentage of cross-sectional microtubule profiles in each bundle that had been decorated.

studies, we used the affinity-purified anti–M-APC antibody. However, immunofluorescence staining with another anti–C-APC antibody (Midgley et al., 1997) gives identical results in cultured cells, and the staining pattern with either the anti–M-APC or anti–C-APC antiserum is independent of fixation conditions.

Microtubule positioning in supporting cells

The association of APC with microtubules in epithelial cells has been well documented and suggests that APC associates primarily with microtubule ends. However, microtubule polarity has only been inferred and never been directly demonstrated relative to APC accumulations (Näthke et al., 1996; Mimori-Kiyosue et al., 2000). Cultured colonic tumor cells, the subject of previous investigations, polarize their membrane domains when grown to confluency on glass coverslips, however, they rarely polarize their microtubule network under these conditions, making it impossible to identify specific microtubule ends and associated proteins unambiguously (unpublished data). To establish the localization of endogenous APC protein in polarized epithelial cells with a well-defined microtubule organization, we used supporting cells isolated from the organ of Corti (Fig. 1 A, schematic). These epithelial cells contain an apico-basal array of several thousand microtubules that provides a large target for end-associated proteins allowing their unambiguous detection.

We examined microtubule polarity in all three types of supporting cells in the organ of Corti: inner pillar cells, outer pillar cells, and Deiters cells. All three contain large microtubule arrays. Mature supporting pillar or Deiters cells contain two microtubule arrays whose ends are anchored at the apex and base of the cell. The apical end of one of the arrays in each cell is situated near the apical centrosome and its centrioles (Fig. 1 A, dark blue). The apical end of the other array is remotely located with respect to the centrosome (>10 μm distant; Fig. 1 A, light blue). The largest arrays occur in the pillar cells and include several thousand microtubules (Henderson et al., 1995; Tucker et al., 1995). In these cells, the microtubules splay at the cell base to either side of cone-shaped fibrous meshworks rather than terminating within them. The ends of many of these microtubules are situated within 0.5 μm of the basal membrane (see Fig. 4 D).

Table III. **Hook curvature for beam microtubules viewed looking away from the bundle's centrosomal end in the outer pillar cells of a 21-d-old mouse**

	MT with counterclockwise hooks/hooks per MT[a]					MT with clockwise hooks/hooks per MT[a]		
	1	2	3	4	Total	Total	MT with ambiguous hook decoration[b]	Percent decorated MT[c]
Bundle								
A	139	33	10	0	182	0	50	65
B	87	28	12	0	127	3	24	56
C	92	20	7	0	119	3	63	45
D	62	24	3	0	89	4	39	41
E	30	18	5	1	54	0	15	53
Totals	410	123	37	1	571	10	191	Mean = 52

All microtubules with clockwise hooks possessed one hook each. 98% of the unambiguously decorated microtubules bore counterclockwise hooks.
[a]Details of the number of counterclockwise, or clockwise, hooks per microtubule are shown for each bundle. Most microtubule profiles possessed one hook each.
[b]Details of the ambiguous patterns of decoration are given in the text.
[c]The percentage of cross-sectional microtubule profiles in each bundle that had been decorated.

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Table IV. **Hook curvature for apico-basally viewed microtubules in the lower portion of Deiters cells of a 21-d-old mouse**

	MT with counterclockwise hooks/hooks per MT[a]					MT with clockwise hooks/hooks per MT[a]		
	1	2	3	4	Total	Total	MT with ambiguous hook decoration[b]	Percent decorated MT[c]
Bundle								
A	14	2	0	1	17	0	8	26
B	8	1	0	0	9	0	0	16
C	5	2	1	0	8	0	6	29
D	4	1	3	0	8	0	2	16
E	4	1	0	0	5	0	2	21
Totals	35	7	4	1	47	0	18	Mean = 22

None of the microtubules bore clockwise hooks. 100% of the unambiguously decorated microtubules bore counterclockwise hooks.
[a]Details of the number of counterclockwise, or clockwise, hooks per microtubule are shown for each bundle. Most microtubule profiles possessed one hook each.
[b]Details of the ambiguous patterns of decoration are given in the text.
[c]The percentage of cross-sectional microtubule profiles in each bundle that had been decorated.

