Oncogene (2001) 20, 3185–3192

www.nature.com/onc

ORIGINAL PAPERS

Regulation of microtubule assembly by human EB1 family proteins

Wen Bu[1] and Li-Kuo Su*,[1]

[1]Department of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas, TX 77030, USA

The EB1 family proteins are highly conserved microtubule-associated proteins. The EB1 protein in yeast has been shown to play an important role in regulating microtubule dynamics and chromosome segregation. Human EB1 family proteins include EB1, RP1 and EBF3. Although EB1 and RP1 have been shown to associate with microtubules, the subcellular localization of endogenous EBF3 had not been characterized. The function of human EB1 family proteins was also not clear. We therefore investigated the cellular localization of EBF3 and the regulation of microtubule organization by EB1 family proteins. As do EB1 and RP1, EBF3 was found to colocalize with microtubules, preferentially at their plus ends, throughout the cell cycle. Moreover, there was a very strong EBF3 signal at the centrosome in interphase cells and at the spindle poles in mitotic cells. When EB1 family proteins were overexpressed, they associated with the entire microtubule cytoskeleton. In addition, EB1 and EBF3 induced microtubule bundling in some cells overexpressing these proteins. These microtubule bundles were more resistant to nocodazole and were more acetylated than regular microtubules. Our results demonstrate for the first time that human EB1 family proteins could regulate microtubule assembly and stability. *Oncogene* (2001) **20,** 3185–3192.

Keywords: EB1; EBF3; microtubule-associated protein; microtubule; microtubule bundle

Introduction

Microtubules are dynamic, noncovalent polymers of α- and β-tubulin. Microtubules are essential for many cellular processes, including cytoplasmic organization, intracellular membrane traffic, generation and maintenance of cell polarity, chromosome segregation and cell division. Many cellular proteins associate with microtubules and regulate microtubule dynamics (Desai and Mitchison, 1997; Cassimeris, 1999). Centrosomes play a critical role in organizing both cytoplasmic microtubules in interphase cells and mitotic spindle in mitotic cells. Abnormal centrosome formation and duplication can lead to abnormal mitotic spindle formation and result in aberrant chromosome segregation (Doxsey, 1998; Salisbury *et al*., 1999). Drugs that interfere with microtubule assembly and remodeling, such as taxanes and vinca alkaloids, have been used as antimitotic agents in treating cancers, and new cancer therapeutics targeting microtubules are being developed (Jordan and Wilson, 1998; Dumontet and Sikic, 1999).

Human EB1 protein is a member of a highly conserved family of microtubule-associated proteins. The cDNA for EB1 was isolated in a yeast two-hybrid screen for proteins that associated with the carboxyl terminus of the protein product of the adenomatous polyposis coli (*APC*) tumor suppressor gene. *In vitro* and *in vivo* experiments confirmed the interaction between EB1 and APC (Su *et al*., 1995; Morin *et al*., 1996; Su and Qi, 2001; Askham *et al*., 2000). Several other potential human EB1 family proteins have been reported (Tirnauer and Bierer, 2000). Our analysis showed that there are most likely three genes (*MAPRE1*, *MAPRE2* and *MAPRE3*) that encode three major forms of EB1 family proteins (EB1, RP1 and EBF3) in human (Su and Qi, 2001). EB1 protein is also evolutionarily conserved. The *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* EB1 homologs have been identified and named Bim1p and Mal3, respectively (Schwartz *et al*., 1997; Muhua *et al*., 1998; Beinhauer *et al*., 1997). Potential EB1 homologs also have been identified in the marine invertebrate *Botryllus schlosseri* (Pancer *et al*., 1996) and in *Caenorhabditis elegans* and *Drosophila melanogaster* (Tirnauer and Bierer, 2000).

EB1 family proteins have been shown to interact with microtubules (Beinhauer *et al*., 1997; Schwartz *et al*., 1997; Berrueta *et al*., 1998; Morrison *et al*., 1998; Juwana *et al*., 1999; Tirnauer *et al*., 1999). The *S. pombe* gene for EB1, *mal3*$^+$, was identified as a gene required for appropriate segregation of minichromosomes (Beinhauer *et al*., 1997). It was then found that Mal3 associates with microtubules and that *mal3* mutants are very sensitive to the microtubule-depolymerizing drug thiabendazole. *S. pombe* cells without functional Mal3 have altered interphase microtubules, and *S. pombe* that overexpress Mal3 have elongated mitotic spindle and defects in chromosome segregation (Beinhauer *et al*., 1997). The *S. cerevisiae* EB1, Bim1p, was identified in a search for proteins that associate with yeast α-tubulin. Yeast cells without functional Bim1p were found to be hypersensitive to the

*Correspondence: L-K Su
Received 27 November 2000; revised 13 February 2001; accepted 26 February 2001

antimicrotubule drug benomyl (Schwartz *et al*., 1997). Detailed analysis of Bim1p's function in *S. cerevisiae* showed that Bim1p promotes dynamic of microtubules in G1 phase cells. However, Bim1p can promote overall microtubule assembly because it increases the time that microtubules spend growing and the frequency of microtubule rescue (Tirnauer *et al*., 1999). Yeast cells without wild-type Bim1p have abnormal spindles and cell division. On the other hand, overexpression of Bim1p blocks spindle formation and division of the nucleus and leads to cell death (Schwartz *et al*., 1997). Bim1p was recently shown to be essential for correct mitotic spindle positioning by interacting with Kar9p (Korinek *et al*., 2000; Lee *et al*., 2000; Adames and Cooper, 2000; Miller *et al*., 2000). Bim1p also cooperates with the motor protein dynein to ensure appropriate cytokinesis (Muhua *et al*., 1998).

