JB Minireview—Signal Transduction Underlying Cell Morphogenesis *J. Biochem.* **134**, 321–326 (2003)
DOI: 10.1093/jb/mvg148

"Search-and-Capture" of Microtubules through Plus-End-Binding Proteins (+TIPs)

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Received June 6, 2003; accepted June 13, 2003

The generation of a polarized microtubule organization is critically important for proper cellular functions, such as cell division, differentiation and migration. Microtubules themselves are highly dynamic structures, and this dynamic property is temporally and spatially regulated within cells, especially at their plus ends. To explain how microtubules set up and make contacts with cellular structures, a "search-and-capture" mechanism has been proposed, in which the microtubule plus ends dynamically search for and capture specific sites, such as mitotic kinetochores and cell cortex. To date, several classes of proteins have been shown to be associated with microtubule plus ends in a wide range of organisms from fungi to humans and to play critical roles in the "search-and-capture" mechanism. In this review, we overview our current understanding of the "plus-end-binding proteins" (+TIPs), including APC (adenomatous polyposis coli) tumor suppressor protein, cytoplasmic linker proteins (CLIPs), CLIP-associating proteins (CLASPs), cytoplasmic dynein/dynactin, and EB1, an APC-interacting protein.

Key words: APC, microtubule, plus-end-binding proteins, +TIPs, search-and-capture, "Search-and-capture" of microtubules.

Abbreviations: +TIP, plus-end binding protein; APC, adenomatous polyposis coli; CLIP, cytoplasmic linker protein; CLASP, CLIP-associating protein; CAP-Gly, cytoskeletal associated protein-glycine rich motif; GEF, guanine nucleotide exchange factor; GSK, glycogen synthase kinase; EB1, end-binding protein 1; MAP, microtubul associated protein; PAR, partition-defective mutant.

The plus ends are the primary sites of growth and shortening of microtubules, exhibiting so-called "dynamic instability" (Fig. 1a, reviewed in Ref. *1*). Microtubule dynamics vary considerably depending on the region of the cell and the stage of the cell cycle: they are spatially and temporally controlled, and thereby their networks are polarized within cells. Based on intensive observations of the dynamic behavior of microtubules, Kirschner and Mitchison proposed a 'search-and-capture' mechanism: during inter-conversion between the growth and shortening of their plus ends, microtubules search for sites (*e.g.*, plasma membranes, chromosomes, organelles *etc.*) to interact with and capture; and this process is followed by stabilization and reorientation of the microtubule-based cytoskeleton (*2*) (Fig. 1a). At the leading edges of cells undergoing active migration, the plus ends of microtubules showed persistent growth when compared with microtubules in less motile cells (*3*, *4*). During mitosis, the major microtubule-capturing sites are the kinetochores, DNA-protein complex that connects microtubules to chromosomes, and the cell cortex. Recent studies have demonstrated that many signaling molecules such as small GTPases are implicated in the regulation of microtubule orientation (reviewed in Refs. *5* and *6*), but the molecular components involved in search-and-capture at the plus ends of microtubules have not been identified.

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Microtubule Plus-End-Binding proteins (+TIPs)

An important step towards a better understanding of the dynamics of the microtubule plus ends was provided by GFP technology, which enabled us to identify specific proteins that accumulate at the plus ends (Fig. 1b). To date, two distinct classes of such end-binding proteins have been described: end-binding proteins associating with growing microtubule ends, and end-binding microtubule destabilizers that induce their depolymerization. (Table 1). In this review, we focus on the former class of proteins, which can be further classified into two groups. The first group includes APC (adenomatous polyposis coli) tumor suppressor protein (Fig. 1b, left) and yeast kelch-repeat protein Tea1p. They are translocated along microtubules toward the plus ends by a kinesin-based motility. The second group comprises so-called "plus-end-tracking proteins" (*7*), which are specifically and directly concentrated at the growing plus ends of microtubules (Fig. 1b, right) (*8*). These proteins are thought to be copolymerized with tubulin dimers at plus ends of growing microtubules, where they remain for a while, and then dissociate from the tubulin in microtubules. Consequently, these proteins are exclusively concentrated in the specialized transient segments at the plus ends of microtubules only in the growth phase (reviewed in Ref. *9*). This group consists of heterogeneous populations of microtubule-associated proteins such as cytoplasmic linker proteins (CLIPs), CLIP-associating proteins (CLASPs), cytoplasmic dynein/dynactin complex including $p150^{Glued}$, and EB1, an APC-interacting protein. No

