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REVIEW

EB-Family Proteins: Functions and Microtubule Interaction Mechanisms

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Abstract—Microtubules are polymers of tubulin protein, one of the key components of cytoskeleton. They are polar filaments whose plus-ends usually oriented toward the cell periphery are more dynamic than their minus-ends, which face the center of the cell. In cells, microtubules are organized into a network that is being constantly rebuilt and renovated due to stochastic switching of its individual filaments from growth to shrinkage and back. Because of these dynamics and their mechanical properties, microtubules take part in various essential processes, from intracellular transport to search and capture of chromosomes during mitosis. Microtubule dynamics are regulated by many proteins that are located on the plus-ends of these filaments. One of the most important and abundant groups of plus-end-interacting proteins are EB-family proteins, which autonomously recognize structures of the microtubule growing plus-ends, modulate their dynamics, and recruit multiple partner proteins with diverse functions onto the microtubule plus-ends. In this review, we summarize the published data about the properties and functions of EB-proteins, focusing on analysis of their mechanism of interaction with the microtubule growing ends.

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MICROTUBULES AND PLUS-END INTERACTING PROTEINS

Microtubules are one of three filament types of the cytoskeleton. They are present in all eukaryotic cells. The microtubule is a hollow cylinder built of 13 protofilaments, which are linear chains of tubulin protein heterodimers consisting of α- and β-subunits. The microtubule diameter is approximately 25 nm, and the length varies from tens of nanometers to tens of microns. The flexural rigidity of microtubules is significantly higher than the rigidities of all other cytoskeleton elements [1]. The microtubule network is very dynamic; it is constantly being renewed and rebuilt due to changes in the length of individual microtubules in its structure. Each microtubule is alternatively in growth or shrinkage phases and stochastically switches between these phases [2, 3]. This behavior of microtubules has been called "dynamic instability". The transition from growth to shrinkage phase and the transition back are called, respectively, "catastrophe" and "rescue".

Functions of microtubules are very diverse [4]. Microtubules from flagella and cilia, which allow cells to move, to create and sense fluid flows in the intercellular space [5]. Microtubules are also involved in intracellular transport, and they serve as main lines for transfer of various loads, such as organelles, vesicles, and signaling molecules. Microtubules can perform many functions due to their unusual dynamics. During interphase, their dynamic instability allows microtubules to robustly accommodate the network produced by them for transport and support and to effectively fill the intracellular space even in the case of frequent changes in cell shape, e.g. during axon growth [6]. Dynamic microtubules play an important role in the spreading and polarization of cells [7, 8]. Dynamic instability of microtubules is extremely important during

Abbreviations: APC, adenomatous polyposis coli (tumor suppressor protein); Bim1, binding microtubules protein 1 (yeast *S. cerevisiae* EB analog); CH, calponin homology domain; EB-proteins, end-binding proteins (family of proteins binding to growing microtubule ends); EBH, EB-homology domain; Mal3, microtubule integrity protein (yeast *S. pombe* EB analog); XMAP215, *Xenopus* microtubule-associated protein 215 kDa.

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mitosis for search and capture of chromosomes and their accurate distribution between the daughter cells [9].

Polarity of microtubules is manifested in the different dynamic properties of their ends. This difference is especially pronounced in living cells, but even in *in vitro* systems of purified tubulin one end of the microtubule grows more quickly than the other [10]. The fast-growing end of the microtubule with β-subunits of tubulin exposed is usually called the plus-end, and the opposite end is called the minus-end. In living cells, the minus-ends are usually stabilized and located nearer to the center, close to the centrosome, whereas the plus-ends are highly dynamic and oriented to the periphery. Dynamics of the microtubule plus-ends are regulated through many proteins interacting with tubulin or with the microtubules. The so-called plus-tip interacting proteins are a special class of microtubule-associated proteins. They are microtubule regulators different in structure and functions, which are mainly located on the growing ends of microtubules [11-13]. Plus-tip interacting proteins can be exemplified by microtubule polymerase XMAP215 (*Xenopus* microtubule-associated protein 215 kDa) [14], the kinesin family members that promote polymerization or depolymerization of microtubules [15-18], several kinetochore proteins, such as the yeast protein complex Dam1 [19], and many other proteins binding to microtubules auto-nomously or through adaptor proteins. One of the most important and abundant among plus-tip interacting proteins is the family of end-binding proteins (EB-proteins), which can autonomously bind to the microtubule growing ends.