Human EB1 and RP1 have been shown to associate with microtubules both *in vivo* and *in vitro* (Morrison *et al*., 1998; Berrueta *et al*., 1998; Juwana *et al*., 1999). These proteins preferentially associate with the plus ends of microtubules in the peripheries of cells as well as with the centrosome in interphase cells. In mitotic cells, EB1 and RP1 interact with mitotic spindle fibers and spindle poles. EB1 also associates with some components of the dynactin complex, a microtubule-associated motor protein complex (Berrueta *et al*., 1999).

The evolutionary conservation of EB1 family proteins and their similar associations with microtubules suggest that the EB1 family proteins play a similar and important role in microtubule functions such as microtubule dynamics and assembly, mitotic spindle formation and chromosome segregation.

We identified the third human EB1 family protein, EBF3 (Su and Qi, 2001). EBF3 is identical to EB3, which was identified as a protein associated with APCL, a brain-specific APC homolog (Nakagawa *et al*., 2000). In this report we describe the cellular localization of endogenous EBF3 and the induction of microtubule bundling by human EB1 family proteins.

Results

Distribution of EBF3 in interphase cells

FLAG-tagged exogenous EBF3 has been shown to be continuously associated with microtubule cytoskeleton (Nakagawa *et al*., 2000). However, the cellular localization of endogenous EBF3 has not been characterized. We investigated the subcellular distribution of EBF3 by using immunofluorescence staining. The EBF3 antibody used in this experiment was a purified polyclonal antibody and was specific to EBF3 (Su and Qi, 2001, and Figure 1). In interphase HCT116 cells, a human colon cancer cell line expressing wild-type APC, the strongest EBF3 antibody signals stained were at the centrosomes and often appeared as doublets (Figure 2a). There were also relatively weak punctate cytoplasmic signals in these cells (Figure 2b).

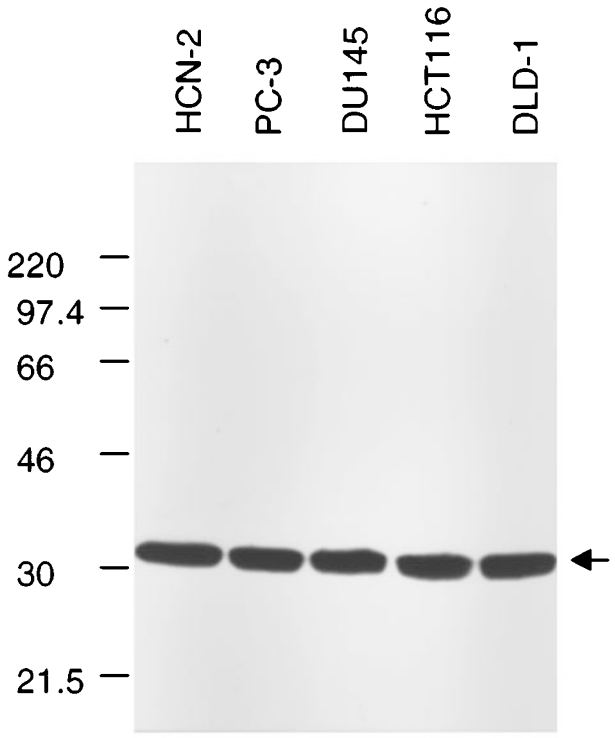

Figure 1 Specificity of EBF3 antibody. Lysates were prepared from the indicated cell lines and EBF3 protein was detected by immunoblot analysis. The arrow indicates EBF3

Even in the centers of cell, which had stronger but more fuzzy signals than did the peripheries, when the cell was observed at different focal planes, it was clear that the fuzzy signal was composed of many punctate signals (data not shown). The punctate EBF3 signals were found to colocalize to microtubule cytoskeleton when the cells were also stained for α-tubulin. However, the EBF3 signals were not distributed along the entire microtubule; instead, they were often near the plus ends of microtubules. Unlike the α-tubulin staining, which revealed cytoplasmic microtubules emanating from the centrosome, EBF3 staining did not show these obvious continuous aster-like signals emanating from the centrosome. To determine whether EBF3 staining depended on wild-type APC, we stained another human colon cancer cell line, DLD-1, which expresses a mutant of APC lacking the C terminus. The staining pattern of EBF3 in DLD-1 was similar to that of HCT116 (Figure 2c). The similar EBF3 staining pattern was also observed in several other human cell lines, including MCF7, which expresses wild-type APC, and HT29, which expresses only mutant APC (data not shown). Therefore, the EBF3 subcellular distribution pattern was not affected by APC status.

