Microtubules and actin filaments “cross-talk” in cell migration: role of the formin mDia and the tumor suppressor APC

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Abstract

Cell migration is a fundamental process throughout the lives of multicellular organisms as well as in the onset of several diseases including cancer formation and metastasis. Microtubules (MTs) and filamentous actin, the two major components of the internal scaffolding of all cell types, are important in several steps of the cell migration cycle and understanding their mutual regulation or “cross-talk” may lead to the development of treatments for controlling tumor invasion or severe developmental aberrations such as some forms of mental retardation. In this paper, I will present an overview on the importance of studying the biochemistry of molecules involved in the regulation of both types of cytoskeleton and summarize my results on the characterization of two key modulators of cell architecture: the actin nucleator mDia and the tumor suppressor Adenomatous Polyposis Coli protein or APC.

Introduction

Cell migration is a critical process throughout the life of every multicellular organism. Upon conception, cell migration is required for the morphogenesis of the embryo. Failure of cells to migrate, or migration of cells to inappropriate locations, can result in severe abnormalities or death. In the developing brain, for example, primitive neuronal cells migrate out of the neural
tube to specific distinct locations, where they send projections (axons and dendrites) to their final targets with which they form connections, called synapses, that allow complex functions such as learning and memory. Developmental processes continue throughout our lifetime as cells in our bodies are born, migrate, mature and die on a daily basis. The continuous renewal of skin cells and intestinal cells are two of many examples. In the adult, migratory cells are also involved in homeostatic processes such as generating an immune response and repairing injured tissues.

To this end, every day in our body, migratory cells move from place to place to complete specific functions such as wound repair, response to infections or cell differentiation. It is not surprising therefore that cell migration occurs in chronic human pathologies such as rheumatoid arthritis, atherosclerosis and cancer [1-4]. In malignant tumors, for example, cells that normally would not migrate start to move uncontrollably. Understanding this behavior, called metastasis, could significantly reduce cancer progression.

Cell migration, defined as a cyclical process in which a cell extends protrusions at its front and retracts its trailing end by exerting traction on an extracellular matrix, requires the integration of extracellular stimuli with changes in cell architecture. The cell front is the motor that pulls the cell forward. The rate of motility is highly variable for different animal cell types, with keratocytes from a fish scale capable of moving one cell diameter in about 2 minutes, as compared to about 1 hour required by a fibroblast, the most common cell in the connective tissue of animals. Cell migration is stimulated by migration-promoting or chemotactic agents, such as lysophosphatidic acid (LPA), which through the binding to their receptors at the cell

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1 the embryo’s precursor to the central nervous system in vertebrates.
2 a complex extracellular scaffold composed of structural and functional proteins (such as collagen, fibronectin and laminin) and proteoglycans.
3 cell types from the epithelia of fish epidermis.
periphery, lead to a cascade of intracellular signals. Transmission of these signals results in the initial reorganization of the cell along the axis of migration, a process known as polarization [5-7]. Members of the family of small G-proteins, PAR proteins (PAR6 and PAR3) and atypical protein kinase (aPKC) are involved in the initial polarization process. This is typically achieved by rearrangements of cell adhesiveness and organization of the internal scaffolding of the cell (the cytoskeleton) to define a cell’s front (or leading edge) and its tail [3].

Actin filaments and MTs, the two major filamentous components of the cytoskeleton, contribute to the establishment and maintenance of cell polarity downstream of small-G proteins activation [8]. Both actin filaments and MTs are polymeric filamentous structures generated by the linear addition of subunits, actin monomers in the case of actin filaments and α/β-tubulin heterodimers in the case of MTs. Because actin monomers and α/β-tubulin heterodimers are intrinsically polarized molecules, their multimerization results in the formation of polarized structures defined by a fast-growing “barbed” end and a slow-growing “pointed” end in the case of an actin filament or a fast-growing “plus” end and a slow-growing “minus” end in the case of a MT.