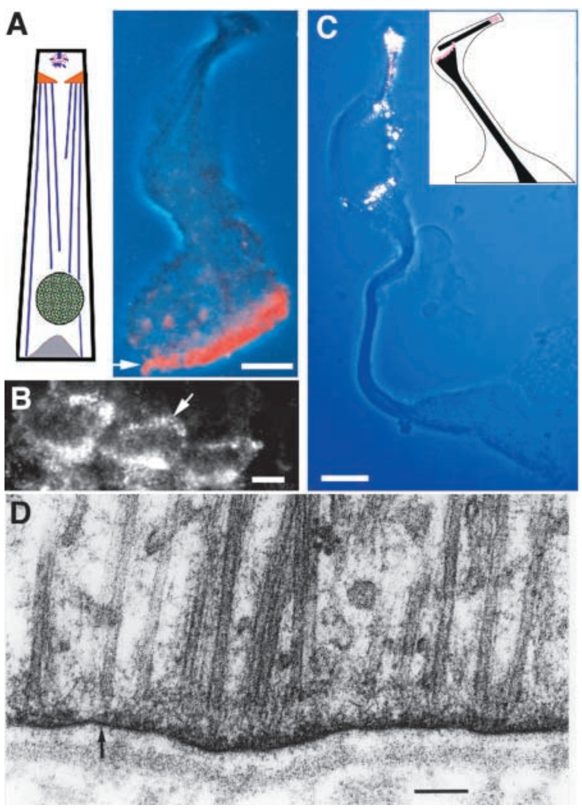

Figure 4. **APC protein concentrates at the basal surface of supporting cells.** (A) Schematic of microtubule organization in a 6-d-old inner pillar cell is shown on the left with the centrosome in pink, microtubules in blue, apical sites in yellow, and the nucleus in green. Immunolabeling of a 6-d inner pillar cell (right) reveals that APC is concentrated at the basal end of these cells (arrow). (B) Cross section through the basal region of three inner pillar cells stained with anti–M-APC antibodies confirming APC concentration near the base of the cell (arrow). (C) Mature guinea pig outer pillar cell stained with antininein antibodies shows localization of ninein to the centrosome and the apical region of noncentrosomal microtubule bundles. The inset shows the microtubule organization in outer pillar cells and ninein localization in pink. (D) Longitudinal section through part of the base of a microtubule bundle in a 6-d inner pillar cell shows microtubule ends embedded in a layer of dense material associated with the plasma membrane (arrow). Bars: (A–C) 5 μm; (D) 0.1 μm.

The microtubules in inner and outer pillar cells are arranged in apico-basal arrays with the plus ends at the basal membrane

To determine the orientation of the microtubule array in the supporting cells we performed hook decoration experiments in the organ of Corti from three mature and one 6 d cochlea when assembly of the apico-basal array is still progressing (for a detailed description of these results see online supplemental material available at http://www.jcb.org/cgi/content/full/jcb200203001/DC1). In summary, hook decoration revealed uniform polarity in all of the four types of microtubule bundles that were investigated with microtubule minus ends located at the apical cell surface sites and the plus ends at the cell base (Figs. 2 and 3 and Tables I–IV). The beam of mature outer pillar cells is an exception as it runs parallel to the apical surface. Beam microtubules reveal minus ends at the centrosomal region and plus ends distally. The microtubule polarities reported here are consistent with previous information obtained from spatio-temporal analyses of microtubule assembly and elongation in supporting cells (Henderson et al., 1994, 1995; Tucker et al., 1992, 1995).