Distribution of EBF3 in mitotic cells

EBF3 also colocalized to microtubules in cells in every stage of mitosis (Figure 3). However, unlike the α-tubulin staining signals, which continuously radiated

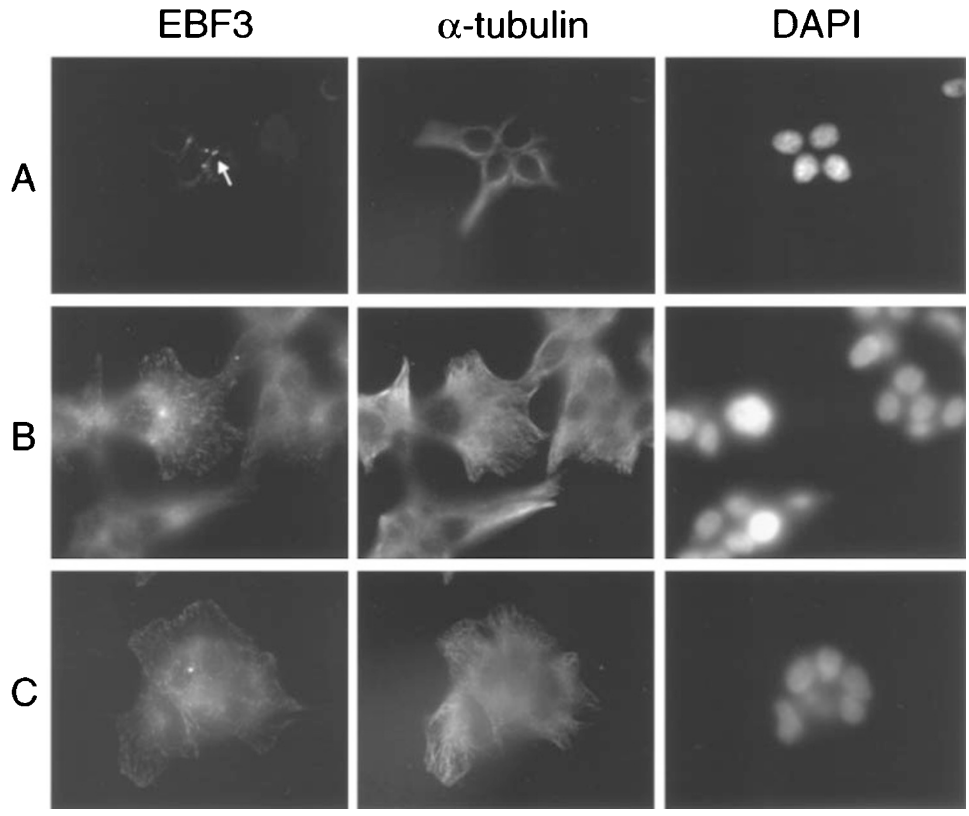

Figure 2 Subcellular localization of EBF3 in interphase cells. (**a**) Localization of EBF3 at centrosomes in HCT116 cells. The arrow indicates a doublet. (**b**) Localization of EBF3 at microtubule plus ends in HCT116 cells. (**c**) Localization of EBF3 at microtubule plus ends in DLD-1 cells

from the centrosome, the EBF3 antibody staining signals were not continuous but punctate. There were also more EBF3 signals around the microtubule plus ends. During metaphase and anaphase, the middle of the spindle, where the microtubule plus ends were, had stronger signals than the area around the spindle poles. In contrast, α-tubulin signals were stronger around the spindle poles, where all the spindle microtubules congregated, than in the middle of the spindle. As in interphase cells, there was no difference in the EBF3 staining patterns of mitotic cells expressing wild-type or mutant APC (data not shown).

Distribution of EBF3 in nocodazole-treated cells

To further confirm that EBF3 associated with microtubules, we examined the distribution of EBF3 in cells treated with the microtubule depolymerization drug nocodazole. HCT116 cells treated with 10 μM nocodazole at 37°C for 1 h had no polymerized microtubules. In these cells, the punctate cytoplasmic EBF3 signals at microtubule plus ends were also lost. However, the centrosome EBF3 staining remained (Figure 4a), even in cells treated with 66 μM nocodazole at 37°C for 1 h (data not shown). Microtubules began to repolymerize in the centrosome 20 min after the nocodazole was washed off of HCT116 cells that had been treated with 10 μM nocodazole (Figure 4b). The microtubule structure as well as the punctate cytoplasmic EBF3 staining pattern recovered completely 30 min after nocodazole was removed (Figure 4c).

In vitro *interaction between EBF3 and microtubules*

Immunofluorescence staining studies presented above showed that EBF3 associated with microtubules *in vivo*. To determine whether EBF3 bind microtubules *in vitro* like EB1 and RP1 do (Berrueta *et al*., 1998; Juwana *et al*., 1999), we carried out sedimentation analysis. As shown in Figure 5, EBF3 was precipitated in the presence of microtubules but was not precipitated in the absence of microtubules. Bovine serum albumin (BSA), which does not bind to microtubules, was not precipitated in the presence of microtubules (data not shown).