GENERAL CHARACTERISTICS OF EB-FAMILY PROTEINS

EB-family proteins are present in the cells of all eukaryotic organisms [20]. They are one of the most numerous proteins binding to the microtubule plus-ends. For example, in cultures of fibroblasts their amount is 7-30 times higher than the amount of protein of the next most abundant family of plus-tip interacting proteins [21]. EB-proteins of yeast and some other model organisms have been studied rather extensively. The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have one protein from this family, Bim1 (binding microtubules 1) and Mal3 (microtubule integrity protein 3), respectively. The nematode *Caenorhabditis elegans* has two, and the fruit fly *Drosophila melanogaster* has three representatives of EB-proteins. In mammals, EB-proteins are also represented by three proteins: EB1, EB2, and EB3 [22].

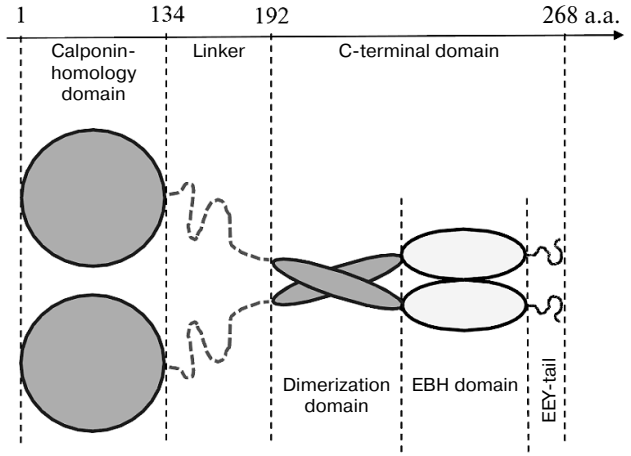

Fig. 1. Scheme of the structure of human protein EB1.

EB-proteins are conservative globular proteins containing about 300 a.a. [23-25]. They consist of three structural domains: the N-terminal calponin-homology domain, the linker, and the C-terminal domain [25-29]. The structure of a typical EB-protein, human protein EB1, is presented in the scheme in Fig. 1.

Calponin-homology domain of protein EB1 consists of 133 a.a. This part of the protein determines its affinity for the microtubule growing ends [24, 26, 28-30]. The N-terminal domain of the EB-family proteins is called "calponin-homology" because of its homology with domains found in actin-binding and signaling proteins [31, 32]. In accordance with this, it has been shown that the EB proteins also have affinity for actin [33]. A calponin-homology domain is also present in some other microtubule-binding proteins, such as the protein Clamp (calponin-homology and microtubule-associated protein) and the kinetochore protein Hec1 [34, 35]. EB1 protein region from 134 to 192 a.a. forms a linker, which is an unstructured and presumably flexible part of the protein that serves a binding link between the N- and C-terminal structural domains. Notwithstanding its relatively low evolutionary conservativeness, the linker of EB-proteins essentially contributes to efficiency of the binding to the microtubule growing ends [24, 36]. The C-terminal domain of EB1 is formed by residues 193-268 a.a. and includes a coiled coil domain responsible for protein dimerization, the unique EB-homology domain (EBH), and the unstructured flexible acidic tail ended by amino acids EEY/F. EB-family proteins interact with many partner proteins through the EBH-domain [13, 26, 37]. This domain recognizes a short polypeptide motif Ser-x-Ile-Pro (SxIP) that is necessary for binding of several plus-end proteins, including, for instance, the APC (adenomatous polyposis coli) tumor suppressor protein, the endoplasmic reticulum calcium-sensitive protein STIM1 (stromal interaction molecule 1), the microtubule depolymerase from the kinesin-13 family MCAK (mitotic centromere-associated kinesin), etc. [37].

In the literature, it has been proposed that EB-proteins can be present in both an active and autoinhibited state when the N- and C-terminal domains interact with

each other. Similar behavior is well known for kinesins [38]. However, the existence of autoinhibition among EB-proteins is not reliably established and remains a matter of controversy. This hypothesis was first proposed by Hayashi et al. [39] based on data on tubulin polymerization in solution in the presence of a high concentration of EB1. The mutant protein EB1 lacking 20 a.a. on the C-end was shown to be more favorable for tubulin polymerization than the full-length protein EB1. This observation became the basis for the hypothesis of EB1 autoinhibition through a direct intramolecular interaction between the calponin-homology domains and flexible C-terminal tails [39]. But later it became clear that removal of the negatively charged C-terminal tail increased the affinity of EB1 for the whole microtubule body, lowering the "preference" for the microtubule growing end [32]. This leads to a simpler alternative explanation of the influence of the EB1 C-terminal tail on the rate of tubulin polymerization. Moreover, if the C-terminal tail interacted with the calponin-homology domain of EB1, it would be reasonable to expect that the full-length protein would be "folded" due to the intramolecular interaction, whereas the mutant protein deprived of the C-terminal tail would be "unfolded". However, this has not been confirmed by data of small angle X-ray scattering [32]. Nevertheless, recent studies using nuclear magnetic resonance have revealed an interaction between EB1 protein fragments containing the calponin-homology and the EBH-domains [40]. Additional experimental studies are required to clarify whether the detected interaction is realized in the context of full-length EB1 protein, and if such interaction occurs what functions and properties of EB1 it can regulate.

