The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules

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Adenomatous polyposis coli protein (APC) is a well-characterized tumor suppressor protein [1–3]. We previously showed that APC tagged with green fluorescent protein (GFP) in *Xenopus* A6 epithelial cells moves along a subset of microtubules and accumulates at their growing plus ends in cell extensions [4]. EB1, which was identified as an APC-binding protein by yeast two-hybrid analysis [5], was also reported to be associated with microtubules [6–8]. To examine the interaction between APC and EB1 within cells, we compared the dynamic behavior of EB1–GFP with that of APC–GFP in A6 transfectants. Time-lapse microscopy of live cells at interphase revealed that EB1–GFP was concentrated at all of the growing microtubule ends throughout the cytoplasm and abruptly disappeared from the ends when microtubules began to shorten. Therefore, EB1 appeared to be co-localized and interact with APC on the growing ends of a subset of microtubules. When APC–GFP was overexpressed, endogenous EB1 was recruited to APC–GFP, which accumulated in large amounts on microtubules. On the other hand, when microtubules were disassembled by nocodazole, EB1 was not co-localized with APC–GFP, which was concentrated along the basal plasma membrane. During mitosis, APC appeared to be dissociated from microtubules, whereas EB1–GFP continued to concentrate at microtubule growing ends. These findings showed that the APC–EB1 interaction is regulated within cells and is allowed near the ends of microtubules only under restricted conditions.

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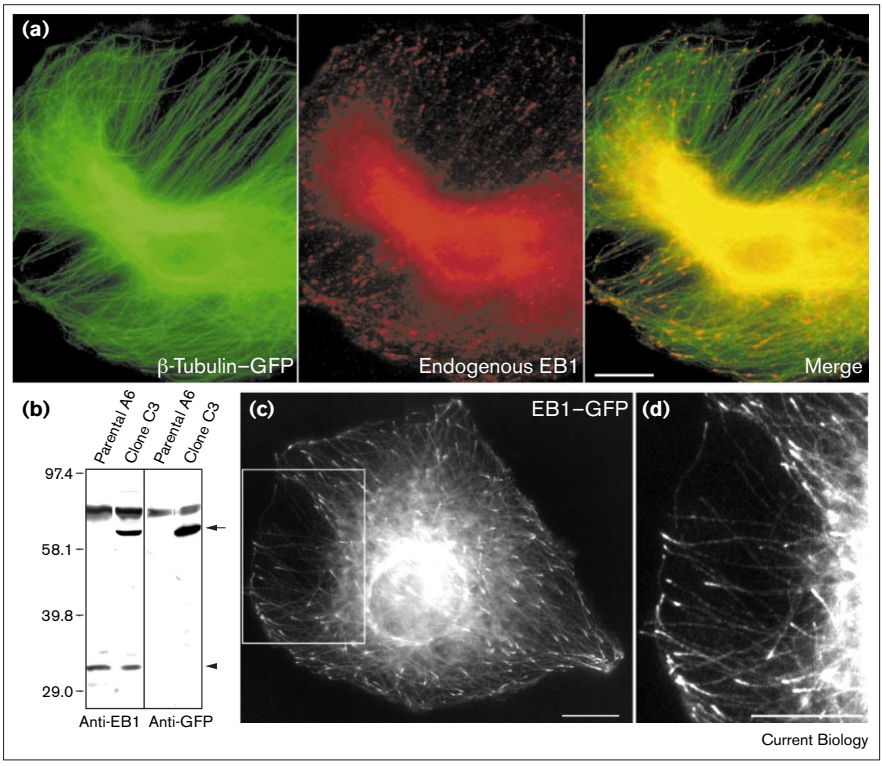
Results and discussion