Microtubule bundling induced by overexpression of EB1 family proteins

To investigate whether EB1 family proteins regulated dynamics and/or assembly of microtubules, we overexpressed these proteins in cells by transient transfection. Immunofluorescence staining showed that in most transfected cells, EB1 family proteins were distributed all along the microtubule cytoskeleton, instead of only at the microtubule plus ends (Figure 6a and data not

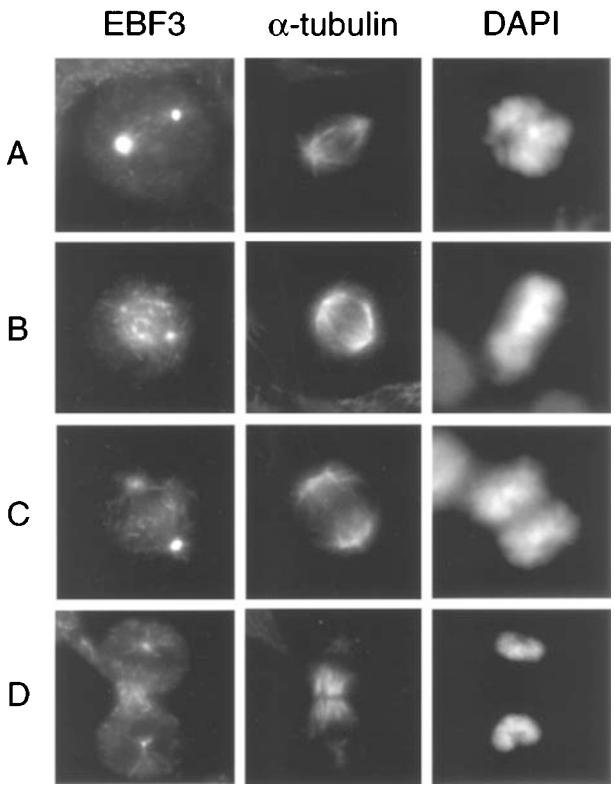

Figure 3 Subcellular localization of EBF3 in mitotic HCT116 cells. (**a**) Prometaphase. (**b**) Metaphase. (**c**) Anaphase. (**d**) Cytokinesis

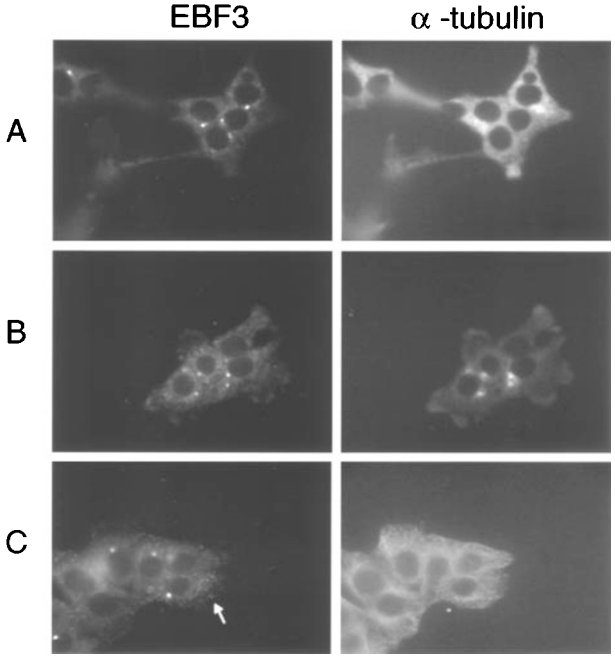

Figure 4 Subcellular distribution of EBF3 in nocodazole-treated cells. (**a**) HCT116 cells treated with 10 μM nocodazole at 37°C for 1 h. (**b**) HCT116 cells 20 min after nocodazole was removed. (**c**) HCT116 cells 30 min after nocodazole was removed. The arrow indicates some EBF3 signals associated with microtubule plus ends

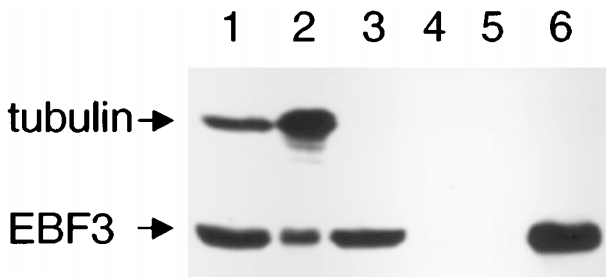

Figure 5 *In vitro* interaction between EBF3 and polymerized tubulin. EBF3 expressed *in vitro* was precipitated in the presence (lanes 1, 2) or absence (lanes 3, 4) of microtubules. Lanes 1 and 3 are supernatants and lanes 2 and 4 are pellets. Lane 5 is the rabbit reticulocyte lysate used for *in vitro* transcription-translation. Lane 6 is the *in vitro* transcription-translation product of EBF3. EBF3 and α-tubulin were detected by immunoblotting