Table 1. **Proteins that bind to microtubule plus ends.**

1) Plus-end-binding proteins (+TIPs)
Proteins transported along microtubules toward the plus ends.
APC (adenomatous polyposis coli), transported by kinesin II complex KAP3/KIF3A/KIF3B
Tea1p, transported by kip2-kinesin Tea2p (*S. pombe*)
Proteins accumulated at growing microtubule plus ends (plus-end-tracking proteins).
Cytoplasmic linker proteins (CLIPs)
CLIP-170, CLIP-115, CLIP-190 (*Drosophila*), Tip1p (*S. pombe*)
CLIPs associating proteins (CLASPs)
CLASP1, CLASP2, MAST/Orbit (*Drosophila*)
Dynein/dynactin complex and binding proteins
Dynein, dynactin including p150^{glued} and Dynamitin/p50
Lis1, Pac1p (*S. cerevisiae*), NudF (*A. nidulans*)
EB/PR family proteins (APC- and dynactin-binding proteins)
EB1, RP1, Bim1p (*S. cerevisiae*), Mal3p (*S. pombe*)
2) Plus end-binding microtubule destabilizers
MCAK (mitotic centromere-associated kinesin) /KIF2 family proteins
MCAK, KIF2A, KIF2C, XKCM1 (*Xenopus*)
Dis1/XMAP215 family proteins
XMAP215 (*Xenopus*), Stu2 (*S. cerevisiae*), dCP224 (*Dictyostelium*)

Mammalian proteins and their orthologues from different organisms (indicated in parentheses) are listed.

structural similarity was found among these proteins, except that CLIPs and p150^{Glued} share a conserved CAP-Gly (cytoskeletal associated protein-glycine rich) motif in their microtubule-binding regions (*10*). These proteins autonomously bind to microtubule plus ends by themselves and, in many cases, stabilize microtubules while they are growing. For example, p150^{Glued} has potent activity for microtubule nucleation, and EB1 has an ability to elongate microtubules (*11*). In addition to their autonomous ability to bind to microtubules, they seem to bind to each other to form multimolecular complexes at the plus ends. Recently, the distal segment of microtubules was described as a "plus-end raft" that allows a cascade of protein interactions, as with lipid rafts (*9*).

Although the term "+TIPs" was originally coined to designate the plus-end tracking proteins, in this review we use it for all the plus-end-binding proteins including APC and Tea1p. As +TIPs are well-placed to regulate microtubule dynamics and the attachment of microtubules to various cellular structures, it is now speculated that +TIPs play a crucial role in the search and capture of microtubules.

Microtubule-Cortex Interaction in Yeast

The first direct evidence for the involvement of +TIPs in microtubule-cell cortex interaction was obtained in budding yeast. Bim1p, a yeast homologue of EB1, was identified as a tubulin-binding protein, the deletion of which caused defects in orienting spindles (*12*). In budding yeast, the spindle microtubules search for and capture the tip of daughter buds to align spindles and thereby segregate the nucleus correctly (Fig. 1c). Genetic analyses have revealed that orientation of spindles requires several proteins, including Kar9p, that localize to the tip of buds (*13*). Interestingly, Bim1p directly interacts with Kar9p and recruits it to microtubules *in vitro* (*14*, *15*). Therefore, it is now believed that Bim1p on the plus ends of microtubules captures Kar9p at the tips of buds to assist with spindle orientation and faithful cell division.