EB1, with a molecular mass of 30 kDa, was initially identified as an APC-binding protein [5], and reported to be localized at the ends of a subset of microtubules in COS-7 and SW480 cells [6]. In *Xenopus* A6 epithelial cells expressing β-tubulin–GFP immunofluorescently stained with anti-EB1 monoclonal antibody, a similar subcellular localization of EB1 was observed (Figure 1a). Mouse EB1 cDNA was then isolated, and EB1 with a GFP tag at its carboxyl terminus (EB1–GFP) was expressed in A6 cells. We isolated several independent clones stably expressing distinct amounts of EB1–GFP. In this study, we used one clone, C3, in which the expression level of EB1–GFP was approximately fourfold higher than that of endogenous EB1 (Figure 1b). When this clone was fixed and observed by fluorescence microscopy, weak diffuse GFP signals were detected along the entire length of all microtubules and were significantly concentrated at their distal ends (Figure 1c) and at centrosomes (Figure 2c). This distribution was very similar to that of endogenous EB1. At higher magnification (Figure 1d), the staining of distal ends of microtubules showed a comet-like pattern of bright fronts with dark tails along the microtubule.

We examined the dynamic behavior of EB1–GFP at microtubule distal ends in live cells at interphase (Figure 2a and Supplementary material). These cells were characterized by continuous centrifugal movements of numerous GFP signals. The concentrations of EB1–GFP signals on microtubule ends moved for some distance toward the cell periphery, after which they abruptly disappeared. At higher magnification, but only when microtubules continued to grow, the distal ends of microtubules appeared to be highlighted by the GFP signal (Figure 2b and see Supplementary material). The concentration of EB1–GFP in stretches along the microtubule distal ends moved for several seconds at the rate of 14.1 ± 2.9 μm/minute (n = 89, five cells), slowed down, then disappeared, while microtubules were still visible owing to weak diffuse GFP staining. Interestingly, the length of each stretch appeared to correlate well with the growth rate of the microtubule. This correlation is represented quantitatively in Figure 2d for two microtubules (A and B in Figure 2b). Next, to block the assembly/disassembly dynamics of microtubules at their plus ends without changing their polymer mass or microtubule arrangements, cells were treated with a low concentration of nocodazole (100 nM) [4,9]. Within 1 minute of incubation with nocodazole, the concentration of EB1–GFP became undetectable at microtubule ends, but was still detectable at centrosomes (Figure 2c and Supplementary material) as well as at the tips of some of the cellular extensions (data not shown). These findings indicate that EB1–GFP is specifically associated with the plus

Figure 1



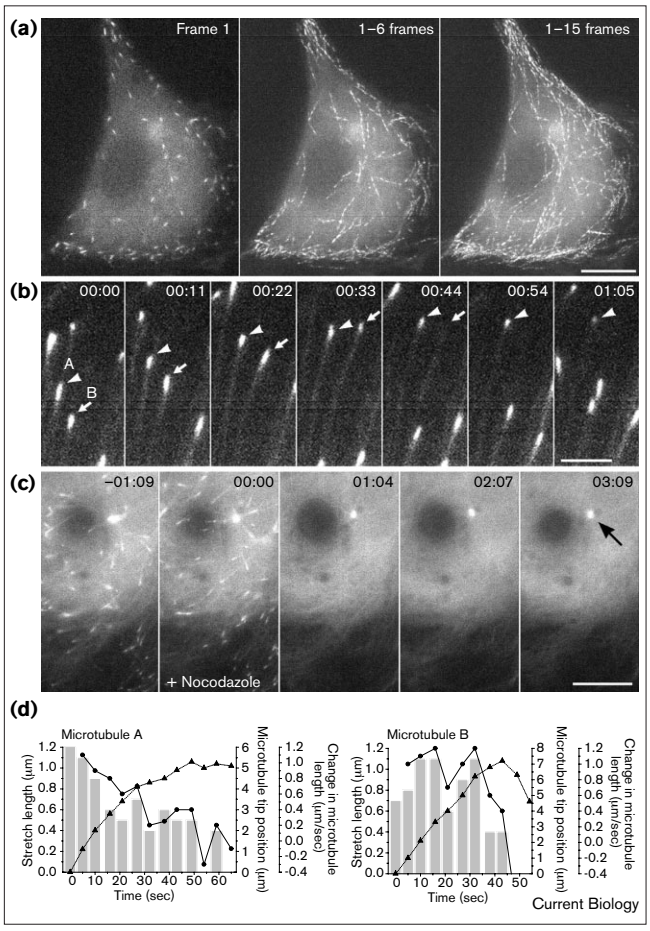
EB1 in *Xenopus* A6 cells. **(a)** A6 cells expressing β-tubulin–GFP (green) were fixed with ethanol and stained with anti-EB1 monoclonal antibody (red). Endogenous EB1 was concentrated at distal ends of some, but not all microtubules, throughout the cytoplasm. **(b)** Expression of endogenous EB1 (arrowhead) and EB1–GFP (arrow). Total cell lysates from parental A6 cells and transfectants expressing EB1–GFP (clone C3) were immunoblotted with polyclonal anti-EB1 or anti-GFP antibody. The uppermost band in each lane was detected non-specifically by tertiary antibodies. **(c)** Distribution of EB1–GFP in clone C3. Cells were fixed with ethanol and observed by fluorescence microscopy. **(d)** An enlargement of the boxed area in (c). Note the characteristic concentration pattern of EB1–GFP on microtubule ends, which was very similar to that of endogenous EB1 (a). The scale bar represents 10 μm.