shown). However, there were unusually thick microtubule bundles in some cells expressing exogenous EB1 or EBF3, probably those expressing very high levels of those proteins (Figure 6). In each cell, there was usually only one long, curved microtubule bundle located around the cell periphery. There were very few microtubules other than those in the bundle in these cells. EB1 and EBF3 associated with these microtubule bundles in these cells. The microtubule bundling induced by EB1 and EBF3 has been observed in several human and rodent cell lines, including HCT116, DLD-1, DU145, PC-3, CHO and NIH3T3. On the other hand, although RP1 also associated with the entire microtubule cytoskeleton continuously when it was overexpressed, RP1 did not cause microtubule bundling in any of these cell lines (data not shown). Because DLD-1 expresses only a truncated mutant APC, the microtubule bundling caused by overexpression of EB1 or EBF3 was not dependent on APC.

We previously showed that there are two forms of EBF3 encoded by two alternatively spliced mRNAs (Su and Qi, 2001). However, there was no detectable difference in how these two forms of EBF3 associated with microtubules and induced microtubule bundling (data not shown). We have also shown that there are two forms of RP1 resulting from translation using different initiation codons (Su and Qi, 2001). Because both the long and short forms of RP1 are translated from the long cDNA, which contains both methionine codons, we could not express the long RP1 alone. However, there appeared to be no difference in microtubule binding between the two forms of RP1 because the cellular localization of RP1 in cells overexpressing only the short RP1 was similar to that in cells overexpressing both forms of RP1 (data not shown).

Enhanced stability of microtubule bundles induced by EB1 and EBF3

Microtubule bundles have been shown to be induced in fibroblast cells expressing the microtubule-associated proteins MAP2 or tau, and these microtubule bundles

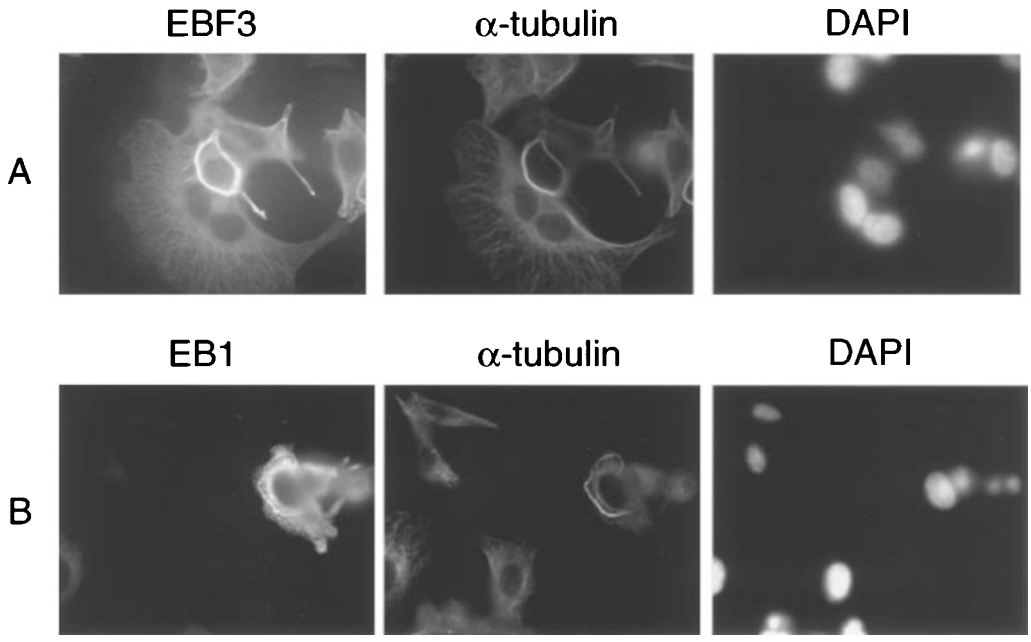

Figure 6 Microtubule bundles induced by EBF3 and EB1. (**a**) Microtubule bundles induced by EBF3. (**b**) Microtubule bundle induced by EB1. EB1 and EBF3 associate with microtubules, bundled or unbundled, continuously when they are overexpressed. Microtubules in some cells overexpressing EB1 or EBF3 form bundles

are more resistant to nocodazole (Lewis *et al*., 1989; Takemura *et al*., 1992). Microtubule bundles induced by tau and MAP2 were also shown to be highly enriched with acetylated α-tubulin (Takemura *et al*., 1992), which is more likely to present in stable microtubules (Piperno *et al*., 1987; Schulze *et al*., 1987).