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iv a specialized tissue that is rich in extracellular matrix and that surrounds other more highly ordered tissues and organs.
v by homology to the oncogenic small guanine nucleotide triphosphate binding protein (GTP) RAS. Small G-proteins can cycle between active and inactive states.
vi a regulatory enzyme that catalyzes the transfer of phosphate groups from a donor molecule to an acceptor molecule. In cell biology they are involved in turning on and off protein activities.
vii in cell biology, downstream and upstream refer to the temporal order of events in a given cellular process.
In a polarized fibroblast, the actin cytoskeleton, which also provides the basic machinery for cell protrusion, is organized in different forms (Fig. 1): 1) a meshwork of branched actin filaments with associated unbranched bundles (filopodia) is typically found in the most anterior membrane extension of a migrating cell (the lamellipodium); 2) contractile bundles of actin filaments (stress fibers) are formed in the cell body and at the cell edge; 3) a loose network of thin actin filaments is localized throughout the cell. Sites of adhesion of the cell with the substrate are referred to as focal complexes or focal adhesions and are associated with lamellipodia and filopodia and the termini of actin stress fibers bundles. In migrating cells, the MT array, the other type of cytoskeleton, is polarized in two distinct ways (Fig. 1, 2): a subset of MTs is stabilized in the lamella viii at the leading edge, and the MT organizing center (MTOC) ix reorients so that it lies between the nucleus ix and the leading edge [9-12]. Locations of the MT organizing center (MTOC) and MTs radiating from it are thought to determine cell polarity by serving as linear tracks for the traffic of vesicles xi and for the localization of cytoskeletal regulators at the leading edge. However, how cells transmit external signals into changes in MT

Figure 1. Schematic diagram of the cytoskeletal and adhesion machinery in a migrating fibroblast

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vi an broader region right behind the lamellipodium.
ix the organelle composed of two orthogonally arranged structures (centrioles) nucleating all the MTs in the cell.
xii the organelle that contains the cell's hereditary material and controls the growth and reproduction of the cell.
xi small intracellular compartments delimited by a membrane and involved in trafficking protein cargos from one location in the cell to another.
arrays to bring about cell polarization and how these changes occur is only poorly understood.
Also, little is known about the molecules that directly modify and coordinate both types of cytoskeleton to regulate cell shape. It’s long been thought that several key regulators of the cytoskeleton are associated either with the actin or the MT cytoskeleton. Recently, however, the actin and the MT cytoskeletons are being seen as two interacting cellular structures that communicate and coordinate. Work from several laboratories is highlighting the importance of studying master regulators of cell shape in their effects on both MTs and actin filaments as a means to better understand the fine tuning that underlies any change in cell geometry. My work presented in this paper aims at testing whether two regulators of one type of cytoskeleton can also directly modulate the dynamics of the other.

**mDia**

Our laboratory developed a mammalian system in which wounded monolayers of mouse fibroblasts (NIH 3T3 cells) are induced to polarize their MT cytoskeleton toward a wound edge in order to migrate into the wound if triggered by the addition of external factors (LPA or serum) or if microinjected\(^{xii}\) with the active forms of the proteins involved in the pathway of MT polarization (Fig. 2). Through this experimental approach, the protein mDia (mouse Diaphanus)

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\(^{xii}\) a technique used to insert DNA or protein into a single cell using a microneedle.
was identified as the downstream effector of the small G protein Rho in the signaling pathway\textsuperscript{xiii} downstream of LPA that leads to the formation of oriented Glu MTs [10].

Glu MTs are a subset of stable (long-lived) MTs typically characterized by a residue of glutamine at the COOH-terminus\textsuperscript{xiv} of the $\alpha$-tubulin subunit (Glu MTs), as opposed to more dynamic (short-lived) MTs (Tyr MTs) where a residue of tyrosine\textsuperscript{xv} is included at the same position [13, 14]. In serum starved cells Glu MTs are induced by serum or LPA (both Rho activators) additions, or by activated forms of Rho alone [9]. mDia is a member of a family of proteins called formins containing one evolutionarily conserved domain\textsuperscript{xvi}, the Formin Homology 2 domain (FH2) which assembles unbranched actin filaments from the fast growing end [15, 16]. mDia’s ability to remodel the actin cytoskeleton has been implicated in cytokinesis\textsuperscript{xvii} and tissue morphogenesis as well as in the establishment of cell polarity and adhesion during cell migration, where it is required for filopodia formation and focal adhesion turnover [17-26]. mDia also regulates endocytosis, a process whereby cells ingest material from the extracellular space. It does so presumably by promoting actin coat formation around the engulfing membrane, a step necessary in the generation of endocytic vesicles (endosomes) [27-29].