APC protein concentrates near the basal membrane in highly polarized inner ear epithelial cells

In 6-d-old mice, pillar cells are still completing their differentiation, so that the phalangeal processes are not yet formed. At this stage, each inner pillar cell contains a single apico-basal array of ~3,000 microtubules. Staining of isolated inner pillar cells with anti–M-APC antibodies revealed that APC concentrates near the basal surface (Fig. 4 A). Cross sections near the base of inner pillar cells show APC in a ring-like configuration at the level of the cone-shaped fibrous meshwork that forms at the basal region of these cells (Fig. 4 B). Ninein, a microtubule minus end binding and anchoring protein is evident at the centrosome and at the apical sites where thousands of microtu-

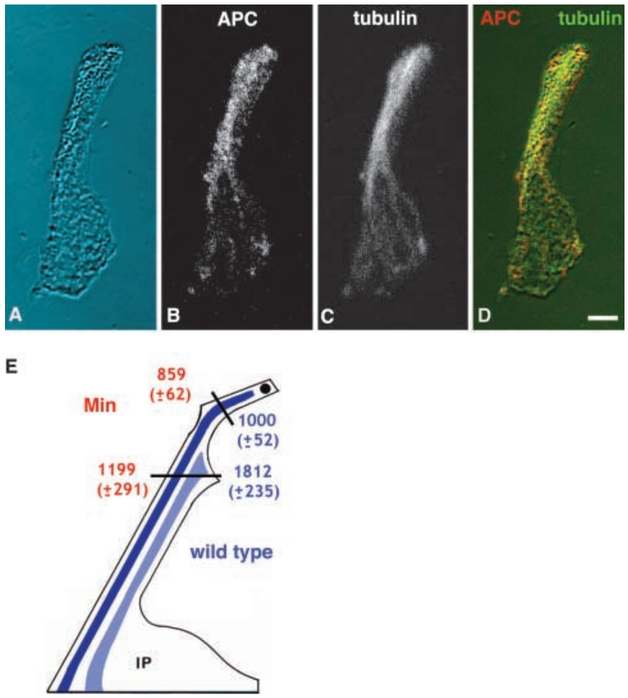

Figure 5. **APC localization in developing supporting cells.** (A) Phase-contrast image of developing inner pillar cells immunolabeled with antibodies against APC (B) and tubulin (C). (D) Staining of the incompletely extended microtubule bundles decorated with APC (red) and tubulin (green). (E) Comparison of microtubule bundles in supporting cells from normal and mice heterozygous for APC (*Min*) revealed a reduction in the number of microtubules in the microtubule arrays in *Min* mice. Positions of the different sections that were used to quantitate the number of microtubules in the bundles are indicated by red lines. The mean of the number of microtubules detected in equivalent regions in wild-type and *Min* littermates is shown in blue and red, respectively, and standard deviations are indicated in parentheses. For each region, microtubules in 3–10 individual sections taken from different cells in two different *min* or control animals were counted.

bule ends are concentrated in the pillar cells at this stage (Mogensen et al., 2000). Here, we show that ninein localizes to centrosomes and the apical ends of both the centrosomal and noncentrosomal arrays of the pillar cells in mature guinea pigs (guinea pigs were used as they contain larger cochlea that simplify isolation of the very delicate mature pillar cells; Fig. 4 C). This localization of ninein confirmed the hook decoration data and provides additional data for those bundles that could not be analyzed by hook decoration due to accessibility problems.

The localization of APC to the basal membrane of these highly polarized cells shows that APC protein concentrates at the plus end of microtubules. Based on its ability to stabilize microtubules and the consistent localization to microtubule plus ends, it is possible that APC is important for the selective stabilization and capture of microtubule plus ends to allow the formation of the highly ordered microtubule arrays in polarized epithelial cells. Once the microtubule network is fully established in supporting cells, the microtubule ends near the basal membrane are embedded in a dense matrix (Fig. 4 D) that may be involved in anchoring these ends and stabilizing them to maintain the organization of the microtubule array.

