Graphical Abstract

Interactions between the microtubule binding protein EB1 and F-actin

Emily O. Alberico^a, Zhiqing C. Zhu^{a,1}, Yueh-Fu O. Wu^a, Melissa K. Gardner^b, Dave R. Kovar^c and Holly V. Goodson^{a,*}

^aDepartment of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556;

^bDepartment of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN 55455

^cDepartment of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637

¹Present address: Biogen 125 Broadway St. Cambridge, MA 02142

*Corresponding author: Holly V. Goodson 251 Nieuwland Hall University of Notre Dame, Notre Dame, IN 46556 hgoodson@nd.edu (574)-631-7744

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KEYWORDS: EB1, actin, microtubules, cytoskeleton, plus-end tracking protein¹

¹Abbreviations: EB1, end binding protein 1; MT, microtubule; G-actin, globular actin; F-actin, filamentous actin; APC, adenomatous polyposis coli, +TIP, plus end tracking protein; CH domain, calponin homology domain; GTP, guanine triphosphate; ATP, adenosine triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; WT, wildtype; KCl, potassium chloride

ABSTRACT: Many cellular processes including cell division and cell migration require coordination between the actin and microtubule (MT) cytoskeletons. This coordination is as-yet poorly understood, but proteins such as formins and IQGAP1 are known to be involved. We show that the MT binding protein EB1, a key regulator of microtubule dynamics, can bind directly to F-actin. We determined that the EB1:F-actin interaction is salt sensitive and weak under physiological salt concentrations but might be relevant in contexts where the local concentration of actin is high. Using bioinformatics and mutagenesis we found that the EB1:F-actin binding site partially overlaps the well characterized EB1:MT binding interface. Congruently, competition experiments indicate that EB1 can bind to F-actin or MTs but not both simultaneously. These observations suggest that EB1:F-actin interactions may negatively regulate EB1:MT interactions, and we speculate that this interaction may assist cells in differentially regulating microtubule stability in the actin-rich cortex as opposed to the cell interior.

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4 Many important cellular and physiological processes (e.g. cell division, cell migration, wound
5 healing, axon guidance) involve both the actin and microtubule (MT) cytoskeletons, and they
6 require coordination between these two dynamic filament systems.[1] While the proteins that
7 control and regulate actin and MTs are relatively well characterized, the processes coordinating
8 these filament systems are less clear.
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14 One reasonable suggestion is that actin:MT coordination is mediated by the use of common
15 signal transduction pathways that control major regulators of both the actin and MT
16 cytoskeletons. For example, the GSK3 kinase regulates both the actin and MT cytoskeletons
17 through phosphorylation of actin and MT binding proteins, such as APC and CLASPs.[2,3]
18 Another explanation (not mutually exclusive) is that proteins described as cytoskeletal
19 integrators (e.g. IQGAP1 and spectraplakins) mediate communication between the filament
20 networks by binding directly to both networks.[4,5] Both of these mechanisms are well-
21 substantiated. However, one could also imagine that actin and MTs might regulate each other
22 more directly, with core elements of one filament system responding to the other. Evidence for
23 this idea is provided by the observation that the crucial actin assembly factors known as formins
24 also bind MTs, regulate MT dynamics, and can bundle MTs in vitro.[6,7] We were intrigued by
25 the possibility that EB1, the core MT plus-end tracking protein (+TIP) [8,9], might be similarly
26 involved in direct actin:MT coordination.
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38 The idea that EB1 might bind actin is suggested by several pieces of circumstantial evidence.
39 First, EB1 contains a CH domain at its N-terminus (Figure 1a). CH domains are typically
40 considered to be actin binding structures,[10] although other MT binding CH-domains besides
41 EB1 do exist (e.g., NDC80 [11]). Second, other MT +TIPs such as APC and spectraplakins can
42 themselves bind actin.[12,13] Lastly, EB1 depletion causes defects in actin based processes in
43 mouse tissue culture cells as well as in *Dictyostelium*, [14,15] and EB1 partially co-localizes with
44 F-actin in *Dictyostelium*. [16] These observations suggest that EB1 has an evolutionarily
45 conserved role in actin-related functions. However, any EB1:F-actin interaction has thus far been
46 assumed to be indirect, likely mediated by EB1 binding proteins such as formins.[7,12,14] We
47 sought to investigate whether EB1 can bind directly to F-actin. Through a combination of
48 biochemical and biophysical techniques we have found that EB1 can bind F-actin *in vitro* and
49 that the F-actin binding site on EB1 overlaps with the well-characterized EB1:MT binding site.
50 Competition experiments support the conclusions from our mutagenesis data and suggest that
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EB1:F-actin binding and EB1:MT binding are mutually exclusive. We speculate that this interaction may assist cells in differentially regulating microtubule stability in the actin-rich cortex as opposed to the cell interior.

