JCB Mini-Review

Where is APC going?

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Adenomatous polyposis coli (APC) protein has been thought to function as a tumor suppressor through its involvement in the Wnt/β-catenin signaling pathway. However, its connections to the cytoskeleton and microtubules in particular are becoming apparent, and the discovery of these new functions for APC is leading to a reevaluation of its role not only in tumorigenesis, but also in normal physiology.

Defects in the *adenomatous polyposis coli* (*APC*)* tumor suppressor gene are linked to the development of colorectal cancer (for review see Nakamura, 1993; Kinzler and Vogelstein, 1996). Somatic mutations in the APC gene occur in the majority of sporadic colorectal tumors, and germline mutations in APC are responsible for familial adenomatous polyposis, an autosomal dominant inherited disease. The APC protein (APC) is a large multidomain protein with a molecular mass of 300 kD (Fig. 1). APC's modular architecture led to attempts to identify binding partners for each domain, and the discovery that APC binds to β-catenin, a protein that functions in cell adhesion and Wnt-based signal transduction, provided the first significant insight into the biological function of APC in molecular terms (Rubinfeld et al., 1993; Su et al., 1993).

There have since been numerous exciting findings regarding the functional relationship between APC and Wnt signaling (for review see Cadigan and Nusse, 1997; Peifer and Polakis, 2000). However, little attention has been paid to the subcellular localization of APC. Recently, another important functional aspect of APC has been highlighted: its association with cytoskeletons. In these studies, the localization of APC has been examined in detail. In this minireview, we provide an overview of this newly emerging field in the study of APC, paying special attention to its subcellular distribution.

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*Abbreviations used in this paper: APC, adenomatous polyposis coli; DGL, *Drosophila* discs large; GFP; green fluorescent protein.

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APC at cell–cell adhesion sites

In various polarized epithelial cells, APC has been found at the sites of cell–cell adhesion. In normal mouse intestinal epithelial cells, immunoelectron microscopy showed APC to be localized in the cytoplasm with a significant concentration along the lateral plasma membrane (Miyashiro et al., 1995). The observation that the COOH terminus of APC directly binds to the PDZ domain of hDLG, a human homologue of the *Drosophila* discs large (DLG) tumor suppressor protein, also favored the notion that APC is associated with cell–cell adhesion sites and/or lateral membranes (Matsumine et al., 1996), since hDLG is distributed along lateral membranes. APC also showed some concentration at cell–cell contact sites in several cultured cell lines (Näthke et al., 1996; Fig. 2 B).

The occurrence of multiple APC proteins in a single species has been reported recently (for review see Dikovskaya et al., 2001), including APC and APC2/APCL with a smaller molecular mass in humans and mice, and dAPC and dAPC2/E-APC in *Drosophila*. Their central portion, containing armadillo repeats and the β-catenin binding region, is fairly conserved, but their COOH-terminal region diversifies significantly. Consequently, in *Drosophila* both dAPC and dAPC2/E-APC lack the PDZ-binding motif at their COOH termini, suggesting that they do not interact with DLG. However, recent studies have suggested a functional relationship between *Drosophila* APC and cell–cell adhesion. In *Drosophila*, dAPC2/E-APC, which is ubiquitously expressed and abundant in epithelial cells, was found to be concentrated at the apicolateral adhesive zones of epithelial cells (McCartney et al., 1999). This junction-specific concentration required an intact actin cytoskeleton, and depletion or mutation of dAPC2/E-APC in embryos resulted in partial defects in the recruitment of Armadillo, a *Drosophila* homologue of β-catenin, to the junctions (Yu et al., 1999; Townsley and Bienz, 2000). These observations suggest a role for *Drosophila* APC in the genesis and maintenance of the integrity of cell–cell junctions.

APC on microtubules

In addition to immunohistochemical analyses, overexpression experiments were also conducted to determine the intracellular localization of APC in several cell lines (Mune-

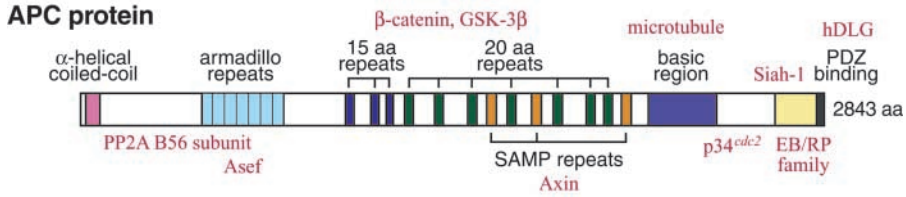

Figure 1. **The domain structure of APC protein.** Linear diagram of 2,843 amino acid human APC protein showing the relative positions of various domains. The known binding partners are presented in red.