Adjacent to the FH2 domain on the mDia molecule is also a Formin Homology 1 domain (FH1) that can accelerate the rate of filament elongation [16, 30-36]. In addition to FH1, FH2 (the domain responsible for both dimerization\textsuperscript{xviii} and actin nucleation), and a poorly defined FH3 domain, the mDia protein is characterized by the presence of two functional motifs\textsuperscript{xix} involved in

\begin{itemize}
\item \textsuperscript{xiii} a process consisting of a temporal sequence of protein-protein interactions.
\item \textsuperscript{xiv} the end of the amino acid sequence terminated by a free carboxyl group (-COOH).
\item \textsuperscript{xv} glutamine and tyrosine are two of the 20 amino acids that constitute proteins in all living organisms.
\item \textsuperscript{xvi} compactly folded element in a protein sequence with its own function.
\item \textsuperscript{xvii} the separation of a single cell into two daughters cells at the end of cell division.
\item \textsuperscript{xviii} the polymerization reaction of two identical molecules.
\item \textsuperscript{xix} a sequence in the protein that can include an entire domain or a recognition signature for other proteins.
\end{itemize}
the activation of the protein upon binding to small G proteins. Mutations in one of these regulatory domains in the sequence of the mDia gene in humans are associated with autosomal dominant\textsuperscript{xx}, nonsyndromic deafness\textsuperscript{xxi} and premature ovarian failure\textsuperscript{xxii} [37, 38]. Formins are also overexpressed in highly metastatic rat osteosarcomas\textsuperscript{xxiii} and leiomyosarcomas\textsuperscript{xxiv} suggesting that they regulate the cytoskeleton at the onset of these malignancies [39, 40]. Recently, our laboratory discovered two additional mDia and MT interacting partners: the MT plus end binding protein EB1 (End Binding 1) and the tumor suppressor protein APC (see below) [41]. Mutations in the APC gene cause 85% of colorectal cancers (both sporadic and familial) raising the question of whether mDia may also be involved in colon malignancies.

There is evidence that the MT stabilization induced by mDia in cells is achieved by the “capping” of MT plus ends, making them refractory to tubulin subunit exchange [10, 42]. As a consequence, MTs are no longer able to either shrink or grow, and are therefore stabilized. The biochemical mechanisms of MT stabilization by Rho/mDia proteins are still poorly understood and no knowledge is available on which proteins in the pathway directly stabilize this class of MTs. To date there is no evidence to support a role for formins in directly modulating the dynamics of the MT cytoskeleton, and it remains possible that MT stabilization is a secondary consequence of mDia mediated assembly and stabilization of actin filaments. My work aims at distinguishing between these two possibilities.

**The tumor suppressor APC**

APC is a large multidomain protein that, as mentioned, is implicated in the majority of colorectal cancers [43-45]. Mutations associated with tumorigenesis almost

\textsuperscript{xx} one only needs to get the abnormal gene from one parent to inherit the disease.
\textsuperscript{xxi} a hearing loss that is not associated with other signs and symptoms.
\textsuperscript{xxii} a syndrome affecting 1 out of 1000 women between 15-29 years of age.
\textsuperscript{xxiii} a type of bone cancer.
invariably delete the COOH-terminus of APC, suggesting that key functions reside in this region of the protein. Whereas COOH-terminal truncations of APC result in deregulated cell morphogenesis and cell proliferation, the earliest detectable defect in the intestinal epithelium from mice carrying this mutant protein is not increased cell proliferation, but rather changes in cell architecture and cell migration [46, 47]. This suggests that loss of cytoskeletal regulation by the COOH-terminus of APC may represent a key step in tumor formation.

APC protein displays a well established localization at clusters of MT plus ends in cells, and purified APC proteins promote the polymerization of tubulin into MTs in vitro [41, 48-51]. The region of the protein that interacts with MTs and tubulin has been localized to the COOH-terminal half of APC and includes the regions deleted by somatic and germline mutations of the APC gene, suggesting a role for this interaction in the onset of malignancies. Interestingly, APC has also been detected at actin-rich regions at the cell periphery and intercellular junctions in highly polarized cells in culture and in mouse intestinal cells [52-55]. More recently, APC has been observed to associate with other regulators of cell migration at the actin-rich leading edge of migrating cells [56], and loss of APC function in the fruit fly disrupts formation of critical actin structures (actin furrows) during embryogenesis [57].