The establishment of the highly organized microtubule array in the supporting cells involves microtubule elongation toward the basal membrane (Tucker et al., 1992; Mogensen, 1999). To distinguish if APC is involved in stabilizing microtubule ends during their extension toward the basal membrane or whether APC is bound to the basal membrane to bind and/or stabilize microtubule ends once they reach the basal membrane, we determined the localization of APC protein in immature supporting cells containing microtubule bundles that had only partially extended toward the basal membrane. In these cells, APC protein localizes along the growing microtubules, and not to the basal membrane (Fig. 5, A–D), confirming that APC associates with growing microtubules. Accumulation of APC at growing ends is impossible to detect in the immature cells because microtubules in the growing bundles are heterogeneous in length, so that ends are distributed at several levels along the bundles. However, the association of APC with these extending microtubules is consistent with the idea that APC aids in the formation of the apico-basal microtubule bundles by stabilizing these microtubules to support their extension.

As described previously, APC has been detected at a number of different intracellular sites in cultured tumor epithelial cells grown at high density: near the apical plasma membrane (Reinacher-Schick and Gumbiner, 2001), at the lateral plasma membrane in an F-actin–dependent pool, and near the basal membrane in microtubule-dependent clusters (Näthke et al., 1996; Rosin-Arbesfeld et al., 2001). In fully polarized cells from isolated tissue, we only detected APC in association with microtubules near the basal membrane, and it is likely that this site corresponds to the microtubule-dependent clusters described in confluent cultured cells. We did not detect APC in association with the apical plasma membrane or the well-developed actin network underlying the intercellular junctions of the supporting cells. These data are different from observations made on cultured cells, although the antibody used for our studies is identical to that used to demonstrate the association of APC with the lateral plasma membrane in cultured cells (Rosin-Arbesfeld et al., 2001). One possible explanation for this discrepancy it that the association of APC with microtubules is dominant and that in the presence of the large microtubule bundles present in supporting cells, APC is not available for F-actin association. An alternative explanation is that the actin filaments that are embedded within the microtubule bundles of the inner ear cells "satisfy" the F-actin binding of APC in these cells. Furthermore, it is likely that differences between cultured tumor cells and cells isolated from tissue also contribute to our observations.

Differences between cultured and tissue cells may also at least partially explain the difference in the localization of APC described in our experiments and those suggesting an apical localization of APC (Reinacher-Schick and Gumbiner, 2001). These studies were performed on cultured colonic tumor cells (HCT116 and DLD1) that were grown to confluency on glass coverslips for 4 d. We found that HCT116 and DLD1 cells do not polarize their microtubules into a parallel array under these conditions, so that the majority of microtubules remain in a radial array (unpublished data). The preferential association of APC protein with microtubules detected in several independent studies (Munemitsu et al., 1994; Smith et al., 1994; Näthke et al., 1996; Mimori-Kiyosue et al., 2000; Zumbrunn et al., 2001) predicts that in such cultured cells APC localization may be different from that observed in tissues where microtubules are fully polarized. In addition, the apical localization of APC reported by Reinacher-Schick and Gumbiner (2001) was determined using the commercially available antibody N15 directed against the NH_2-terminal region of APC. In our study, this antibody does not detect endogenous APC reliably, but reacts strongly with smaller proteins as shown in Fig. 1 (B and C).

Reduction in APC leads to a reduction in the number of microtubules in parallel bundles