mitsu et al., 1994; Smith et al., 1994). In these studies, the exogenously expressed wild-type APC unexpectedly appeared to be distributed along microtubules. Consistant with this observation, APC was shown to bind directly to microtubules throughout its COOH-terminal basic region, and to stabilize microtubules in vitro (Munemitsu et al., 1994) as well as in vivo (Zumbrunn et al., 2001) in a manner similar to conventional microtubule-associated proteins. In MDCK cells, endogenous APC was localized in clusters of puncta near the ends of microtubules at peripheral membrane sites of migrating edges, and this localization appeared to require intact microtubules (Näthke et al., 1996). Despite Näthke et al.'s (1996) influential images, the relationship of APC to microtubules has been largely neglected, because a great deal of attention has been focused on the function of APC that pertains to its ability to regulate β-catenin activity.

Movies were more influential than still images. Analysis of green fluorescent protein (GFP)-tagged APC (APC-GFP) in living *Xenopus* A6 epithelial cells uncovered a peculiar dynamic behavior of APC within cells (Mimori-Kiyosue et al., 2000a): APC-GFP moved continuously along a subset of microtubules toward their distal ends in an energy-dependent manner and accumulated as granular aggregates at the ends (Fig. 2 A). These movies, together with the results of other concurrent genetic and immunofluorescence studies on APC, prompted many APC researchers to turn back to microtubules (for review see McCartney and Peifer, 2000).

Movies showing the dynamic behaviors of APC and EB1 are supplemented in Mimori-Kiyosue et al. (2000a), Mimori-Kiyosue et al. (2000b), and Eccleston, A. 2001. *Trends Cell Biol.* 11:311.

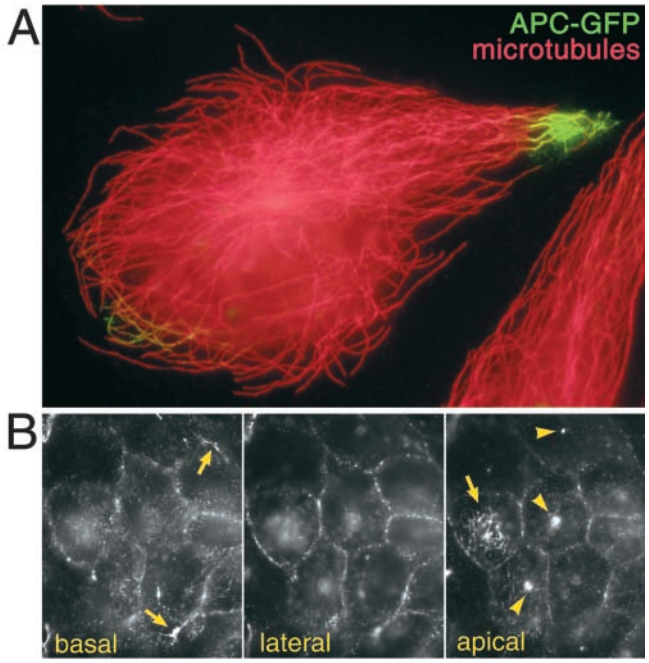

Figure 2. **Localization of APC-GFP (fAPC-mGFP) in *Xenopus* A6 epithelial cells.** (A) A6 cells expressing APC-GFP were fixed and stained for microtubules (red). APC-GFP (green) was localized along the ends of a subset of microtubules at the tip of a cell extension. (B) Confluent culture of A6 cells expressing APC-GFP. Cells were fixed and observed at distinct focus levels (basal, lateral, and apical levels). Most GFP signals were detected along the lateral membranes in a punctate manner. Some APC-GFP was concentrated along microtubules at both the apical and basal levels (arrows). Intense spot-like signals at the center of apical membranes (arrowheads) were derived from the roots of primary cilia.