ends of dynamically growing microtubules, but not with shortening ends.

As described previously, GFP-tagged full-length APC (fAPC–GFP) forms granular aggregates, moves along a subset of microtubules toward their distal ends, and accumulates at the tips of cellular extensions by showing affinity for the growing microtubule ends [4]. Therefore, the dynamic behavior of EB1–GFP described in this study was fairly distinct from that of fAPC–GFP. When A6 transfectants expressing EB1–GFP (clone C3) were immunofluorescently stained with anti-APC polyclonal antibody, APC was shown to be co-localized with EB1–GFP on microtubule ends only at the tips of cellular extensions; in other instances of EB1–GFP concentrations at distal ends, APC was undetectable (Figure 3a). To determine whether EB1 interacts with APC in these areas of co-localization, we examined the distribution of endogenous EB1 in an A6 transfectant clone (clone B4, see [4]) that overexpresses large amounts of fAPC–GFP (23-fold higher level than endogenous APC). In this clone, fAPC–GFP was accumulated in large amounts at the tip regions of cellular extensions, where microtubules were bundled abnormally (Figure 3b). Interestingly, immunofluorescence microscopy of this clone with anti-EB1 revealed that endogenous EB1 was significantly recruited to these bundled microtubules to co-localize with accumulated fAPC–GFP, while the comet-like patterns of EB1 concentration were still detectable throughout the cytoplasm. The same result was obtained with another A6 clone (clone C1, see [4]) overexpressing fAPC–mGFP (GFP sequence inserted within the full-length APC gene). Next, to examine whether intact microtubules are required for the co-localization and interaction of APC with EB1, we treated an A6 clone (clone C1) expressing fAPC–mGFP with a high concentration of nocodazole (33 μM). When most microtubules were disassembled, fAPC–mGFP was distributed at the basal plasma membrane cortex in a striped pattern as previously described [4], whereas endogenous EB1 was not co-localized in these APC concentrations (Figure 3c). These findings indicated that EB1 interacts with APC only on microtubules in the tips of cellular extensions.

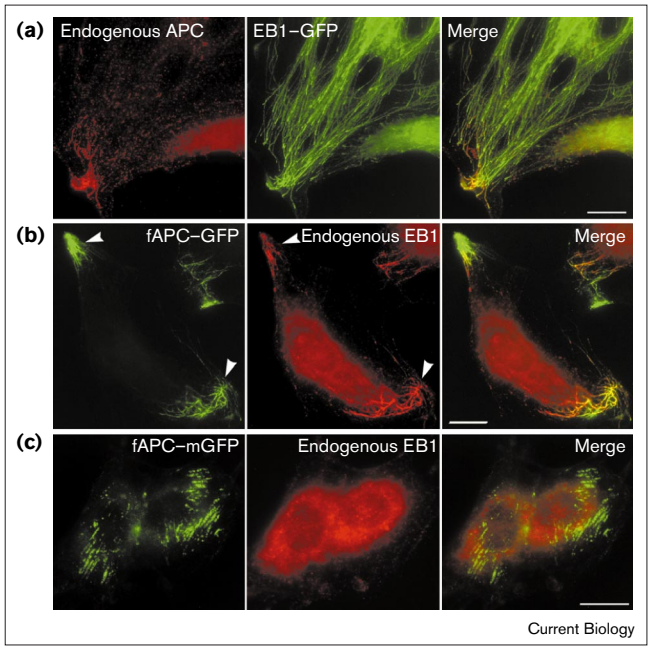
In mitotic cells (clone C3), EB1–GFP also appeared to be concentrated at the microtubule ends, although it was not clear what percentage of microtubule ends were associated with EB1–GFP (Figure 4a). Time-lapse observation of live mitotic cells revealed that EB1–GPF continued to concentrate at the growing microtubule ends during the extensive rearrangement of microtubule networks to form mitotic spindles and asters (see Supplementary material). During mitosis (from nuclear envelope breakdown to the onset of cytokinesis), the EB1–GFP concentrations at the