The assembly, positioning, and maintenance of apico-basal microtubule arrays is an essential feature of the functional design of polarized epithelial cells. Our hypothesis that APC has a role in establishing the cytoskeletal organization of supporting cells predicts that loss of APC may result in the reduced ability of polarized epithelial cells to organize their cytoskeleton. Unfortunately, cells expressing only truncated APC are genetically unstable (Fodde et al., 2001) and are likely to carry mutations in many additional genes. This makes it impossible to unambiguously attribute any observed changes in cytoskeletal organization to loss of APC. Furthermore, deleting APC from mice prevents development past day E6, which is consistent with an important role for APC in epithelial cell organization (Moser et al., 1995). To determine whether animals heterozygous for APC exhibit any defects in the organization of their cytoskeleton, we examined mature cochlea from *Min* mice heterozygous for APC (Moser et al., 1995). The overall organization of microtubule bundles in *Min* mice was normal (unpublished data). However, a marked reduction in the number of microtubules was observed in the bundles (Fig. 5 E). Interestingly, a relatively greater reduction in microtubule number was observed in the middle region (66% of normal) compared with the apical region (86% of normal). There are a number of possible explanations for this observation: reducing the amount of APC may affect the stability of only a proportion of the microtubules, or it may compromise their continued growth so that bundles contain fewer microtubules relative to controls along the apico-basal axis. Alternatively, microtubules in centrosomally anchored bundles may be affected differently than those in noncentrosomally anchored bundles. Furthermore, it is also possible that APC is important for microtubule plus-end anchoring and maintenance of the apico-basal array.

In summary, our results confirm that APC protein plays a role in the organization of cytoskeletal elements in polarized epithelial cells. The organization of ordered cytoskeletal arrays is extremely important for the function of epithelial cells. Loss of the association of APC and the cytoskeleton due to truncation mutations, as those found in colorectal cancers, may contribute significantly to the progression of colon cancer.

Materials and methods

Hook decoration

Sheep brain tubulin protein was prepared by two cycles of depolymerization and polymerization as described previously (Prescott et al., 1992). Cochleas were dissected from CD-1 mice (Charles River Laboratories) as described previously (Henderson et al., 1994). Hook decoration was performed essentially using the procedures described in Mogensen et al. (1989), but using one fifth of the detergent concentration. Cochleas were prepared for transmission electron microscopy as described previously (Tucker et al., 1992; Henderson et al., 1994). For further details please see Online Supplemental Material (available at http://www.jcb.org/cgi/content/full/jcb200203001/DC1.

Immunolabeling of supporting cells

Cochleas from 2- and 6-d-old CD-1 mice and mature guinea pig (Dunkin Hartley strain) were dissected and immunolabeled as described previously (Mogensen et al., 2000) except that pillar cells were isolated by gently teasing the organ of Corti apart using forceps. Dilution of primary antibodies was 1:500 for M-APC (Näthke et al., 1996) and 1:1,000 for ninein (Mogensen et al., 2000). Secondary anti-rabbit Alexa-conjugated antibodies (Molecular Probes) were diluted 1:1,000. Fluorescent images were recorded with a Bio-Rad MRC 600 series laser scanning confocal imaging system operating in conjunction with a Nikon Microphot-SA or a Zeiss 410 LSM microscope. Digital image files were transferred to Adobe Photoshop® for image handling.

Immunoblots

Cell lysates were prepared in RIPA buffer (20 mM sodium phosphate, pH 7.4, 1% NP-40, 1% deoxycholate, 0.1% SDS, and 150 mM NaCl). Total protein content was determined using a protein assay (Bio-Rad Laboratories), and lysate corresponding to 40 μg of total protein was subjected to PAGE using 3–8% gradient gels (Novex and Invitrogen). Proteins were transferred to nitrocellulose in 380 mM glycine, 50 mM Tris, and 0.02% SDS at 30 V for 18 h, and APC was detected using three different primary anti-APC polyclonal antibodies, secondary antibodies coupled to HRP (Scottish Antibody Production Unit) and ECL (Amersham Pharmacia Biotech) as described previously (Zumbrunn et al., 2001). The crude anti–N-APC antiserum (Midgley et al., 1997) was diluted 1:1,000, anti–M-APC (Näthke et al., 1996) was used at 1.3 μg/ml, and N15 (Santa Cruz Biotechnology) was used at 1.5 μg/ml.

Online supplemental material

Details on the hook decoration on cochlea cells and a complete description and discussion of the hook decoration results are available at http://www.jcb.org/cgi/content/full/jcb200203001/DC1.

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