Microtubule search-and-capture mechanism

Microtubules are structurally very dynamic, and their distal (plus) ends are the primary sites of growth and shortening, exhibiting dynamic instability (for review see Desai and Mitchison, 1997). Microtubule organization is highly polar, and microtubule dynamics vary considerably between different regions of the cell and stages of the cell cycle, suggesting that they are spatially and temporally controlled. Through intensive observations of such dynamic behavior of microtubules, Kirschner and Mitchison (1986) proposed a "search-and-capture" mechanism: during interconversion between growth and shortening of their plus ends, microtubules search for the sites (i.e., plasma membranes, chromosomes, organelles, etc.) with which they interact to capture, followed by stabilization and reorientation of the microtubule-based cytoskeleton (Fig. 3 A). However, at that time the molecular components involved in this search-and-capture mechanism remained undefined.

In recent years, mainly using GFP technology, several proteins that are specifically concentrated in transient segments at the growing plus ends of microtubules have been identified (Table I). These proteins are thought to be copolymerized into plus ends of microtubules where they remain for a while before dissociating from microtubules, allowing the existence of specialized transient segments at the plus ends of microtubules only during the growth phase (for review see Sawin, 2000; Schroer, 2001; Schuyler and Pellman, 2001). These findings led to the hypothesis that these transient segments (and also proteins specifically associated with these segments) are crucial for the search-and-capture mechanism of microtubules. Interestingly, from the viewpoint of the APC study, EB1, which was identified as an APC binding protein by yeast two-hybrid screening, was also included in this category of proteins (Su et al., 1995; Mimori-Kiyosue et al., 2000b, reviewed in Tirnauer and Bierer, 2000).

Recent studies in yeast showed that EB1 acts as a cross-linker between microtubule ends and the cell cortex. Bim1p, a budding yeast homologue of EB1, was identified as a tubulin-binding protein whose deletion causes defects in orienting

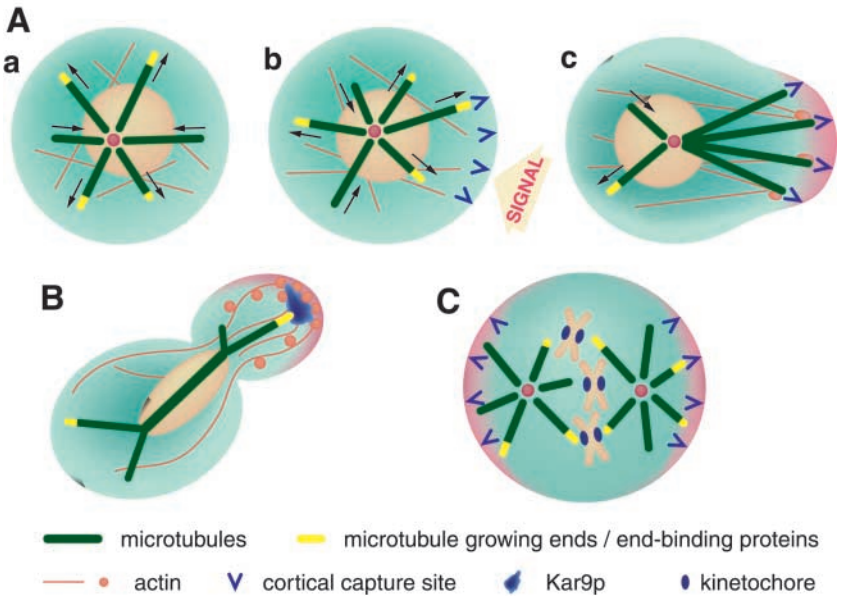

Figure 3. **Microtubules and search-and-capture.** (A) The microtubule search-and-capture mechanism, modified from Kirschner and Mitchison (1986). (a) In unpolarized cells, microtubules interconvert between growing and shortening with no preferred direction by dynamic instability, which enables microtubules to explore all over the cellular space. The growing microtubule ends are always capped by microtubule end binding proteins such as EB1. (b) A local spatial cue activates some microtubule-capturing site at the cell cortex. (c) Some microtubules are captured and selectively stabilized, which induces asymmetric orientation of the microtubule-based cytoskeleton. (B) In the budding yeast, the spindle microtubules originating from the spindle pole body on the nuclear envelope search for and capture the tips of daughter buds. The microtubule attachment to the cortex is mediated by interaction of EB1 (Bim1p) on the growing microtubule ends with Kar9p at the bud tip. (C) Microtubule search-and-capture mechanism during mitosis of higher organisms. Microtubules search for the kinetochores or attachment sites on the cortex to capture the chromosomes or to orient spindle microtubules, respectively.