In fission yeast, the microtubules grow out from the cell center and align along the long cell axis (Fig. 1c). Tea1p, Tea2p and the CLIP-170 homologue Tip1p form a complex at the plus ends of microtubules, and the proper interaction of this complex with the cell cortex is thought to be important for the microtubule organization and cell polarization (for review see Ref. *16*). In *tea1* mutant cells, microtubules failed to stop growing when they reached the cell ends, and, as a result, curved around the cell end. In contrast, in both *tea2* and *tip1* mutants, Tea1p did not concentrate at the microtubule ends, and these cells bore short interphase microtubules, which shrank when they reached lateral cell membranes and could not continue to grow to the cell ends. As a consequence, all the mutants showed a bent or T shape due to the failure to maintain the growth zones at opposite ends. These results indicated that microtubules and/or +TIPs are critical to define the long axis of the cells in fission yeast.

+TIPs in migrating cells of multicellular organisms

In a motile eukaryotic cell, the plus ends of microtubules are stabilized in the protrusive zones comprising lamellipodia and filopodia. APC, an important tumor suppressor in human colon cancer (*17*), binds directly to microtubules and stabilizes them (*18*, *19*), and thus APC can be regarded as a conventional microtubule-associated protein (MAP). However, APC is a peculiar MAP: it is transported by kinesin motor proteins along selected microtubules, clusters at the plus ends of microtubules facing the leading edges (Fig. 1a) (*20–22*), and associates with basal plasma membranes to anchor the microtubules to the cell cortex (*21*, unpublished observations). Similarly, the distribution of CLASPs is polarized in migrating cells (*23*). However, GFP-fused CLASP2 appears to accumulate along selected microtubule ends in a distinct manner from APC. When microtubules grow into a special area (cortical capture site) on basal plasma membranes in the leading lamellae, GFP-CLASP2 attaches firmly to their distal end segments (unpublished observations). CLIP-170 and p150^{Glued} are frequently

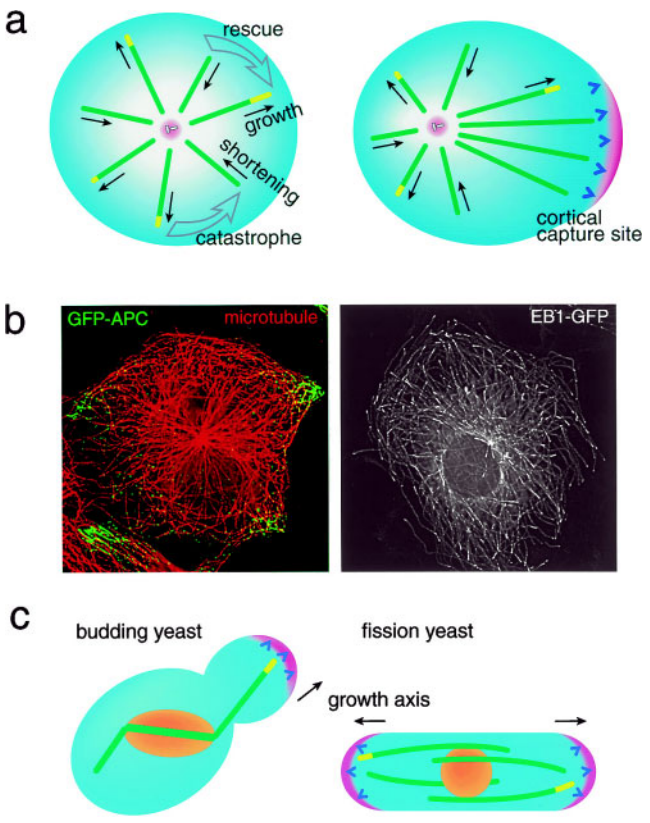

Fig. 1. **Distribution of +TIPs and the microtubule "search-and-capture" mechanism.** (a) Localization of GFP-fused APC and EB1 in *Xenopus* A6 epithelial cells. GFP-APC clusters at selected microtubule ends in the cell periphery, while EB1-GFP associates with every growing microtubule plus end throughout the cytoplasm. (b) The "search-and-capture" of microtubules. Adapted from Ref. *2*. In an unpolarized cell (left), microtubules undergo assembly/disassembly behavior known as dynamic instability, in which microtubules coexist in growing and shrinking populations that inter-convert stochastically. The parameters of MT dynamic instability are defined as growth, shortening, the transition from growth to shortening (catastrophe), and the transition from shortening to growth (rescue). If a signal input activates some microtubule-capturing site at the cell cortex, microtubules are captured and selectively stabilized, which induces an asymmetric orientation of the microtubule-based cytoskeleton. (c) The morphology of microtubules in budding and fission yeasts.