The EB-family proteins are capable of homo- and heterodimerization, and they usually exist as dimers [23, 26, 27, 41]. It is interesting that in mammals, heterodimers can be produced only by two of three types of proteins of this family, namely by proteins EB1 and EB3, whereas EB2 mainly produces homodimers [23]. Dimerization is not necessary for localization of the EB-proteins on the ends of growing microtubules, at least in the cells of mammals and *in vitro* [24, 42]. However, the dimerization can be important for the interaction with partner proteins and for influence on microtubule dynamics [23-25].

PARTNER PROTEINS AND EB REGULATORS

EB-family proteins form a basis (platform, central node) of the functional group of proteins acting on the microtubule plus-end. The literature contains an abundance of information on partners of EB1 and EB3 and significantly less data on partners of EB2 protein. The table shows that the partner proteins can be divided into two global groups depending on their interaction with EB-proteins: regulatory partners binding to EB-proteins beyond the microtubule plus-ends, and partners interacting with EB-proteins on the microtubule plus-end. These groups, in turn, can be divided formally depending on the interaction site (e.g. SxIP or Cap-Gly); however, the functional role of these proteins does not correspond to the interaction site used. It should be noted that the motif SxIP is represented rather widely among different proteins [43] and has a simple structure of a small polypeptide region of serine-x-isoleucine-proline [37]. It can be presumed that, from the evolution standpoint, this allows this motif to relatively easily arise on different proteins and thus introduce an ability to bind to the microtubule end due to the acquired affinity for EB-proteins. However, the formal presence in the partner protein of the SxIP motif does not in all cases result in interaction with EB just through this region [44, 45].

Listing all properties of the known EB partner proteins is beyond the limits of this review; however, we can exemplify some pronounced and well-studied partner proteins, such as protein APC (protein EB1 was initially identified by interaction with it), protein p150glued (protein 150 kDa dynein-associated dynactin 1), and protein XMAP215. Protein APC is a tumor suppressor and regulates mitotic spindle dynamics, binding of microtubule plus-ends with kinetochore, cell migration, cell proliferation, neurogenesis, and development of axons/dendrites [46-57]. Protein p150glued is a part of the dynactin complex and regulates the loading and transport of cargos that are transferred with involvement of dynein [58]. XMAP215, in turn, is tubulin polymerase [59], and in the presence of EB1 it increases the microtubule polymerization rate up to physiological values [60]. However, it should be noted that XMAP215 does not now have an identified interaction motif with EB. Thus, it seems to be an example of a protein that does not interact with EB1 directly, but the effect of XMAP215 on the microtubule polymerization rate is sensitive to the presence of EB1.

FUNCTIONS OF EB-PROTEINS IN VARIOUS CELLULAR PROCESSES

Because EB-family proteins are key elements of infrastructure of the microtubule network in the cell, they directly or through partner proteins participate in virtually all processes, which in one way or another are associated with functioning of this network. EB-proteins are present in mammalian cells during all phases of the cell cycle, but their individual activities are different for different tissues [61]. Among the three mammalian proteins (EB1, EB2, and EB3), the role and functions of protein EB1 are studied in more detail, the role of EB3 is studied mainly in neurons, and protein EB2 is studied the least. The yeast homology proteins Bim1 and Mal3 are now considered functional analogs of protein EB1.

Classification of EB partner proteins

Location of a functional interaction of a partner protein with EB	Mechanism of direct binding/interaction with EB-proteins	Some characteristic representatives of this type of partner proteins	Functions and role of interaction of this type of partner proteins with EB
Mainly on the growing microtubule plus-ends	binding through calponin-homology domain and linker	α- and β-tubulin [29]	EB location onto microtubule plus-ends
	binding through EBH-domain (mediated through SxIP motif on partner protein)	APC [46, 47], MCAK (mitotic centromere-associated kinesin) [37], CLASP1/2 (cytoplasmic linker-associated protein) [111]	modulation of microtubule dynamics, regulation of binding and interaction with kinetochore, cell proliferation, etc.
	binding through EEY/F-tail (mediated through Cap-Gly motif on partner protein)	CLIP-170 (cytoplasmic linker protein 170 kDa) [112], p150glued (protein 150 kDa dynein-associated dynactin 1) [112]	modulation of microtubule dynamics, regulation of load transport, etc.
	binding through dimerized EBH-domain	yeast (*S. pombe*) protein – polymerase of microtubule Dis1 (defective in sister chromatid disjoining 1) [86]	regulation of chromosome segregation
Outside of microtubule plus-ends	binding through calponin-homology domain and linker	actin [33]	regulation of microtubule stability in actin-rich zones of cell cortex
	binding through C-terminal domain of EB	MAP1B (microtubule-associated protein 1B) [113], Tau MAP2 (Tau microtubule-associated protein 2) [44], ATIP3 (angiotensin II type 2 receptor-interacting protein 3) [45]	regulation of EB amount in cytosol, regulation of EB binding kinetics