However, for a variety of reasons, APC association with the actin cytoskeleton has remained controversial. First, difficulties with specificity in detecting the protein in cells have led to skepticism about non-MT-associated APC localization patterns. Second, molecular mechanisms connecting APC protein to the actin cytoskeleton have not been clearly defined, and

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xxiv a rare cancer arising from muscle tissue.
xxv Refers to an experiment conducted in a test tube as opposed to in vivo where the experimentation is carried out in living cells or a in a whole organism.
xxvi refers to any cell type in the body with the exception of the reproductive cells in the gonads (germline cells).
xxvii Development of the fertilized egg into an embryo.
no functional data have yet linked APC protein directly to the regulation of actin dynamics and/or organization.

Results

The formin mDia stabilizes MTs independently of its actin nucleation activity

To test whether actin nucleation and MT stabilization by mDia are two separate cellular functions, we generated two mutants (K853A and I704A) that were predicted to disrupt actin binding in a constitutively active version of mDia (consisting only of the FH1 and the FH2 domains). Both K853A and I704A mutants did not efficiently nucleate actin filaments in vitro, and did not induce actin polymerization or “surf” at the ends of actin filaments in vivo. However, both mutants still induced stable MTs in serum-starved cells and bound to the MT plus end binding proteins EB1 and APC, which have also been implicated in MT stabilization. A dimerization-impaired mutant of mDia (W630A) that failed to nucleate actin in vitro and in vivo also generated stable MTs in cells. Because dimerization is a necessary requirement for processive actin filament association by mDia, these results altogether show that the abilities of mDia to promote actin assembly and induce MT stability can be functionally uncoupled.

We examined whether mDia had direct activity on MTs in in vitro assays and found that both wild type and actin nucleation mutants bound to MTs and stabilized them against cold- and dilution-induced depolymerization. We mapped this stabilization activity in the FH2 domain, the region also involved in actin filament nucleation. Video microscopy of individual MTs nucleated from sea urchins-axonemal fragments showed that FH1FH2mDia decreased the

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xxviii refer to the positions of the introduced amino acid substitutions.
xxix Refers to the standard version of the gene or protein.
xxx structure from the flagellum of sperm cells that is composed of MTs aligned in parallel. It can nucleate MTs in vitro.
rates of MT polymerization and depolymerization indicating a mechanism of stabilization different from conventional MT stabilizing proteins (see discussion). These results indicate that mDia2 has a direct MT stabilization activity which is mechanistically novel and separate from its actin nucleation activity. Interestingly, we found that this activity maps within the domain (FH2) that is also involved in actin polymerization, suggesting that there may be competition between the two cytoskeletal elements for binding to mDia.

**The actin and the MT cytoskeleton compete for mDia binding**

We hypothesized that if MTs competed with actin filaments for mDia binding in cells, then the depolymerization of the actin cytoskeleton induced by drugs should redistribute mDia onto MTs and induce MT stabilization. We found that incubation of serum-starved 3T3 cells with different drugs capable of disrupting the structure or the dynamics of the actin cytoskeleton could also induce MT stabilization. Latrunculin A (Lat A)xxxi elicited the strongest response, and its mechanism of function was further examined. We tested whether induction of MT stability by Lat A could be positioned downstream of Rho activation and whether Lat A effect on MT stability was indeed dependent on mDia and its redistribution onto MTs. To this end, we first analyzed the effects of Lat A on MTs upon Rho inhibition or upon microinjection of EB1-C, a fragment of EB1 previously shown to block the formation of stable MTs downstream of mDia [41]. We found that Rho inhibition had no effect on the induction of MT stability by Lat A. However, injection of EB1-C, but not its functionally dead mutant version EB1-C KR, was sufficient to inhibit MT stabilization induced by Lat A. We concluded that the effect of Lat A on MT stability can be positioned in the LPA signaling pathway downstream of Rho activation and upstream of EB1, suggesting a direct modulation of the Rho effector mDia. To test whether

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xxxi a potent actin filaments depolymerizing drug that acts by reducing the pool of available monomeric actin.
mDia was necessary for induction of stable MTs by Lat A, mDia expression was silenced by siRNA technology\textsuperscript{xxxii}, and the Lat A effect on MT stability was tested in the absence of mDia. We found that cells silenced for mDia expression lost their ability to stabilize MTs upon incubation with Lat A, strongly indicating that mDia is a necessary requirement for induction of MTs stability induced by the disruption of the actin cytoskeleton. Accordingly, when the pattern of localization of mDia was analyzed in cells upon Lat A treatment, we found that a significantly larger population of mDia preferentially localized onto MTs compared to untreated cells where the majority of mDia appeared more randomly distributed. Re-localization of mDia to MTs upon actin disruption was confirmed by biochemical analyses of the levels of mDia associated to MT pellets from cells in culture treated with Lat A.