colocalized with CLASPs along the same microtubules, probably forming a multimolecular complex, whereas APC usually appears to be excluded from this complex. These findings suggest that CLASPs, CLIP-170, dynein and dynactin are accumulated at microtubule ends by a distinct mechanism from APC.

In cells, EB1 is found in both the CLASPs/CLIP-170/dynein/dynactin complex and APC clusters only in the presence of an intact microtubule (*24*, unpublished observations). Interestingly, EB1 is always concentrated at growing microtubule plus ends, even when CLIPs and dynein/dynactin are removed from microtubules (*25*), or in cancer cell lines expressing a truncated APC lacking the ability to bind to EB1 (*26*). Taking the characteristic behavior of EB1 during mitosis (see below) into consideration, it is likely that growing microtubule plus ends always carry EB1, and that these EB1-positive tips interact with other +TIPs to subsequently modulate the dynamics of microtubule ends. Possible networks of +TIPs are presented in Fig. 2a.

Several lines of evidence indicate that +TIPs associate with the cell cortex. A component of the dynactin complex, p62 subunit, associates with the actin cytoskeleton in the cell cortex (*27*), and CLASPs directly bind to the plasma membrane through palmitoylated NH_2 termini (*23*). These proteins may provide molecular linkages between microtubule ends and the cell cortex. Interestingly, dynein was reported to be recruited to cell-cell adhesion sites by β-catenin (*28*), and the dynein intermediate chain was shown to bind directly to a 24-kDa protein (PLAC-24) that is specifically recruited to sites of cell-cell contact together with components of adherens junctions (*29*). These findings suggested a crucial role for dynein in capturing microtubules at adherens junctions, although this hypothesis remains to be tested experimentally.

The question has naturally arisen as to how the interaction of +TIPs with MTs and/or plasma membranes is spatially regulated within migrating cells. To date, several attractive findings have been reported, but the information is still largely fragmentary. Inhibition of PI-3 kinase reduces the polarized localization of CLASP2, and overexpression of the constitutively active form of a glycogen synthase kinase 3β (GSK-3β) blocks the association of CLASP2 with microtubule plus ends throughout the cytoplasm (*23*). The microtubule-binding properties of CLIP-170 and $p150^{Glued}$ are also regulated by their phosphorylation, although their effects are complicated: PKA downregulates the interaction of $p150^{Glued}$ with microtubule ends (*30*), whereas the phosphorylation of CLIP-170 by rapamycin-sensitive kinase (FRAP) increases its association with microtubules (*31*). Furthermore, CLIP-170 was recently reported to interact with IQGAP1, an effector of the small G proteins Rac1 and Cdc42, at the leading edge of cells through its NH_2-terminal region including the CAP-Gly motif (*32*). Considering that CLIP-170 is an anti-catastrophe factor for microtubule ends (*33*), it would be interesting to further investigate the action of IQGAP1 on CLIP-170 as well as other CAP-Gly motif proteins such as CLIP-115 and $p150^{Glued}$. APC interacts with many signaling molecules, including Wnt signal-related components such as β-catenin, Axin, GSK-3β and a Rac-GEF called Asef (*34*, *35*). At least in part, its behavior is probably regulated by the Wnt signaling. Moreover, recently, Cdc42 in a Par6-atypical protein kinase C (aPKC) complex was reported to regulate the localization of APC through phosphorylation of GSK-3β at the migrating edges of cells (*36*).