To illustrate the variety of the EB-family proteins, we list some examples of their functions and properties in different cell types and at different stages of the cell cycle.

The yeast protein Bim1 from the EB-family at normal expression level is localized only on the mitotic spindle and on the microtubule plus-ends [62], whereas in the case of increased expression it covers the entire length of the microtubule [63]. Its removal does not completely suppress the ability of yeast cells for budding, but it makes them extremely sensitive to temperature or to presence in the medium of additional substances influencing microtubule dynamics, such as benomyl [63]. Note that the removal of Bim1 influences cell phenotype, which is manifested by a decrease in microtubule network dynamicity, especially in the G1 phase, and in defects of the mitotic spindle position at the budding [62].

In *S. cerevisiae* cells, the only representative of EB-family, protein Mal3, can be found on the dynamic ends of cytoplasmic microtubules and on the mitotic spindle microtubules. Removal of this protein leads to major disorders in morphology of the network of cytoplasmic microtubules, making them shorter and often not reaching the cell edges. This is associated with disturbance in polarity and shape of the yeast cells, changes in the usual position of their nuclei, and with increase in frequency of errors during chromosome segregation [64, 65].

In higher organisms, protein EB1 is also located mainly on centrosomes and microtubule plus-ends [66, 67]. During the whole cell cycle, EB1 accompanies the ends of polymerizing microtubules; therefore, its levels on the plus-ends, as well as the amount of such active plus-ends, reflect changes in the activity of the microtubule network. Thus, analysis of the number of EB comets in LLCPK cells (pig kidney epithelial-like cell line) revealed that the nucleation rate of microtubules increases fourfold between the G2 phase and prophase, continues to grow during the anaphase and telophase, and becomes sevenfold higher compared with the interphase [68]. In general, the presence of EB1 is necessary for correct mitosis in mammalian cells. EB1 together with the EB1-dependent plus-end partner proteins is present on mammalian kinetochores, and it contributes to establishing and maintaining interactions between dynamic microtubules and chromosomes [69]. The absence of EB1 leads to anaphase-specific cortical blebbing and asymmetry of the mitotic spindle position. This is associated with inheritance by cells with more pronounced asymmetry of more pronounced changes in the number of microtubules, which indicates an

important role of EB1 in mitotic spindle functioning and in regulation of cortical contractility of the cell during the late stages of mitosis [70].

It is interesting that the mechanism of search and capture of intracellular cargos by plus-ends of dynamic microtubules with involvement of EB1 and EB1-dependent partner proteins is not limited to the search and capture of chromosomes during the early stages of mitosis. A similar principle is also realized in interphase cells at the initiation of the minus-end transport of membrane organelles, such as Golgi apparatus membranes or melanosomes [58, 71].

Functions of EB-family proteins in developing neurons have been studied rather extensively (this theme is covered in a review by van de Willige et al. [21]). The microtubule network plays a key role in neurons during both their maturation and in the matured state, because microtubules are both directing elements during the growth of axons and dendrites and main tracks providing the transport along their length. As a rule, EB-proteins support more stable and processive growth of microtubules and decrease the number of catastrophes [24]. As a result, they play an important role during the elongation of nerve cells. In neuroblastoma cells, the stage of axon growth coincides with an increase in EB1 expression [72]. In hippocampus cells, EB3 expression increases concurrently with the decrease in EB1 expression. Microtubule plus-ends decorated with EB3 come into dendrite spines, and the appearance of EB3 in the spines is accompanied by their growth, whereas EB3 removal leads to loss of the spines [73]. In the cells of drosophila, EB1 loss leads to a strong shortening of the axons and to general disorganization of the microtubule network [74]. It is interesting that effects of EB1/3 and EB2 can be opposite when EB2 knockout results in an increase (instead of decrease) in the length of the young dendrite/axon. This result can be caused by a different affinity of these proteins for partner proteins and by their competition for binding sites on the microtubules [75].