We therefore investigated whether the microtubule bundles induced by EB1 or EBF3 were also more stable. To examine the sensitivity of bundled microtubules to nocodazole treatment, cells transiently transfected with plasmids expressing EB1 or EBF3 were treated with nocodazole and then stained with α-tubulin antibody. While unbundled microtubules were completely depolymerized in cells treated with 10 μM nocodazole at 37°C for 1 h, most microtubule bundles induced by EB1 or EBF3 were intact (Figure 7a and data not shown). We then investigated whether the microtubule bundles induced by EB1 or EBF3 were enriched with acetylated α-tubulin. We found that the microtubule bundles that formed in cells overexpressing EBF3 or EB1 were strongly stained by antibody against acetylated α-tubulin. In contrast, acetylated microtubules were almost undetectable in the neighboring untransfected cells (Figure 7b). These results showed that microtubule bundles induced by EB1 and EBF3, similar to those induced by MAP2 and tau, were more stable than unbundled microtubules.

Discussion

We demonstrated in this report that EBF3 associated with microtubules *in vitro* and that endogenous EBF3 localized at the plus ends of microtubules and centrosomes *in vivo*. EB1 and RP1 have been shown to bind to both monomeric tubulin and microtubules (Berrueta *et al*., 1998; Juwana *et al*., 1999). The N-terminal 198 amino acid fragment of EBF3 (RP3) has also been shown to interact with purified monomeric tubulin (Juwana *et al*., 1999). Using sedimentation analysis we showed that EBF3 associated with microtubules *in vitro*. Immunofluorescence staining was used to investigate the cellular localization of endogenous EBF3. The results showed that there were weak cytoplasm signals distributed along the microtubule cytoskeleton in interphase cells. However, the staining was punctate rather than continuous and was predominantly near the cell membrane where the plus ends of the microtubules were. Co-immunostaining of EBF3 with α-tubulin confirmed that EBF3 localized at the plus ends of the microtubules. The punctate EBF3 signals disappeared when microtubules were depolymerized by treatment with nocodazole and reappeared when the microtubules reassembled after nocodazole was removed. This result confirmed the association of EBF3 with microtubules. The preferential localization of EBF3 at microtubule plus ends is similar to that of EB1 and RP1 (Berrueta *et al*., 1998; Morrison *et al*., 1998; Juwana *et al*., 1999; Mimori-Kiyosue *et al*., 2000b).

Immunofluorescence staining with EBF3 antibody also revealed very strong signals at the centrosomes, which often appeared as doublets. Different from the α-tubulin signals, there was no obvious aster-like EBF3 signals surrounding centrosomes. The strong EBF3 signal at the centrosomes could simply reflect the EBF3 associated with the minus ends of the microtubules and that all microtubules emanated from

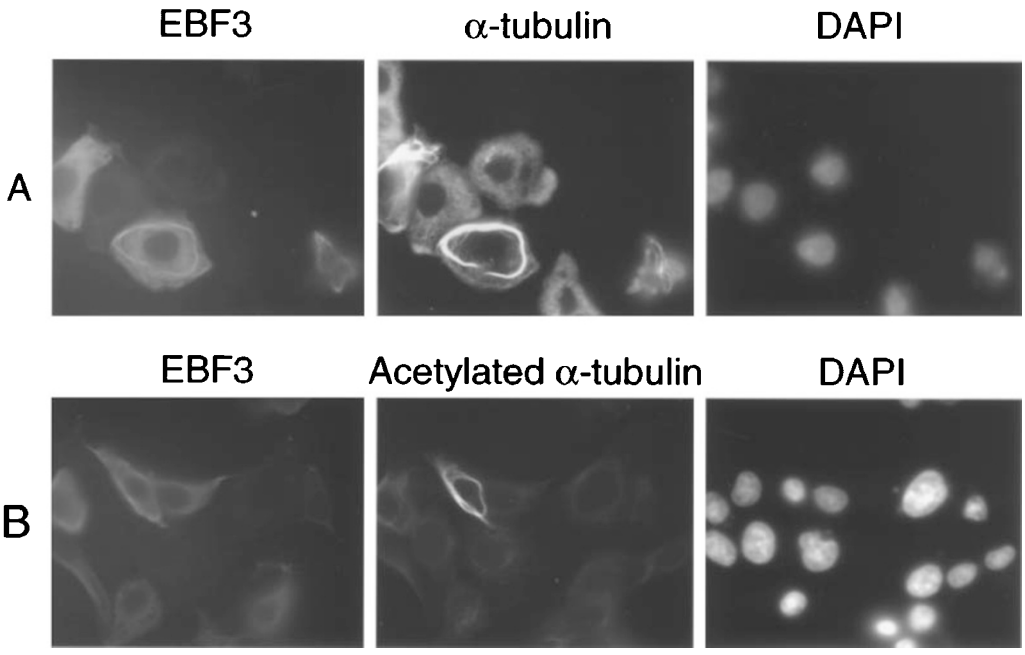

Figure 7 Enhanced stability of microtubule bundles induced by EBF3. (**a**) Microtubule bundles induced by EBF3 are more resistant to nocodazole. HCT116 cells transfected with an EBF3-expressing vector were treated with 10 μM nocodazole at 37°C for 1 h. The bundled microtubules remained while unbundled microtubules completely depolymerized. (**b**) Microtubule bundles induced by EBF3 contain high level of acetylated α-tubulin

centrosomes. However, the EBF3 signals at the centrosomes remained after the microtubules were completely depolymerized and punctate cytoplasmic EBF3 signals disappeared in cells treated with nocodazole. This result strongly suggests that the presence of EBF3 at the centrosome is not simply due to the convergence of the microtubules. It is likely that EBF3 binds to the centriole or to proteins in the centrosome and may play an important role in the organization and function of the centrosome. Because the two centrioles within a centrosome are too close to be distinguished by light microscopy, the doublet EBF3 signals at the centrosomes probably reflect duplicated centrosomes rather than the two centrioles in the centrosome.