These findings are only just beginning to shed light on the possibility that the signaling molecules somehow regulate the function of +TIPs. In future studies, the regulatory mechanism of the functions as well as the dynamic behavior of +TIPs in migrating cells should be further clarified.

+TIPs in dividing cells of muticellular organisms

During mitosis in multicellular organisms, many types of +TIPs accumulate at the kinetochore. It is widely accepted that dynein/dynactin are components of the kinetochore outer domain (*37*, *38*). Dynein/dynactin are

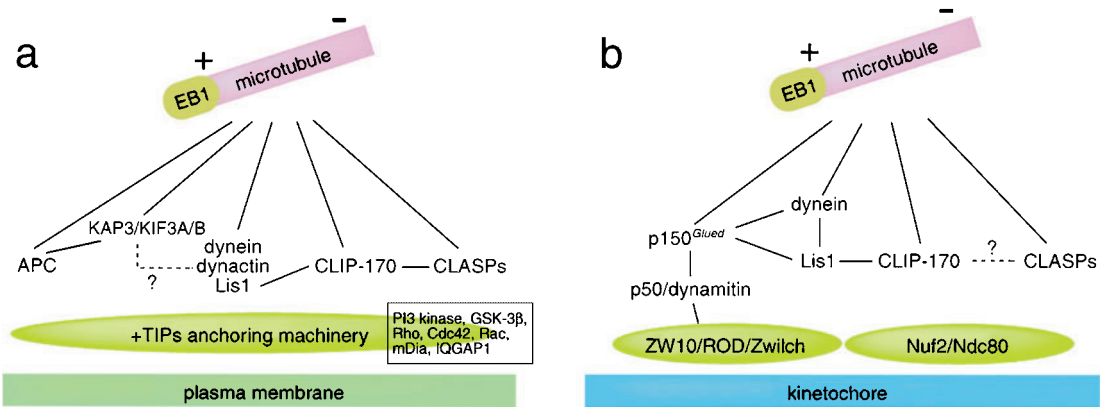

Fig. 2. **Interaction networks of +TIPs at the leading edges of cells (a) and at the mitotic kinetochores (b) in multicellular organisms.** Major +TIP components have an ability to autonomously bind to microtubules, but they also interact with other +TIP proteins at the microtubule ends. Among them, EB1 is unique in terms of its interaction with microtubules, as discussed in detail in the text. (a) At the leading edges of cells, +TIPs are concentrated at the cortex through unknown machinery, the formation/destruction of which may be regulated by various signaling molecules such as PI3 kinase, small GTPases, mDia, IQGAP1 *etc.* (b) At the mitotic kinetochores, the dynein/dynactin/Lis1/CLIP-170 complex is recruited to these structures by the ZW10/ROD/Zwilch complex prior to the microtubule capturing. CLASPs are also recruited to the kinetochores at the same time, although the targeting mechanism has not yet been clarified. EB1 is not recruited to the microtubule-unattached kinetochores, but time-lapse observation revealed that EB1 is transiently concentrated at the microtubule-kinetochore attachment sites when growing microtubule ends make contact with the kinetochores.

concentrated at the kinetochores by the ZW10/ROD/Zwilch complex (*39*, *40*), and in turn recruit Lis1 and CLIP-170 to kinetochores (*41*). At the onset of the prometaphase of mitosis, when nuclear membrane breakdown occurs, these molecules as well as CLASPs immediately begin to accumulate at microtubule-unattached kinetochores. In contrast, EB1 continuously marks the growing microtubule ends throughout mitosis and does not accumulate at microtubule-unattached kinetochores. However, when growing microtubule ends with EB1 reach the kinetochores by chance, EB1 appears to transiently accumulate at kinetochores (*42*, unpublished observations). These observations suggest that, at the kinetochores, EB1 and p150^{Glued}/CLIP-170/CLASPs have distinct functions, *i.e.*, as a searching probe or tag at microtubule plus ends and as the capture machinery on kinetochores, respectively. Then, the question arises as to which protein in the capture machinery of kinetochores is the binding partner for EB1. EB1 was shown to associate directly with dynein/dynactin, but, even when dynein/dynactin and CLIP-170 were removed from the kinetochores by overexpression of p50/dynamitin, EB1 was still transiently localized at the kinetochores (*42*). Recently, APC was reported to be concentrated at the kinetochores and to recruit EB1 to these structures (*43*, *44*). However, in our hands, APC was undetectable at kinetochores with APC-specific antibodies, and the transient recruitment of EB1 to the kinetochores was not abolished in several cancer cell lines expressing only a truncated APC lacking EB1 binding ability (unpublished observations). These results indicated that there should be binding partner(s) for EB1 other than dynein/dynactin and APC at the kinetochores, although it remains possible that a small amount of APC plays an important role at the spindle checkpoint by associating with BubRI at the kinetochores (*43*).