The role of EB-proteins and their partners is important far beyond the presented examples of regulation of microtubule dynamics in interphase cells, contribution to building the correct mitotic spindle, interaction of chromosomes and microtubules in mitosis, and initiation of the minus-end transport of organelles. EB-proteins and their partners can also promote coordination of dynamics of microtubules and their filaments, anchoring of microtubule ends on the cell cortex, accumulation of signaling molecules on the microtubule ends, etc. [76].

INFLUENCE OF EB ON MICROTUBULE DYNAMICS *in vivo* AND *in vitro*

As we already mentioned, the integral effect of EB-proteins and their partners on microtubule dynamics *in vivo* usually manifestes itself by suppressing catastrophes and increasing the rate of microtubule growth [24]. Moreover, there are some data indicating that EB1 also can somehow increase the frequency of microtubule rescues, despite the absence of this protein on the microtubule end during depolymerization [77].

Do EB-proteins themselves contribute directly to the regulation of microtubule plus-ends dynamics, or is their role limited to the delivery of regulatory proteins onto the microtubule plus-ends? Experiments with living cells usually do not answer this question, because the removal of EB-proteins from the microtubule ends inside the cells inevitably disturbs functioning of their numerous partners that are localized to the microtubule growing end by EB-proteins. Therefore, studies *in vitro* on microtubule dynamics in the presence of purified EB-proteins are very interesting. Such studies have revealed that the EB-family proteins increase, rather than decrease the frequency of microtubule catastrophes [24, 60, 77-79]. Concurrently, EB-proteins also increase the rate of microtubule polymerization [24, 77, 80, 81]. However, this effect in the presence of only EB-proteins is rather low and cannot be an explanation of the difference between the rates of microtubule polymerization *in vitro* and *in vivo* without consideration of the influence of additional protein factors. At tubulin concentration of 15 μM that seems to be close to its concentration in cells [82], the rate of microtubule polymerization is usually only 3-4 μm/min *in vitro* [10], whereas the rate of polymerization *in vivo* is up to 20-40 μm/min [24, 83]. The EB1 content of 400 nM in the *in vitro* experiment (at tubulin concentration of 7 μM) leads only to slight increase in the rate of microtubule polymerization (<50%). The recombinant purified protein polymerase XMAP215 in turn increases several-fold the rate of microtubule growth *in vitro*, from 1 to 6-7 μm/min [60]. The combination of EB1 with XMAP215 has a synergistic effect on microtubule polymerization that allows it to reach *in vitro* growth rates up to 20 μm/min, which is close to values observed in cells [60]. It is also known that EB1 and XMAP215 binding sites on the microtubules are different and not overlapping, which is evidenced by different profiles of microtubule ends decoration with these proteins *in vivo* [84] and *in vitro* [85]. Although a recent study revealed binding of the EB1 and XMAP215 homolog in drosophila cells [86], no direct interaction was detected between EB1 and XMAP215 in mammalian cells [60]. Therefore, it can be concluded that the role of EB-proteins is not limited to attracting regulatory partner proteins onto the microtubule ends, but it can also include a direct and rather pronounced influence on microtubule dynamics in cells. Speaking about the progress in experiments for *in vitro* reconstitution of the cellular dynamics of the microtubule network, it is important to mention a study of Moriwaki et al. [82]. This study revealed that addition to the EB1 and XMAP215 (Msps in *D.*

melanogaster) of a combination of three purified recombinant proteins (kinesin-13 (Klp10A), CLASP (cytoplasmic linker-associated protein), and Sentin) resulted in the *in vitro* reconstitution of a system, where changes in the microtubule dynamics upon removal of individual protein components corresponded to effects observed upon the knockout of these proteins in cells.

MECHANISM OF INTERACTION OF EB-FAMILY PROTEINS WITH MICROTUBULE GROWING ENDS

A fundamental question about the mechanism of the surprising ability of EB-proteins to locate on the microtubule growing ends arose from the moment of the discovery of this phenomenon. Two principal ideas have been suggested – a hypothesis of tubulin copolymerization together with EB-proteins, and a hypothesis of "recognition" by EB-proteins of some structural or chemical features of the microtubule end compared to its body. The situation became clear after the first observations of the *in vitro* interaction of EB-proteins and microtubules. First, gel filtration failed to find a direct interaction of the yeast protein Mal3 with unpolymerized tubulin [78]. Second, the duration of individual interaction acts of EB-protein molecules with the microtubule growing end was significantly shorter than expected according to the copolymerization hypothesis [87, 88]. This argument and others led to the convincing rejection of the hypothesis that EB-proteins were located on the microtubule growing ends due to copolymerization with tubulin and dissociated later from the microtubule body.