MATERIALS AND METHODS:

Bioinformatics

To find the sequences for the amino acid alignments, a BLASTp search was performed with human EB1 or human calponin as the query. The EB1 or calponin sequences of 10 vertebrates were chosen as representatives of vertebrate diversity and independently aligned with ClustalX (for this initial work, we used only this subset of organisms in order to avoid misinterpretations caused by the varied gene duplications that have happened in many organisms, particularly at the base of vertebrate radiation). Similar alignments were obtained with other alignment programs such as Muscle (not shown). The model vertebrates include: human (GI: 6912494), chicken (GI: 71894959), cow (GI: 115496618), zebrafish (GI: 41054091), frog (GI: 147905448), mouse (GI: 7106301), lizard (GI: 327289281), pig (GI: 113205898), fish (GI: 348517001), and elephant (GI: 344279857). The conservation of EB1 or calponin was aligned on to the crystal structure of the CH domain of human EB1 (PDB ID: 1PA7) or human calponin (PDB ID: 1WYP) using the structure analysis and presentation program Chimera.[17]

For the analysis of the more divergent EB1 proteins, we identified from NCBI a set of early-diverging organisms (both basal animals and model unicellular eukaryotes) that have only one EB1 protein, again chosen to avoid misinterpretations caused by gene duplications: *S. kowalevskii*, *H. vulgaris*, *M. brevicollis*, *S. rosetta*, *C. intestinalis*, *N. vectensis*, *A. queenslandica*, *L. gigantea*, *S. pombe*, *N. crassa*, *D. discoideum*, *G. intestinalis*, *S. cerevisiae*. The sequences for the first 8 organisms were aligned against the model EB1 vertebrate sequences. A separate alignment of the last organisms was generated. Both alignments were made in ClustalX. The amino acids are colored based on percent identity in Jalview.

Mutagenesis and Purification

Highly conserved surface residues were selected either in the known microtubule binding interface as determined from previous electron microscopy and mutagenesis[18] or in the face opposite the microtubule interface. Amino acids were either mutated to an alanine or a charged amino acid that resulted in a charge reversal. Mutants were generated through site-directed mutagenesis (Agilent QuickChange II) and confirmed by DNA sequencing. Proteins (WT or mutant) were purified through previously described methods.[19] Briefly, non-tagged proteins were isolated using classical ion exchange techniques. Lysates were exchanged on to sequential cation and anion exchange columns with a desalting column in between. The protein was dialyzed into PEM buffer (100 mM Pipes, 1 mM EGTA, 2 mM MgCl₂, pH 6.8), flash frozen and stored at -80°C. EB1 proteins for the low salt experiments were dialyzed into TK buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM DTT, pH 7.5).

APC-C1 was bacterially expressed and purified using a nickel-NTA column.[20] APC-C1 was dialyzed in to PEM buffer, flash frozen, and stored at -80°C.

Actin was purified from rabbit muscle acetone powder through filament cycling[21] and stored in calcium buffer G (2mM Tris-HCl, 0.2mM ATP, 0.5mM DTT, 0.1mM CaCl₂, 1mM sodium azide, pH 8). For F-actin studies, G- actin was converted to MG-actin by adding 5 mM MgCl₂ and 0.2 mM EGTA for 5 minutes at room temperature. To polymerize the actin, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, and 10 mM Imidazole-HCl (pH 7) were added and allowed to incubate 1 hour at room temperature. After polymerization 0.8 μM phalloidin was added to stabilize the filaments.