Once microtubules are attached to the kinetochores, the dynamics of microtubules appear to be regulated by the end-binding proteins to move, congress and separate the chromosomes. Recent studies revealed that the microtubules are stabilized by Mast/Orbit, a CLASP orthologue of *Drosophila*, to maintain a normal spindle bipolarity (*45*, *46*), and that MCAKs (mitotic centromere-associated kinesins) depolymerize the filament to pull the chromosome toward the pole (reviewed in Ref. *47*). Furthermore, dynein/dynactin are thought to be crucial for kinetochore proteins such as Mad2 and BubR1 to be transported to spindle poles along microtubules, which is an important process to inactivate the spindle checkpoint in mitosis (*48*).

During mitosis, a major pool of APC is found at the cell cortex, suggesting that APC is involved in orientating spindles along the axis of cell division. Indeed, in *Drosophila*, APC is important for both symmetrical and asymmetrical epithelial cell division, and also for anchoring spindles to the cell cortex: *Drosophila* neuroepithelial cells divide in a symmetric manner, but when adherens junctions are destroyed, they divide in an asymmetric manner (*49*). Similarly, when dAPC2/E-APC or the *Drosophila* homologue of EB1 (dEB1) was downregulated using the RNA interference (RNAi) method, asymmetric cell division was induced. In dividing neuroblasts, dAPC2/E-APC, which is asymmetrically localized at the cortex in a crescent adjacent to one spindle pole, is thought to be involved in asymmetric cell division (*50*). Moreover, during the syncytial mitoses of early *Drosophila* embryos, dAPC2/E-APC and its binding partner, Armadillo, were both shown to play important roles in anchoring spindles to the cortical actin cytoskeleton under regulation by Zeste-white 3 (GSK-3β) (*51*).

Dynein/dynactin/Lis1 complex, CLIP-170 and CLASPs are also distributed along the cell cortex of dividing cells, suggesting that they are involved in capturing microtubules to orient mitotic spindles. Indeed, when Lis1 was overexpressed or suppressed by antibody injection, spindles were misorientated in MDCK epithelial cells (*25*). In

Drosophila, MAST/Orbit was reported to be required for asymmetric division of stem cells: It recruits CLIP-190 to the microtubules and to the fusome, a membrane-rich cytoplasmic structure that anchors one of the spindle poles at mitosis (*52*).

Perspectives

Microtubule end-binding proteins are evolutionarily conserved as essential factors for microtubule orientation in various cellular events. However, knowledge is still fragmentary about how these proteins are involved in the search and capture of microtubules and how this process is spatially and temporarily regulated within cells. It is rapidly becoming clear how various polarity-related molecules such as small GTPases and PAR (partition-defective mutant) proteins work on the actin-based cytoskeletons in the cell cortex in different cellular systems, but the molecular linkage between these polarity-related molecules and microtubule end-binding proteins is still unclear. With more tools now in hand, the missing links should be elucidated in the near future. This field is only just beginning to open up.

During the preparation of this manuscript, interestingly KIF2A was reported to regulate microtubule dynamics by depolymerizing microtubules at growth cone edge to suppress axonal collateral branch extension [Homma, N *et al*. (2003) Kinesin Superfamily Protein 2A (KIF2A) Functions in Suppression of Collateral Branch Extension. *Cell* **114**, 229–239].

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