\textbf{Regulated binding of APC protein to actin}

We found that purified COOH- domain of human APC protein (APC-basic) bound directly to and bundled actin filaments and associated with actin stress fibers in microinjected cells. Actin filaments and MTs competed for binding to APC-basic \textit{in vitro} and \textit{in vivo}, but APC-basic also could cross-link actin filaments and MTs at specific concentrations, suggesting a possible role in cytoskeletal coordination. APC interactions with actin \textit{in vitro} were inhibited by its ligand EB1, and co-microinjection of EB1 prevented APC association with stress fibers. Mutations in EB1 that disrupted APC binding relieved the inhibition \textit{in vitro} and restored APC localization to stress fibers \textit{in vivo}, demonstrating that EB1-APC regulation is direct.

\textbf{Discussion}

\textsuperscript{xxxii} the technique based on the insertion of small inhibitory RNA molecules into a cell in order to silence the expression of a protein without eliminating the gene that codes for it.
Altogether our data provide evidence for a novel mechanism of induction of MT stability upon polarity cues and establish two solid precedents to start analyzing the MT and actin cytoskeletons as a whole structure rather than as two functionally independent entities.

**mDia**

Formins have been extensively characterized for their ability to nucleate and elongate actin filaments [58]. However, their regulation of the MT cytoskeleton, which has been documented both in dividing and migrating cells, is poorly understood. In migrating cells mDia is both necessary and sufficient to stabilize a subset of MTs (Glu MTs) arranged in the direction of migration downstream from Rho signaling [10, 59-61]. However, given the potent direct effects of mDia on actin assembly, it has remained unclear whether the MT effects might occur as a secondary consequence of mDia-induced actin assembly in cells. Here we show that promotion of actin polymerization and induction of stable Glu MTs by mDia can be functionally separated, as two actin polymerization-defective mutants of mDia were still capable of inducing stable Glu MTs in cells. We also demonstrate that a stable mDia dimer is not required to generate stable Glu MTs. As the stable dimer is thought to be the functional state of formins during actin assembly [33, 62, 63], this finding reinforces the notion that actin assembly and induction of MT stability are functionally separable. The finding that actin remodeling by mDia was not required for stabilizing MTs in cells prompted us to test whether formins could regulate MT stability directly. Indeed, we observed that an FH1FH2 fragment of mDia stabilized MTs assembled from purified tubulin against depolymerization induced by either cold or tubulin dilution. As induction of MT stability by mDia was also observed in assays with
perfusion chambers where the concentration of free tubulin is essentially negligible after dilution, we suspected that stabilization would be mediated by inhibition of polymer dynamics rather than by stimulation of dimer assembly. Consistent with this idea, we found no evidence for mDia interacting with tubulin dimers and observed a small, but significant, decrease in the rate of MT polymerization when mDia was incubated with tubulin during assembly. An even larger effect of mDia was observed on rates of MT shrinkage and in a small percentage of cases this effect led to MTs that exhibited shrinkage rates near zero. The ability of mDia to strongly slow MT shrinkage rates is presumably related to mDia’s role in generating stable MTs *in vivo* that do not shrink for extended intervals and behave as if they are capped [9, 10, 42]. In this respect mDia’s effect on MTs is distinct from traditional MAPs, which increase MT assembly rates without significantly interfering with MT shrinkage rates under similar experimental conditions [64-66].