For Figure 1c, actin was purchased from Cytoskeleton Inc. (Denver, CO) and stored in G buffer (10 mM Tris-HCl, 0.1 mM CaCl₂, 0.2 mM ATP, 0.5 mM DTT, pH 7.5). Actin was centrifuged (200,000 × g, 15 minutes 4°C) immediately prior to use to remove any aggregates.

Tubulin was purified as previously described[22]; briefly the tubulin was purified from porcine brain through two rounds of polymerization and depolymerization. MTs were polymerized with Taxol (paclitaxel, Sigma) as previously described.[22] Tubulin and MTs were frozen and stored at -80°C.

To measure EB1 (calculated as EB1 monomer) and MT (calculated as tubulin dimer) concentration, we used BSA standard curves in conjunction with Bradford dye (Bio-rad). To

determine the actin concentration, we measured an optical density at 290 nm and used an extinction coefficient of $26,600 \text{ M}^{-1} \text{ cm}^{-1}$.

Binding Studies

Low salt F-actin binding

Initial experiments utilized $2 \mu\text{M}$ F-actin and $2 \mu\text{M}$ any other proteins (EB1, EB1 fragments, or controls as indicated, stored in TK buffer, see above). For the initial co-sedimentation assays, proteins as indicated were incubated with F-actin in Mg-G buffer (10 mM Tris-HCl, 0.1 mM MgCl_2 , 0.2 mM ATP, 0.5 mM DTT, pH 7.5) in the presence of phalloidin at room temperature for 1 h. After centrifugation ($200,000 \times g$), supernatant (top $50 \mu\text{l}$) and pellet fractions were separated by SDS-PAGE. The bottom $50 \mu\text{l}$ supernatant was discarded and not used for analysis.

Salt Sensitivity

EB1₂₄₈ was diluted to $2 \mu\text{M}$ in calcium buffer G along with $7 \mu\text{M}$ F-actin in the presence of $0.8 \mu\text{M}$ phalloidin. The amount of KCl added to each reaction was calculated so that the total amount of KCl from the F-actin, EB1 and buffer equaled 25 mM , 50 mM , 75 mM , 125 mM , or 175 mM . The reactions were incubated for 25 minutes at room temperature and then centrifuged at $150,000 \times g$ for 15 minutes at 22°C to sediment the F-actin. The supernatant and pellet fractions were separated as above and analyzed by SDS-PAGE. Densitometry (ImageJ) was used to determine the fraction of EB1₂₄₈ bound; in some experiments a small amount of EB1 sedimented by itself and this amount was subtracted from the fraction of EB1 bound to F-actin.

Microtubule and actin comparison study:

For the MT binding experiments, EB1_{FL} WT or mutant proteins ($2 \mu\text{M}$) were incubated with $5 \mu\text{M}$ MTs stabilized with $10 \mu\text{M}$ Taxol. For the F-actin binding experiments, EB1_{FL} WT or mutant proteins ($2 \mu\text{M}$) and APC-C1 ($2 \mu\text{M}$; added to release EB1 autoinhibition) were incubated with $7 \mu\text{M}$ F-actin stabilized with $0.8 \mu\text{M}$ phalloidin. In all the comparison study experiments, the binding reactions were incubated in PEM 50 (50 mM Pipes, 2 mM MgCl_2 , 1 mM EGTA pH 6.8) for 25 minutes at 37°C . Proteins were then centrifuged at $150,000 \times g$ for 15 minutes at 37°C to sediment any filaments (F-actin, MTs, or both) present in the reaction. The supernatant and pellet fractions were separated and analyzed by SDS-PAGE. Densitometry

(ImageJ) was used to determine the fraction of EB1_{FL} WT or mutant bound to MTs or F-actin. Experiments with EB1_{FL} WT or mutant proteins by themselves were included to ensure that the proteins did not sediment on their own. Both F-actin and MT concentrations were chosen so that approximately 50% of the EB1_{FL} WT was bound in each case. The fraction bound data were normalized against the fraction of EB1_{FL} WT bound under the indicated conditions.