Figure 2



The dynamic behavior of EB1–GFP in live A6 transfectants at interphase. **(a)** The EB1–GFP concentrations on distal microtubule ends moved centrifugally toward the cell periphery (see Supplementary material for movie). The first frame image (left panel) and accumulated images of frames 1–6 (elapsed time = 27 sec; middle panel) or frames 1–15 (elapsed time = 76 sec, right panel) are presented to trace the paths of EB1–GFP movement. **(b)** The behavior of EB1–GFP at the distal ends of microtubules (see Supplementary material for movie). Elapsed time is indicated at the top in min:sec. The EB1–GFP-positive stretches (see two stretches A and B marked by arrowheads and arrows, respectively) moved for several seconds, slowed down, and disappeared. **(c)** Effects of a low concentration of nocodazole (100 nM) on the behavior of EB1–GFP (see Supplementary material for movie). Within 1 min of incubation with nocodazole, EB1–GFP disappeared from the distal ends of microtubules, but not from the centrosome (arrow), leaving weak and diffuse staining along microtubules. Elapsed time is indicated at the top in min:sec. **(d)** Correlation between the length of EB1–GFP stretches and the growth rate of microtubules. The stretch length (bars), position of microtubule tips (triangles) and microtubule growth rate (circles) of stretches A and B in (b) were plotted as a function of time. The scale bars represent (a) 10 μm; (b) 3 μm; and (c) 5 μm.

Figure 3

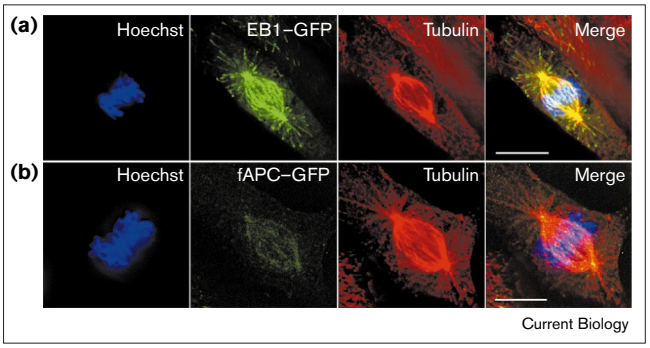


Comparison of subcellular distribution between EB1 and APC. **(a)** A6 transfectants (clone C3) expressing EB1–GFP (green) were fixed and stained with anti-APC polyclonal antibody (red). Endogenous APC was concentrated and co-localized with EB1–GFP on the microtubule ends only at the tips of cellular extensions. In addition, EB1–GFP-positive stretches were scattered throughout the cytoplasm. **(b)** A6 transfectants (clone B4) expressing large amounts of fAPC–GFP (green) were fixed and stained with anti-EB1 monoclonal antibody (red). Overexpressed fAPC–GFP was concentrated at the tips of cellular extensions to bundle microtubules, where endogenous EB1 was recruited (arrowheads). **(c)** A6 transfectants (clone C1) expressing fAPC–mGFP (green) were incubated with a high concentration of nocodazole (33 μM) to disassemble microtubules, fixed and stained with anti-EB1 (red). fAPC–mGFP was localized along the basal plasma membrane in a striped pattern, but endogenous EB1 was not recruited to these concentrations of fAPC–mGFP molecules. The scale bar represents 10 μm.