Thus, it has been shown that EB-proteins interact with the microtubule growing ends mainly through binding to the ends of growing but not of depolymerizing microtubules. What structural features of the microtubule growing end make them different from the microtubule body and are "recognized" by EB-family proteins?

Before answering this question, the literature data concerning the structure and chemical composition of the ends of growing and depolymerizing microtubules should be addressed. In 1991, it was shown by cryoelectron microscopy that the ends of the microtubules depolymerizing *in vitro* had outwardly curved protofilaments with broken lateral bonds [89]. Tubulin oligomers, which dissociated during depolymerization, were also curved, with the curvature radius of ~20 nm. These observations were confirmed many times by studies performed both *in vitro* [90, 91] and *in vivo* [92]. However, the shape of the microtubule growing ends is still under discussion. According to the first cryoelectronic studies *in vitro*, the microtubule growing ends are mainly cylindrical, blunt, or slightly flared [89]. However, Chrétien's group, using the same method, systematically found long slightly curved structures on the growing microtubule ends *in*

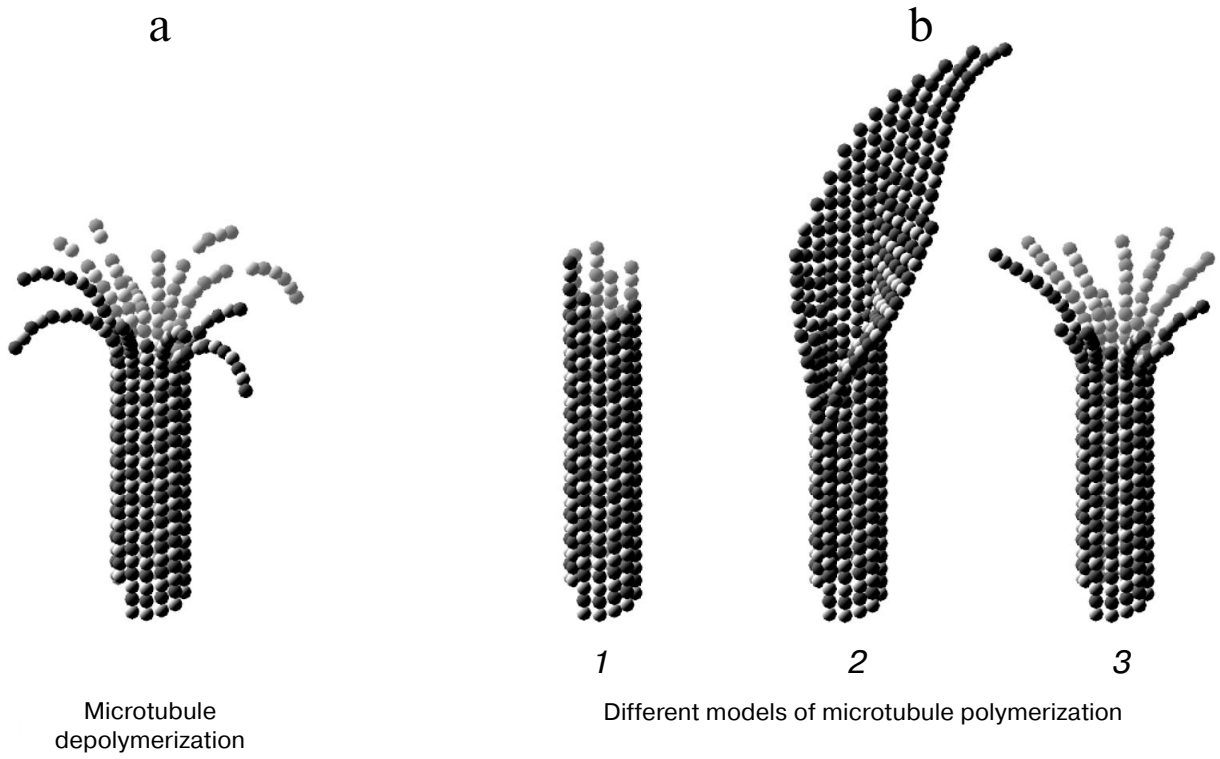

Fig. 2. Schematic illustration of shapes of dynamic microtubule ends: a) shape of depolymerizing microtubule end; b) possible different shapes of growing microtubule ends: *1*) blunt end; *2*) open sheet; *3*) funnel-like end.