spindles (Schwartz et al., 1997). In the budding yeast, the spindle microtubules search for and capture the tip of daughter buds to align spindles and thereby segregate the nucleus correctly (Fig. 3 B). Genetic analyses have revealed that spindle orientation requires several polarity proteins that localize to the tip of buds, including Kar9p which was originally identified in a screen for karyogamy mutants as a protein involved in nuclear migration (Miller and Rose, 1998). Interestingly, Bim1p was found to directly interact with Kar9p and to recruit it to microtubules (Korinek et al., 2000; Lee et al., 2000). Therefore, it is now believed that Bim1p on the plus ends of microtubules captures Kar9p at the tips of buds to assist spindle orientation and faithful cell division. It is interesting to note that EB1 is conserved in a wide range of organisms from yeast to human, but no Kar9p homologues have yet been found in other species. This raises the question of the identity of the counterpart of Kar9p in higher vertebrates. Given the affinity between EB1 and APC, as well as the subcellular localization of APC, it is tempting to speculate that APC is one of the functional counterparts of Kar9p in multicellular organisms, although Kar9p and APC show no structural similarity.

APC during mitosis

If APC is one of the counterparts of Kar9p, it is likely that in higher organisms, the APC–EB1-based microtubule-capturing process occurs during cell division (Fig. 3 C). This led Jan and colleagues (Lu et al., 2001) to test the function of dAPC2/E-APC in epithelial cell division in *Drosophila* embryos. *Drosophila* neuroepithelial cells divide in a symmetric manner, but when adherens junctions were destroyed, they divided in an asymmetric manner. Similarly, depletion of dAPC2/E-APC or *Drosophila* homologue of EB1 (dEB1) by the RNA interference (RNAi) method induced asymmetric cell division. Although the direct binding between dAPC2/E-APC and dEB1 was not detected in vitro, these findings suggested the involvement of dAPC2/E-APC and dEB1 in determining spindle orientation. Moreover, in dividing neuroblasts, dAPC2/E-APC was shown to be asymmetrically localized at the cortex in a crescent adjacent to one spindle pole (McCartney et al., 1999), suggesting that dAPC2/E-APC is also involved in asymmetric cell division.

Recently, two independent groups reported that APC is localized at kinetochores in mitotic cells, the microtubule attachment sites of chromosomes, and that APC mutant cells are defective in spindle formation and chromosome segregation (Fodde et al., 2001; Kaplan et al., 2001). Furthermore, Kaplan et al. (2001) showed that APC forms a complex with cell cycle checkpoint proteins Bub1 and Bub3 at kinetochores, and they proposed a model in which APC monitors the accurate attachment of microtubule ends to kinetochores. These findings led to the intriguing hypothesis that APC (and probably EB1) is essential for microtubules to search for and

Table I. **Proteins concentrated at growing microtubule plus ends in multicellular organisms**

EB/RP family proteins	Cytoplasmic linker proteins and its binding proteins	Dynein, dynactin complex and their binding proteins
EB1	CLIP -170	Dynein
RP1	CLASPs (CLIP-associating proteins)	$p150^{Glued}$
		Dynamitin/p50
		Arp1

capture the kinetochores during cell division. In contrast, it was reported that the APC–EB1 interaction is downregulated in mitotic cells (Askham et al., 2000). Thus, the above hypothesis should be evaluated experimentally in future studies.

APC during cell migration

The search-and-capture mechanism of microtubules could also work in migrating cells. During migration, microtubules are asymmetrically organized with their plus ends facing the leading edge of the cell. When the wound-healing process was observed using A6 cells expressing GFP-APC, in the front row of cells GFP-APC began to gradually concentrate at the distal ends of a subset of microtubules which appeared to grow continuously toward the wounded region (Mimori-Kiyosue et al., 2000a). EB1 appeared to be colocalized with APC only at these ends of microtubules (Mimori-Kiyosue et al., 2000b). Furthermore, APC appeared to associate with microtubules preferentially in migrating epithelial cells, but not in highly polarized cells (Näthke et al., 1996) (Fig. 2 B). These findings suggested that in migrating cells the APC–EB1 interaction has some important role in guiding microtubule plus ends to specific cortical sites, as observed in the tips of daughter buds of yeast.