While mDia’s ability to stabilize MTs *in vitro* is consistent with its activity in generating stable MTs *in vivo*, the *in vitro* dilution experiments showed that mDia only inefficiently caused MTs to stop shrinking completely, as is observed for stabilized MTs *in vivo*. This may reflect the experimental design of our experiments where MT depolymerization was rapidly induced by diluting MTs into solutions containing mDia. Another possibility is that full length mDia has significantly greater activity toward MTs than the FH1FH2 fragment we used in the *in vitro* experiments. It also seems likely that the capping effect observed *in vivo* requires additional cellular factors that function with mDia. Indeed, APC and EB1, two MT binding proteins themselves, interact with mDia and have been implicated in generating stable MTs induced by LPA [11]. Also, mDia
regulation of the kinase GSK3β, another modulator of MT stability, has been recently shown to promote MT stabilization in migrating fibroblasts [60]. It is possible that these factors and maybe others yet to be discovered contribute to cap MTs in vivo. This could be accomplished by the formation of a multiprotein complex at MT plus ends, in which all the players contribute to the stabilization activity. It will be important to determine whether these additional factors enhance the activity of mDia toward MTs.

FH1FH2mDia fragments can stabilize MTs directly by inhibiting MT dynamics and the stabilization activity maps to the FH2 domain of mDia, the most conserved region among formins. These observations indicate that induction of MT stability is likely to be a shared feature of many formins. It will be important to test whether other formins can stabilize MTs directly and whether this activity is required in other cellular processes in which formins have already been implicated, such as cell adhesion, cell division or organelle trafficking.

Our results show that the MT stabilization domain of mDia maps within the same region that is involved in actin polymerization (FH2 core domain). This finding raises the possibility that there is competition between the two cytoskeletal systems for mDia. Moreover, the activity of mDia for actin is in the high affinity range, whereas that for MTs is in the low affinity range. A simple competition model would predict that if actin and MTs exhibit mutually exclusivity for binding to mDia, then redistribution of mDia to the MT cytoskeleton can only occur if the affinity to MTs is significantly increased. Potentially, this may be mediated by any of several regulatory proteins already implicated in stable Glu MTs formation downstream of LPA signaling, including the MT plus end binding proteins APC, EB1[11], and the kinases PKC and GSK3β [60].
Selective activation of these mDia partners could localize mDia to MTs by preferential association of the complexes to MTs or by blocking high affinity actin binding sites on the formin itself. Indeed, when we mimicked an increase in the affinity of mDia to MTs by disrupting the actin cytoskeleton, the high affinity substrate of mDia in the cell, we observed a strong induction of MT stability and found that this effect was downstream of Rho and upstream of mDia. More importantly, because by silencing mDia expression we no longer detected an increase in MT stability upon Lat A incubation, we conclude that mDia is required for this process. Accordingly, we observed a significant redistribution of mDia to the MT cytoskeleton under the same experimental conditions, strongly suggesting the mDia is directly responsible for the increase in MT stability. Currently, we are examining several mDia interacting proteins, such as EB1 or the tumor suppressor APC and members of the kinesin and myosin families of motor proteins, in their ability to shuttle mDia from actin filaments to MTs and vice versa downstream of a migratory stimulus from LPA.

**APC**

Our results identify a new activity for APC protein and provide a functional explanation for APC protein localization to actin-rich cellular regions. EB1 directly controls APC protein association with MTs versus actin filaments. Our work raises a number of questions about the APC mechanism of interaction with actin filaments and its role in regulating actin dynamics and organization in cells. Future experiments will be directed at mapping APC residues that interact with actin, and identifying sites of direct APC-actin association *in vivo*. In addition, coordination of the actin and MT cytoskeletons by APC protein is likely to be regulated on many additional levels,
including the APC ligand mDia [41], and possible intramolecular interactions between the NH-xxxiii and COOH- termini of APC [29]. Because the APC protein COOH- terminus is deleted in most tumorigenic APC mutations, we suggest that this cytoskeletal function is involved in APC tumor suppressor function. In particular, we note that APC protein has been localized to actin-rich intercellular junctions [54, 55], which can be found in the intestinal epithelium. The ability of APC protein to bundle actin filaments and to link the actin and MTs cytoskeletons may be required for maintenance of cell-cell adhesion, and disruption of these activities may contribute to uncontrolled cell migration, with profound implications for tumor formation.

Bibliography


xxxiii the end of the amino acid sequence terminated by a free amino group (-NH2)


48. Mogensen, M.M., et al., *The adenomatous polyposis coli protein unambiguously localizes to microtubule plus ends and is involved in establishing parallel arrays