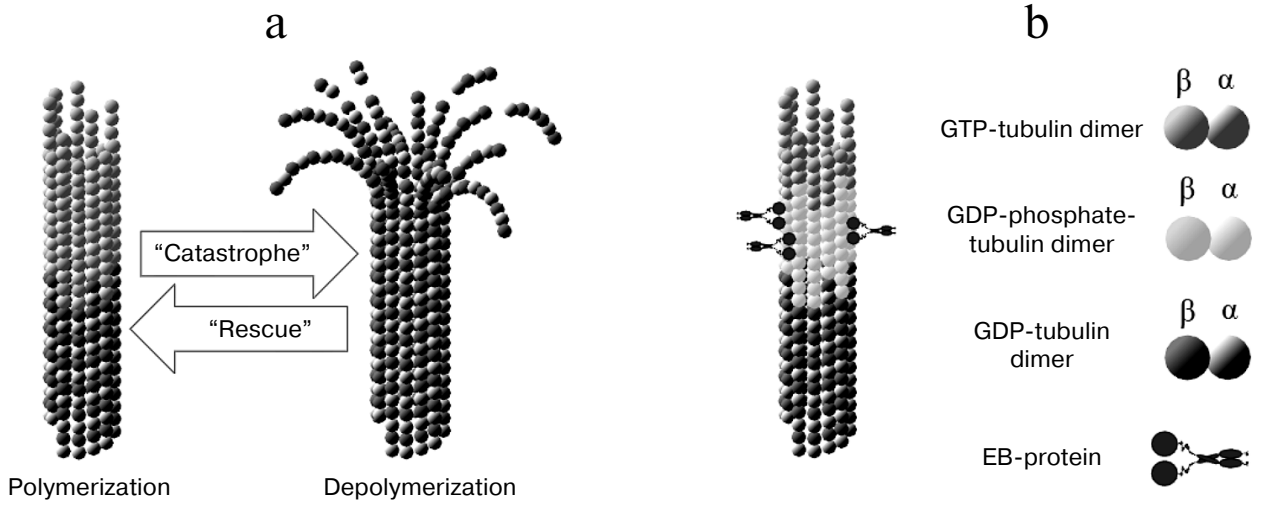

Fig. 3. Schematic illustration of GTP-cap model (a), of GDP-phosphate cap potentially recognized by EB-proteins (b).

vitro. Those structures were interpreted as open sheets often consisting of an incomplete number of laterally bound protofilaments [77, 93, 94]. The picture became even more complicated by findings *in vivo* that in yeast cells the microtubule growing ends had structures similar to funnels of slightly curved protofilaments (Fig. 2) [95].

Chemical composition of the growing microtubule ends is now believed to be different from both the microtubule body and the depolymerizing ends due to the presence of GTP molecules in pockets of the β-subunits of tubulin dimers. The presence of GTP molecules only at the end of growing microtubules is the basis for the classical model explaining the dynamic instability of microtubules (Fig. 3a).

That model was proposed more than 30 years ago and was called the GTP-cap model [3, 96]. According to this model, the microtubule is polymerized by addition of tubulin dimers in complex with a GTP molecule to its growing end. We call such dimers GTP-tubulins. Sometime after the incorporation into the microtubule, the GTP molecule in the tubulin β-subunit pocket is hydrolyzed and the phosphate group is detached from it. Then this phosphate group leaves the tubulin pocket retaining in it the GDP molecule. This destabilizes the microtubule structure. Thus, the microtubule can grow only if it has on its end a stabilizing cap of a certain number of GTP-tubulins. If the GTP-cap is lost for some reason, the GDP-tubulin body of the microtubule begins to depolymerize by dissociating GDP-tubulin oligomers from the microtubule end. Due to the curved shape of the GDP-tubulins at the ends of depolymerizing microtubules, characteristic outwardly curved protofilaments are produced.

Existence of the stabilizing cap on the microtubule growing plus-end was confirmed experimentally by cutting off the microtubule plus-end using a focused ultraviolet beam, which resulted in depolymerization of the remaining part of the microtubule [97, 98]. The importance of GTP for polymerization of microtubules is evidenced by the inability of microtubules to polymerize in its absence. The necessity for GTP hydrolysis for switching the microtubules from growth to disassembly is confirmed by the inability of *in vitro* grown microtubules to depolymerize in the presence of GMPCPP, which is a very slowly hydrolysable GTP analog [99]. Attempts to directly detect the GTP-cap and measure its size were unsuccessful; therefore, it was concluded that the GTP-cap was small and possibly could be presented only by one layer of laterally bound tubulins [100, 101]. In accordance with that assumption, it was shown that one layer of tubulins bound to the slowly hydrolysable analog of GTP was sufficient for stabilizing the microtubule against disassembly [102, 103].