Recently, the relationship between APC and the actin-based cytoskeleton has been investigated. Asef, APC-stimulated guanine nucleotide exchange factor, was identified as one of the binding partners for the armadillo repeat region of APC (Kawasaki et al., 2000). APC was shown to enhance the guanine nucleotide exchange factor activity of Asef, resulting in the activation of Rac, a small G protein. Rac activation was followed by actin polymerization at the cell periphery, manifest by membrane ruffling and lamellipodia formation in MDCK cells. This finding may provide an important clue to understand how APC is involved in the regulation of microtubule- and actin-based cytoskeletons.

APC in nucleus and apical membranes[99]

We should point out that there have been several reports in which APC has been localized to subcellular sites other than those described above. For example, APC was reported to be localized in the nucleus in various cellular systems (Neufeld and White, 1997), but this nuclear localization still remains somewhat controversial (Näthke et al., 1996). Furthermore, endogenous APC was recently reported to be concentrated at apical plasma membranes in a variety of polarized epithelial cells (Reinacher-Schick and Gumbiner, 2001). In contrast, in our own experiments in highly polarized A6 cells expressing APC-GFP, no intense signal was detected from apical membranes (Fig. 2 B), although exogenously expressed APC-GFP in culture cells does not always mirror the behavior of endogenous protein. In general, we have often encountered difficulty in detecting endogenous APC molecules by immunofluorescence microscopy, partly due to the low expression level of endogenous APC and to problems in the specificity of commercially available antibodies.

The cytoskeleton and cancer?

Patients suffering from familial adenomatous polyposis develop hundreds to thousands of polyps in the colon and rectum at an early age, a subset of which invariably progress to malignant tumors if not surgically removed. Polyp formation is initiated by abnormal accumulation of the intestinal epithelium at the crypt-villus boundary where, in the normal intestine, enterocytes migrate up toward the tips of villi maintaining the integrity of a tight layer of cells with concomitant differentiation. These findings suggest that APC may be involved in polyp formation by influencing not only the proliferation and differentiation, but also the migration and adhesion of epithelial cells (for review see Hülsken et al., 1994; Polakis, 1997; Bienz and Clevers, 2000). Indeed, as discussed above, evidence is now accumulating that APC has a crucial role in cellular migration and adhesion through its associations with the cytoskeleton. Therefore, further analyses of these newly identified functions of APC will lead to a better understanding of the molecular mechanism of the APC-based tumorigenesis.

From the viewpoint of cancer research, the idea that APC is directly involved in chromosome segregation as well as microtubule orientation during mitosis is also intriguing. This is particularly attractive when considering the genetic instability and the loss of epithelial polarity during tumorigenesis. In APC mutant mice, intestinal adenomas are polyclonal during the early stage of polyp formation, and this polyclonality appears to be responsible for tumor progression (Merritt et al., 1997). Human colorectal cancer cells were shown to have a marked defect in chromosome segregation (Lengauer et al., 1997). These observations could be explained by the chromosomal instability induced by the missearching and/or miscapturing of kinetochores in dividing cells due to APC mutations. On the other hand, the aberrant orientation of the cell division plane, which could also be induced by APC mutations, may affect the distribution of cells in the intestine, resulting in the loss of normal monolayer organization. Nevertheless, these ideas are still largely speculative, and further experimental support is needed to consolidate this presumptive role of APC in cell division as well as tumorigenesis.

Finally, we should discuss the relationship of the new functions of APC summarized in this review to its "classic" function, the destruction of β-catenin. Several lines of evidence suggest that the function of APC at the ends of microtubules is regulated by Wnt/β-catenin signaling. Without Wnt signaling, wild-type β-catenin is rapidly degraded and undetectable in the APC clusters at microtubule ends. However, an exogenously expressed stable β-catenin mutant, NH_2 terminally truncated β-catenin, accumulated in the APC clusters. Moreover, expression of NH_2 terminally truncated β-catenin impaired the migratory properties and formation of cellular extensions of MDCK cells (Barth et al., 1997; Pollack et al., 1997). These findings led to the speculation that increased stability of β-catenin (and resultant accumulation of β-catenin in the APC clusters) suppresses the APC function at microtubule ends (i.e., the extension of cellular processes through stabilization of microtubules). It has been widely accepted that APC regulates the function of β-catenin, but this speculation implies the reverse, that β-catenin regulates the function of APC.

The APC-based connections between the Wnt/β-catenin pathway and cytoskeleton are not yet apparent, but are likely to emerge in the coming years. We are only just beginning

to seek the missing pieces linking signal transduction to structure and/or vice versa.

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