Labeled microtubules for microscopy

20 μ M pure tubulin, 2 μ M rhodamine labeled-tubulin (Cytoskeleton Inc.) and 1 mM GTP were incubated in BRB80 (80 mM PIPES, 2 mM MgCl₂, 0.5 mM EGTA, pH 6.9) and polymerized with Taxol as previously described.[22] After incubation, the MTs were centrifuged (200,000 x g, 15 min at 37°C). The MT pellet was washed with PEM and then re-suspended by half reaction volume of PEM buffer with addition of 10 μ M of Taxol. The concentration of polymerized rhodamine labeled-microtubules was measured by Bradford assay, frozen by liquid nitrogen, and stored at -80°C.

Microscopy

F-actin and microtubule bundling was observed by fluorescence microscopy. 10 μ M Taxol, 1.5 μ M Alexa-488-phalloidin and 3.5 μ M unlabeled-phalloidin were incubated with 5.0 μ M G-actin and/or 0.5 μ M rhodamine-MTs in the presence or absence of 1 μ M EB1 (EB1₂₄₈ or EB1_{FL} as indicated) for 25 minutes at room temperature in PEM buffer. Calcium buffer G and PEM were added as buffer controls. EB1_{FL} was used as a negative control. Experiments were performed in a blinded manner to prevent biased data interpretation (microscopist did not know the identity of the samples being analyzed). To visualize actin and MT bundles, we used a 60X objective, 1.5X optivar, and 1.4 N.A. on a TE2000 inverted microscope (Nikon). Images were obtained by a Hamamatsu CMOS camera controlled by NIS-Elements BR 413.04 64bit software (Nikon). Data were further processed using Image J (NIH).

RESULTS AND DISCUSSION:

EB1 binds to F-actin

As a first test of whether EB1 binds to F-actin, we examined the ability of various fragments of EB1 to co-sediment with filamentous actin (F-actin) under conditions of low salt, where we

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4 expected that binding to the negatively charged actin filaments would be strongest. We tested
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6 three different EB1 constructs: EB1_{FL} (the full-length human protein), EB1₂₄₈ (the activated form
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8 of the protein with the acidic tail removed), and EB1₁₈₄ (monomeric EB1 that contains the CH
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10 domain and part of the unstructured region) (Figure 1a-b). [22] We found that full-length EB1
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12 (EB1_{FL}) binds weakly to 2 μ M pre-polymerized F-actin: almost all of the EB1_{FL} protein
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14 remained in the supernatant whether or not F-actin was added to the assay (Figure 1c). However,
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16 nearly all the EB1₂₄₈ fragment pelleted in the presence (but not the absence) of 2 μ M F-actin
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18 under these conditions (Figure 1c). These observations demonstrate that EB1 can bind to actin,
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20 and that EB1:F-actin-binding, like EB1:MT-binding, is negatively regulated by the EB1 tail (i.e.,
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22 removal of the tail increases the affinity of EB1 for both actin and MTs).[22,23] In addition, the
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24 monomeric MT-binding “head” domain of EB1 (which contains the CH domain) also pelleted
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26 with F-actin (Figure 1c). This observation indicates that F-actin binding is mediated at least in
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28 part by the microtubule-binding CH domain.

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30 Next, we wanted to get a better understanding of the EB1:F-actin interaction by determining its
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32 salt sensitivity. To address this question, we used EB1₂₄₈ because this construct bound to F-actin
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34 the strongest. We found that as we increased the amount of salt from 25-175 mM KCl, the
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36 fraction of EB1₂₄₈ bound decreased and then leveled off (Figure 1d). This salt sensitivity
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38 indicates that at least part of EB1:F-actin binding is mediated by ionic interactions.

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40 We also tested EB1₂₄₈ for its ability to bind to F-actin without the presence of phalloidin to
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42 ensure that the interaction was mediated by F-actin and not phalloidin or the combination of
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44 phalloidin bound F-actin. We found that the fraction of EB1₂₄₈ bound was the same with or
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46 without phalloidin (Figure S3b). This is consistent with the idea that the interaction between EB1
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48 and F-actin is direct.