In mitotic cells, the EBF3 signals were also colocalized to microtubules and centrosomes. However, unlike the α-tubulin signal, which radiated continuously from the centrosome, the EBF3 signals were not continuous and were more apparent in areas where the microtubule plus ends were. This suggests that EBF3 may regulate the dynamics and/or position of the mitotic spindle. EBF3 may also take part in the capture of the kinetochore by kinetochore microtubules. However, it is difficult to determine whether the EBF3 staining signals were at polar microtubule plus ends or at kinetochore microtubule plus ends.

In contrast to the punctate endogenous EBF3 signals, the overexpressed exogenous EBF3 signal was distributed along the microtubule cytoskeleton continuously. This distribution pattern is similar to that of exogenous FLAG-tagged EBF3 detected by using anti-FLAG antibody (Nakagawa *et al*., 2000). The difference in the distribution of endogenous and exogenous overexpressed proteins also held for EB1 and RP1 (data not shown). In yeast, green fluorescence protein-tagged Bim1p expressed at physiological levels localizes at the distal tips of microtubules, whereas green fluorescence protein-tagged Bim1p expressed at higher levels associates with the entire microtubule cytoskeleton (Tirnauer *et al*., 1999; Schwartz *et al*., 1997). Why the distribution patterns of endogenous and exogenous overexpressed EB1 family proteins were different is still unclear. One possibility is that the EB1 family proteins bind the microtubule plus end more strongly than they bind the other portions of microtubules. Alternatively, EB1 family proteins may interact with other cellular proteins at the plus ends of microtubules. These proteins themselves may serve as high-affinity binding sites for EB1 family proteins or facilitate binding of EB1 family proteins to microtubule plus ends. Several proteins have been shown to be at the plus ends of microtubules including KIN I kinesins (Desai *et al*., 1999), dynactin complex and CLIP-170 (Vaughan *et al*., 1999). EB1 has been shown to interact with some components of the dynactin complex (Berrueta *et al*., 1999). Additional studies will be needed to reveal the functional interactions among these proteins.

Our results demonstrate for the first time that human EB1 family proteins can induce microtubule bundling in mammalian cells. Other microtubule-associated proteins, such as MAP2 and tau, which are expressed only in the nervous system, have been shown to induce microtubule bundling when they are expressed in non-neuronal cells (Kanai *et al*., 1989; Lewis *et al*., 1989; Takemura *et al*., 1992). The

microtubule bundles induced by EB1 and EBF3 were usually single thick curved bundles at the peripheries of the cells (Figure 6) and so were morphologically different from the microtubule bundles induced by MAP2 or tau, which are usually multiple thick bundles radiating from the center of each cell (Lewis *et al*., 1989; Kanai *et al*., 1989; Takemura *et al*., 1992). However, like the bundles induced by MAP2 or tau, the microtubule bundles induced by EB1 or EBF3 were enriched with acetylated α-tubulin and were more resistant to nocodazole. EB1 and EBF3 may normally regulate microtubule stability in a more subtle way rather than inducing microtubule bundling. Additional research will be needed to fully understand how EB1 family proteins normally regulate microtubules organization and stability.

The mRNA for EBF3 has been shown to be preferentially expressed in brain (Nakagawa *et al*., 2000). However, our immunoblot and immunofluorescence staining analyses showed that, like EB1 (Su *et al*., 1995; Morrison *et al*., 1998; Su and Qi, 2001); EBF3 was ubiquitously expressed (Su and Qi, 2001, and this study). These observations suggest that EB1 and EBF3 may regulate the stability of microtubules in many types of cells.

EBF3 has been shown to interact with the brain-specific APC homolog APCL (Nakagawa *et al*., 2000). We have shown that, in addition to binding APCL, EBF3 also bind APC (Su and Qi, 2001). Similar to the distribution of EB1 (Berrueta *et al*., 1998; Morrison *et al*., 1998), the subcellular distribution of EBF3 is not affected by the presence or absence of wild-type APC. The functional importance of the interaction between APC and EB1 family proteins is still not clear. However, accumulating evidence suggests that APC, in addition to regulating β-catenin function (Morin, 1999; Peifer and Polakis, 2000), associates with microtubules (Smith *et al*., 1994; Munemitsu *et al*., 1994; Morrison *et al*., 1997; Mimori-Kiyosue *et al*., 2000a). It therefore remains possible that EB1 family proteins participate in some aspects of APC's function.