Thus, there are some specific features of the microtubule growing end that could be "recognized" by EB-proteins: (i) a special surface of tubulin that is uncovered only on the growing end, e.g. the inner part of the open tubulin sheet; (ii) a special curvature of protofilaments at the growing end; (iii) the nucleotide composition of tubulins of the growing microtubule end.

It is not trivial to test these hypotheses directly because the literature data on microtubule end structure are contradictory, and there is no approach for direct probing of the nucleotide states of tubulin at the microtubule end. An approach for passing over this problem is the use of nonhydrolysable or slowly hydrolyzable analogs of GTP to induce tubulin to take the conformation over the whole microtubule body similar to its conformation at the growing microtubule end. It was shown in works of Dixit et al. and Zanic et al. [88, 104] that EB-proteins ceased to recognize a region on the microtubule growing

end when β-tubulins along the whole microtubule length were bound with identical nucleotides, in particular, the slowly hydrolysable GTP analog GMPCPP. Initially, it was concluded that EB-proteins could bind to GMPCPP-tubulin better than to GDP-tubulin, but later it became clear that the preference for GMPCPP-tubulin more likely was an artifact of the presence of the 6-histidine tag at the end of protein constructions of EB-proteins used *in vitro* [105]. However, it was established that EB-family proteins had high affinity for microtubules polymerized in the presence of another GTP analog, GTPγS [105].

On one hand, from these data it can be concluded that the complex of tubulin and GTPγS molecule really has a structure closely imitating the tubulin conformation that is recognized by EB-protein on the growing microtubule end. However, interpretation of the experiments with GTP analogs could also be less optimistic. These analogs may just induce an artificial nonphysiological conformation of tubulins that coincidentally could have a high affinity for EB-proteins.

In any case, due to the high affinity of EB-family proteins for microtubules polymerized in the presence of GTPγS, the location of the binding site of the calponin-homology domain of EB-proteins on tubulin was established by high resolution cryoelectron microscopy. EB-proteins were shown to be bound in the corner between four tubulin dimers [28]. These data were recently confirmed in work by Zhang et al. [29]. Such position of the EB-protein binding site probably allows these proteins to sense the nucleotide-dependent conformation of tubulins and the local configuration of the microtubule end, i.e. the number of lateral bonds or, possibly, the curvature of tubulin protofilaments, similarly to the protein doublecortin [106]. However, the latter hypothesis contradicts the observations that EB-proteins continue to bind to the growing microtubule ends even in the presence of taxol, which straightens curved protofilaments [106].

If EB-proteins really sense the nucleotide state of tubulin, what is the phase of the GTP hydrolysis cycle preferred by them for the binding? In other words, do EB-proteins recognize the GTP-cap or the GDP-phosphate cap on the microtubule growing end?

Published results of biochemical studies concerning the GTP-phosphate cap indicated that even if such cap was really present on the microtubule end, it failed to significantly influence the frequency of microtubule catastrophes [107]. Therefore, the recent results obtained by the group of Surrey, implying that EB-proteins recognize the area responsible for the microtubule stability [108], more likely indicate that it is not the GDP-phosphate cap that they recognize.

On the other hand, EB-proteins bind to microtubules with higher affinity in the presence of BeF_3 molecules in the solution, which are traditionally considered to be analogs of phosphate molecules [105]. This provides evidence in favor of the recognizing of the GDP-phosphate cap by EB-proteins. The observation that the binding site of EB-proteins does not reach the very end of the growing microtubule [79] could also be interpreted as an indirect sign of binding to the GDP-phosphate cap. Indeed, the GDP-phosphate cap position must be displaced from the very edge of the microtubule, where freshly joined GTP-tubulin molecules are located, which have not yet detached the phosphate (Fig. 3b). To be fair, an alternative explanation of the EB-protein binding site shift from the microtubule end can also be proposed. Namely, this shift can be due to "raggedness" of the microtubule end. We understand "raggedness" as an interchange of short and long protofilaments at the very end of the microtubule, which can arise stochastically during the polymerization and lead to the fact that not all protofilaments are connected laterally at the very end, and, therefore, do not form a full binding site for EB-proteins [109].

Strictly speaking, it has been noted earlier in the literature [110] that other intermediate states can also exist corresponding to various stages of GTP hydrolysis in tubulins, which can impart to tubulin the conformation recognizable by EB-family proteins. Thus, the problem of the binding mechanism of EB-proteins on the microtubule end is not solved completely and must be investigated further. Other important lines of future studies involve problems associated with the mechanism of EB-protein effect on microtubule dynamics, including the nontrivial synergistic action of EB-proteins and the protein XMAP215 on the rate of microtubule polymerization, as well as combined effects of EB-proteins and small-molecule inhibitors of microtubule dynamics [80].

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