51 52 53 **The EB1:F-actin binding surface overlaps the EB1:MT binding surface.**

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55 The conclusion that the CH domain can bind to actin raises the question of how the F-actin and
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57 MT binding surfaces of EB1 relate to each other. To investigate this issue, we mapped onto the
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59 surface of EB1 a set of amino acids previously identified as being critical for tracking MTs [18]
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61 (Figure 2a-b, blue residues; for additional information refer to Figure S1b and Figure 3). Next,
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4 we mapped onto the crystal structure previous mutations that had no effect on MT tracking [24]
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6 (Figure 2 a-b, yellow residues). We then compared these two maps to a map of the surface
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8 residues that are highly conserved, under the assumption that highly conserved surfaces are
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10 involved in functionally significant interactions (Figure 2c-f and Figure S1a).

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12 When we compared these maps, we observed that the whole surface of the EB1 CH domain is
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14 well-conserved across vertebrates, including the face opposite the well-characterized MT binding
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16 interface (compare Figure 2b and d). [18,24] On the basis of these data, we hypothesized that this
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18 highly conserved back surface of EB1 might be involved in F-actin binding. The strong
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20 conservation of the EB1 surface is all the more striking when compared to the much weaker
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22 conservation of calponin, the protein for which CH domains are named (Figure 2g-j). We
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24 initially thought that we might be able to predict the actin binding surface of EB1 by aligning the
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26 EB1 CH domain to the region of the calponin CH that binds to actin, but this surface has not yet
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28 been determined; indeed, it is presently unclear whether the calponin CH does bind to actin
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30 (calponin's actin binding seems to be mediated by other parts of the protein).[25] Comparison to
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32 other actin-binding CH domains also gave ambiguous results (not shown).

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34 Therefore, to test the hypothesis that the back surface of EB1 is involved in binding to F-actin,
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36 we identified and mutated highly conserved, charged, surface residues on the MT binding
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38 surface of EB1 and in the face opposite (Table S1). We specifically mutated charged amino acids
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40 due to the electrostatic nature of the EB1:F-actin interaction (Figure 1d). We made the mutations
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42 in EB1_{FL} as opposed to EB1₂₄₈ because the full-length protein is the most physiologically
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44 relevant construct. However, this introduced a problem with assaying the binding activity of the
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46 mutated EB1 because EB1_{FL} has relatively weak actin binding, likely due to autoinhibition as
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48 concluded from the data in Figure 1c. Therefore, to assay the activity of the EB1 mutants, we
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50 mixed the EB1 with a fragment of APC (APC-C1) that binds to the EB1 tail in order to relieve
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52 its autoinhibition.[20,26]

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54 As predicted, EB1_{FL} WT and APC-C1 co-pelleted significantly (~50%) with F-actin when mixed
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56 together but not when incubated individually with F-actin (Figure 3a). We then used this assay to
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58 test the EB1 mutants for their ability to co-sediment with F-actin. The fraction of EB1 bound was
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60 calculated for each mutant (Figure S2a-b) and normalized against the fraction of EB1_{FL} WT
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bound under these conditions (Figure 3b). We then classified the 20 mutant EB1 proteins according to their effect on F-actin and MT binding (Table S1).

Of the 20 EB1 mutants generated, one was found to bind both F-actin and MTs more weakly (K89D), while six were found to bind MTs more weakly without significantly affecting F-actin binding (K83A, K83D, R84A, Q102E, and (I72,K76,Q79A)) (Figure 3b-f; note that colors in the figure are coordinated across panels according to the effect of the mutant). In addition, some mutants bound one or both filament types more strongly (E26A, D49K, D116R, D123K). Several mutations of conserved surface residues had no effect on binding to F-actin or MTs (E26K, D116A, and (8,910A)) (Figure 3b-f and Table S1). The mutants that showed decreased binding were tested to ensure that were properly folded using circular dichroism. Of the 7 mutants tested 5 of them had indistinguishable spectra compared to EB1_{FL} WT consistent with the idea that they are properly folded (Figure S3a). Two mutants (R84D and (55,58,59A)) showed decreased α -helical content and therefore were not used for EB1 mutagenesis analysis (Figure S3a).

To allow comparison between our mutagenesis data and previous work, we have mapped on to the EB1 crystal structure amino acid residues previously found to be important for the EB1:MT interaction. Residues defined by Maurer et al.[18], Zhang et al.[27], and Slep and Vale[24] are colored purple. Residues defined only by Maurer et al.[18] are colored blue. Those residues not important for MT tracking are colored yellow.[24] (Figure 3g-j). Zhang et al. also noted that part of the EB N-terminus differed between their cryo-EM structure and a previous crystal structure [28], suggesting that some subset of amino acids in this region might be involved in MT binding; this region has been colored dark gray.