The *S. cerevisiae* EB1, Bim1p, has been shown to regulate the dynamics of microtubule plus ends and to take part in the capture of microtubule plus ends to the cell cortex by interacting with Kar9p (Tirnauer *et al*., 1999; Korinek *et al*., 2000; Lee *et al*., 2000; Miller *et al*., 2000; Adames and Cooper, 2000). Because human EB1 family proteins associate with microtubules and can regulate microtubule assembly, they may also have these functions. Although Kar9 homologs have not been identified in any other species (Tirnauer and Bierer, 2000), it is possible that there are functional homologs of Kar9p in other species, including human. Although EB1, EBF3 and RP1 have very similar amino acid sequences and all associate with microtubules, they are clearly different. While we detected microtubule bundles induced by EB1 and EBF3, we have never observed microtubule bundles induced by RP1. RP1 is also different from EB1 and EBF3 in that it does not associate with APC *in vitro* under the conditions in which EB1 and EBF3 associate with APC (Su and Qi, 2001). Moreover, we have shown that EB1 appears to be expressed at constant level in all cell lines, whereas RP1 and EBF3 levels vary among different cell liens (Su and Qi, 2001). Additional studies will be needed to determine which EB1 family proteins participate in and regulate which specific microtubule functions.

Materials and methods

Cell lines

All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116, HT29 and DLD-1 are human colon cancer cell lines. HCN-2 is a human cortical neuronal cell line. MCF7 is a human breast cancer cell line. DU145 and PC-3 are human prostate cancer cell lines. CHO is a Chinese hamster ovary fibroblast cell line, and NIH3T3 is a mouse fibroblast cell line.

Antibodies

The antibodies against the EB1 family proteins used in this study have been described elsewhere (Su *et al*., 1995; Su and Qi, 2001). Mouse (Ab1) and rat (MCAP77) monoclonal antibodies for α-tubulin were purchased from Oncogene Research Products (Cambridge, MA, USA) and Serotec Inc. (Raleigh, NC, USA), respectively. Monoclonal antibody for acetylated α-tubulin (clone 6-11B-1) was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA). Horseradish peroxidase-conjugated secondary antibody was from Bio-Rad Laboratories (Hercules, CA, USA). Fluorescein-conjugated donkey anti-mouse antibody and Texas Red-conjugated donkey anti-rat antibody were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other fluorescein- and Texas Red-conjugated secondary antibodies were from Vector Laboratories Inc. (Burlingame, CA, USA).

Immunoblot analysis

Detection of EBF3 by immunoblotting was performed as described previously (Su and Qi, 2001). Proteins in cell lysate were resolved by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis and then electrophoretically transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). EBF3 was detected by using polyclonal EBF3 antibody (Su and Qi, 2001) and the ECL system (Amersham Pharmacia Biotech, Inc. Piscataway, NJ, USA).

Immunofluorescence staining

Cells were seeded onto sterile 18×18 mm^2 glass coverslips in six-well plates to reach 70–80% confluence after 48 h. After washing with 37°C phosphate buffered saline (PBS), the cells were immediately fixed in methanol at −20°C for 10 min. Fixed cells were rehydrated in PBS and then incubated in 10% serum (horse, goat or donkey, individually or in combination, depending on the primary and secondary antibodies used) in PBS to block nonspecific antibody binding. The cells were then incubated with primary antibodies in the blocking solution for 1 h at room temperature, washed with PBS and then incubated with secondary antibodies in the blocking solution at room temperature for 30 min. After washing with PBS, the cells

were incubated with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min followed by washing with PBS. Coverslips were mounted with VECTASHEILD mounting medium (Vector Laboratories, Inc.). Slides were viewed with a Zeiss Axioplan 2 fluorescence microscope. Images were taken, saved and analysed with the Zeiss KS400 3.0 Imaging System and Adobe Photoshop software.

Cell treatments

To depolymerize microtubules, nocodazole was added to the medium to final concentrations of 10, 33 or 66 μM and the cells were incubated at 37°C for 1 h before immunofluorescence staining. To remove the nocodazole, the nocodazole-treated cells were washed in nocodazole-free medium.

Microtubule binding assay

A 25 μl reaction of *in vitro* transcription-translation of EBF3 was performed as previously described (Su *et al*., 1995; Su and Qi, 2001). Eight μl each of the reaction product was used for the sedimentation assay in the presence and the absence of microtubules. Another 8 μl of the reaction product was immunoblotted directly to detect the expression of EBF3. The microtubule binding assay was carried out using Microtubule Associated Protein Spin Down Assay Biochem Kit (BK029) (Cytoskeleton Inc., Denver, CO, USA) following the manufacturer's instruction. EBF3 and α-tubulin in the supernatant and pellet fractions of this assay were detected by immunoblotting.

Transfection

The plasmids used to express EB1 family proteins have been described elsewhere (Su and Qi, 2001). Transfections were performed with LipofectAMINE PLUS Reagent (Life Technologies, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions.

Acknowledgments
We thank Weizhe Yao for technical assistance and Dr Maureen E Goode for editorial review of this manuscript. This research was supported in part by The Gillson Longenbaugh Foundation and grant CA 70371 and Cancer Center Core Grant CA 16672 from the National Cancer Institute.

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