ends of astral microtubules moved toward the cell cortex at the rate of 19.5 ± 4.8 μm/minute (n = 33, three cells). In contrast, as A6 transfectants (clone B4; see [4]) entered mitosis, the granular structures of fAPC–GFP disappeared and GFP signals along microtubules were significantly decreased (data not shown). During mitosis, fAPC–GFP signals were distributed diffusely in the cytoplasm without detectable concentration at microtubule ends, and only faint GFP signals were detected on mitotic spindles (Figure 4b). At cytokinesis, the fAPC–GFP granular structures re-emerged and began to move along microtubules.

In this study, we determined the dynamic behavior of EB1–GFP in live *Xenopus* A6 epithelial cells. This behavior was very similar to that of CLIP-170, which was also visualized using the GFP tag [10]. Detailed analyses *in vivo* [10] and *in vitro* [11] have suggested that CLIP-170 is localized at growing microtubule ends by co-polymerization with free tubulin dimers. As EB1 directly binds to microtubules [8], a similar molecular mechanism may be responsible for its specific association with growing microtubule ends. On

Figure 4



The dynamic behavior of EB1–GFP in live A6 transfectants during mitosis (see Supplementary material for movies). A6 transfectants (clone C3 or clone A4, see [4]) expressing **(a)** EB1–GFP (green) or **(b)** fAPC–GFP (green), respectively, were fixed and doubly stained with Hoechst 33342 (blue) and anti-tubulin monoclonal antibody (red). In each image, three-dimensional deconvolution was calculated to remove out-of-focus signals, and several optical sections were superimposed. EB1–GFP continued to be concentrated at the growing microtubule ends throughout mitosis, whereas fAPC–GFP was dissociated from microtubule ends, leaving only faint signals on mitotic spindles. The scale bar represents 10 μm.

the other hand, EB1 was recently reported to bind to dynein intermediate chain and p150^{glued} (a component of the dynactin complex) [12], which were also shown to be co-localized with CLIP-170 at microtubule ends [13]. Therefore, it is possible that EB1 associates with growing microtubule ends by binding to these molecules. The list of the proteins associated with growing microtubule ends is increasing, and it is now clear that a large multimolecular complex is associated with these ends. The physiological relevance of this complex remains elusive, and the effects of the proteins on microtubule dynamics need to be examined in detail. It has been shown that Bim1p, a yeast homolog of EB1, promotes microtubule dynamics (both growth and shrinkage) specifically during G1 phase [14], but this kind of analysis is very difficult in A6 cells, which express endogenous EB1 (see Figure 1b).

EB1 was initially identified as an APC-binding protein [5]. EB1 was, however, concentrated at microtubule ends in SW480 cells, which express only truncated APC molecules lacking affinity for EB1 [6]. This indicates that APC–EB1 interaction is not required for the association of EB1 with microtubule ends. As discussed above, the behavior of APC is different from that of EB1 and CLIP-170. When APC molecules select microtubules and arrive at their distal ends, they might encounter EB1 molecules only if the ends are growing. The present observations in fAPC–GFP-overexpressing cells as well as nocodazole-treated cells (see Figure 3b,c), suggested that APC can interact with EB1 only on microtubules at the tips of cellular extensions. These observations raise the question of the significance of the APC–EB1 interaction at such restricted sites. As reported previously, the GFP-tagged carboxy-terminal fragment of APC (GFP–cAPC), which contained the EB1-binding region [5], showed the same dynamic behavior in live cells as EB1 and CLIP-170 [10]. However, *in vitro*, the purified carboxy-terminal fragment of APC itself decorated the entire length of microtubules with no concentration at their ends (data not shown). Therefore, it is tempting to speculate that the EB1–APC interaction confers specific affinity for growing microtubule ends on APC molecules within cells. Further studies, especially *in vitro* reconstitution studies, are required to clarify what happens dynamically at the distal ends of cellular microtubules in molecular terms.

Supplementary material

Supplementary material including including methodological details and movies is available at http://current-biology.com/supmat/supmatin.htm.

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