The lysine at position 89 has been previously found to make important contacts with tubulin and to be a crucial amino acid for MT tracking.[18] We confirmed that the K89D mutation significantly reduced MT binding. Interesting to note, Zhang et al. did not find this residue making any important contacts on their most recent cryo-EM work.[27] We were intrigued to see that the K89D also interfered with F-actin binding. This observation suggests that the F-actin and MT binding interfaces overlap, contrary to our original hypothesis.

We found that some of our other mutations agreed with previously published work, but others did not. More specifically, Q102E and cluster mutant I72,K76,Q79A decreased MT binding (Figure S3b), as expected.[18,24,27] However, when we changed the histidine at position 69 to an alanine, we did not observe the reduction in MT binding expected from the idea that that this residue is in the MT binding interface.[18,27] This difference in results could be due to the differences in functional assays used (co-sedimentation vs. plus-end tracking assays) or the MT analogs used (GDP-Tx-MTs vs. GTP γ S-MTs).[18,27] Another interesting observation is that mutations in the amino acids K83 and R84 were previously found to have no effect on plus end tracking in vivo[24], but when we mutated these residues we found that they displayed decreased MT binding in our in vitro assays. Again this observation could be due to the differences in the functional assay or MT analogs used. The observation that several mutations affect binding to only MTs indicates that the overlap between the actin binding sites and MT binding sites is only partial.

We were surprised to see that some mutants (D49K, D116R, D125A/R) increased binding to MTs, F-actin or to both F-actin and MTs, and we were intrigued to see that these clustered on the conserved face opposite the MT binding interface. We speculate that this surface of EB1 may be making contacts with the C-terminal region of EB1 during autoinhibition, and that the mutations generated disrupt normal autoinhibition.

Upon finalizing our mutant analysis we wanted to ask whether the amino acid positions discussed above are conserved across a broader array of organisms. To do this, we widened our bioinformatics analysis to include several basal animals as well as a few selected unicellular eukaryotes. These sequences were aligned with the EB1 vertebrates and shown in Figure 4. When we examined the conservation at these positions, we found (as expected) that almost all of the residues previously established as being involved in MT binding (Figure 4; K83, R84 boxed red and K89 boxed black) are very highly conserved amongst all of the organisms examined. Q102 is particularly well conserved (Figure 4, red box). Most intriguing was the strong conservation of D49 and D116 (Figure 4, yellow boxes), two positions at which mutations caused increased binding. These two amino acids are conserved to a similar degree as Q102 and are located on the face opposite of the MT binding interface, suggesting that whatever function this surface has, it is highly conserved across eukaryotes.

MTs compete with actin for binding to EB1

The evidence that the F-actin and MT binding surfaces of EB1 overlap suggests that MTs might compete with F-actin for binding to EB1. To test this hypothesis, we had to devise an assay capable of assessing F-actin binding in the presence of MTs; the standard co-sedimentation assay would not work because proteins binding to either filament would end up in the pellet. After trying a series of different approaches (not shown), we developed an assay based on the observation that incubation of EB1₂₄₈ with actin causes formation of F-actin bundles (Figure 5a). It is important to note that the goal of this assay is to determine whether EB1 is binding to F-actin under a given set of conditions ($\pm$ MTs). We are not implying that F-actin bundling is a normal EB1 activity, nor suggesting that Taxol-MTs are the optimal EB1 substrate, only that bundling of F-actin by EB1₂₄₈ requires EB1:F-actin interactions.

Initial experiments established that EB1 does bundle F-actin (Figure 5a, 2nd row). We then tested how adding MTs affected the ability of EB1 to promote F-actin bundling. The idea was that if binding of EB1 to F-actin and MTs is mutually exclusive, the presence of MTs should interfere with the ability of EB1 to bundle F-actin. However, if EB1 can bind both F-actin and MTs at the same time, then addition of MTs shouldn't alter bundling of F-actin by EB1. Moreover, since EB1 also bundles MTs, one would predict that the F-actin and MTs would be co-bundled if EB1 can bind both filaments simultaneously, but that the filaments should remain separate if binding is mutually exclusive.

We chose to perform the experiments in a blinded matter (see Methods) to ensure that observer bias did not influence the results. As expected, bundles were not observed when F-actin and MTs were incubated alone (Figure 5a, top row). When we mixed 5 μ M actin with 1 μ M EB1₂₄₈, we observed frequent bundles in a background of unbundled F-actin (Figure 5a, 2nd row). EB1 also promoted the formation of MT bundles, some of which were extremely large (Figure 5a, 3rd row). However, when EB1₂₄₈ was mixed with both F-actin (5 μ M) and microtubules (0.5 μ M), F-actin bundles became rare (Figure 5a, 4th row, see also Figure 4b). In contrast, the effect of F-actin on MT bundling by EB1 was more difficult to discern (Figure 5a, 4th row, see also Figure 5c). Qualitatively, blinded observers noted that F-actin reduced the size of the MT bundles, though that effect was difficult to quantify (not shown). EB1_{FL} had little effect on bundling of

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4 either F-actin or MTs (Figure 5a, 5th row, see also Figures 5b-c), consistent with the idea that
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6 EB1_{FL} is autoinhibited.

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9 These observations support the hypothesis that F-actin and microtubules compete for binding to
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11 EB1. This hypothesis further predicts that EB1 should fail to co-bundle F-actin and
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13 microtubules. When labeled F-actin and microtubules were mixed in the presence of unlabeled
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15 EB1₂₄₈, the filament networks remained separate, consistent with this idea (Figure 5a, 4th row,
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17 note lack of colocalization between red MTs and green F-actin). The sum of these results lead us
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19 to conclude that EB1 can bind to F-actin or microtubules, but not both simultaneously.

20 21 **Is binding of EB1 to F-actin physiologically significant?**

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23 The affinity of EB1 for F-actin is relatively strong in 50 mM total KCl; examination of Figure 1d
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25 shows that approximately half of the EB1 is bound at 7 μ M F-actin, indicating that the apparent
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27 K_D is in the range of 7 μ M. 50 mM is a salt concentration commonly used in F-actin binding
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29 assays. However, the binding is weaker at more physiological salt concentrations (Figure 1d,
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31 compare 50 mM salt to 75 mM or 125 mM salt, where only ~20% of the protein is bound at 7
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33 μ M F-actin). At first glance, these data might be interpreted to imply that the interaction is not
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35 physiologically significant. However, it is important to remember that the local concentration of
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37 F-actin can be extraordinarily high in some regions of the cell, especially near the cell boundary.
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39 For example, the concentration of F-actin in the lamellipodium of a migrating cell has been
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41 estimated to be >500 μ M. [29,30] A protein with a 50 μ M K_D would be > 90% bound to actin in
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43 an environment with >500 μ M F-actin (assuming simple binding). This reasoning suggests that
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45 EB1:F-actin interactions could be relevant in contexts where the local concentration of F-actin is
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47 high, such as in the leading edge of a migrating cell where one might expect coordination
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49 between the actin and MT cytoskeletons.

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51 This information, together with our evidence (above) that MTs and F-actin compete for binding
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53 to EB1 (Figure 5), leads us to propose a model where EB1 interacts primarily with MTs in the
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55 relatively actin-poor interior of the cell, but is competed away from MTs, onto F-actin, in the F-
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57 actin-rich periphery of cells. In other words, we suggest that high concentrations of F-actin may
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59 act as a passive sink for EB1, helping to promote MT depolymerization in actin-rich areas, e.g.
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61 near the cell edge (Figure 6). We suggest this hypothesis because it has been shown that MTs
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63 grow persistently in the interior of cells and then display dynamic instability near the edge.[30]
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Alternatively, EB1's weak actin binding could become more significant in the context of a complex with other +TIP interacting proteins (e.g. formins, spectraplakins) that themselves bind actin[13,31], or they could be involved in other as-yet uncharacterized aspects of cross-talk between the actin cytoskeleton and components of the +TIP network. These ideas are compatible with the idea that high concentrations of F-actin might compete EB1 off of MTs.

One prediction of this model is that EB1 +TIP localization will be reduced in regions with high actin concentrations, e.g. the lamellipodium. For technical reasons including incompatibilities between EB-labeling methods and lamellipodium-labeling methods, it is difficult to address this question, so whether this prediction will be validated remains a focus for future work. Another question raised here is whether weak binding to actin might be a general feature of MT-binding CH domains. Given that actin-binding CH domains can use different surfaces to bind actin [10], and microtubule-binding CH domains can also use different surfaces to bind tubulin[27], it seems unlikely that one can make generalizations about the binding activities of CH domains. This too remains an interesting question for future study.

Conclusions

We have shown that the microtubule binding CH domain of EB1 can also bind to F-actin, and that contrary to our expectations, the surfaces that bind microtubules and F-actin partially overlap. Consistent with this observation, we have provided evidence that microtubules and F-actin compete for binding to EB1. We have shown that the affinity of the EB1:F-actin interaction is weak, but suggest that it could be significant in the periphery of the cell, where the concentration of F-actin is very high. One possible role for this interaction would be to destabilize MTs by recruiting away EB1 as the MTs approach the F-actin-rich cortex of the cell; it remains possible that the F-actin-binding activity of EB1 also has a more direct role in actin function mediated through formation of tripartite complexes with other proteins that bind both EB1 and actin (e.g. formins and APC).

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Supporting Information: Additional figures and methods can be found in the supporting information.

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Figure Legends:

Figure 1. Activated EB1 binds directly to F-actin. (a) EB1 constructs. EB1 full-length (EB1_{FL}) contains a N-terminal microtubule-binding CH (calponin-homology) domain, a flexible region, a dimerizing coiled coil, and an autoinhibitory acidic tail. EB1₂₄₈ is an activated fragment (also dimeric) that lacks the autoinhibitory acidic tail, while EB1₁₈₄ lacks the entire coiled-coil region and is monomeric.[22] (b) Purified EB1 proteins. An SDS-PAGE gel of the purified bacterially expressed EB1 proteins is shown. (c) F-actin binding assay, 2 μM of the indicated EB1 proteins (calculated as EB1 monomer) and/or 2 μM preformed F-actin (calculated as actin monomer) were incubated and centrifuged, and the resulting supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE. The “activated” EB1₂₄₈ and monomeric EB1₁₈₄ fragments show 100% binding under the assay conditions. In contrast, EB1_{FL} shows minimal actin binding, consistent with autoinhibition. BSA was used as a negative control for F-actin binding. (c) Salt sensitivity binding assay. 2 μM EB1₂₄₈ sedimented with 7 μM F-actin in increasing KCl concentrations. The fraction of EB1₂₄₈ bound to F-actin decreased as the salt concentration increased, consistent

with a salt sensitive interaction. The observation that ~half of the 2 μ M EB1 co-sedimented with 7 μ M F-actin in 50 mM salt indicates that at this salt concentration, the apparent K_D is ~7 μ M.

Figure 2. Functionally significant surfaces of EB1. (a, b) A set of amino acids that are crucial for MT tracking are shown in blue, [18] while those mutated without effect on MT tracking are shown in yellow[24], mapped onto the previously determined EB1 crystal structure (PDB ID: 1PA7.)(32] (c-f) EB1 conservation map, based on 10 vertebrate model organisms (see Methods). Red residues are 100% conserved, while pink and white are 70% and 50% conserved, respectively. These data indicate that the face opposite the MT binding interface is evolutionarily constrained and suggest that this region is important for EB1 function. (g-j) Calponin CH domain conservation (same species as in c-f) mapped on the crystal structure (PDB ID: 1WYP). The increased amount of pink and white surface in these panels as compared to c-f shows that the CH domain of Calponin is not as conserved as that of EB1.

Figure 3. Characterization of EB1 and EB1 mutants. (a) EB1:F-actin binding assay in which 2 μ M EB1_{FL} and/or 2 μ M APC-C1 were incubated with/without 7 μ M preassembled F-actin (all concentrations calculated as monomer), centrifuged, and the resulting supernatant (S) and pellet (P) fractions were analyzed by SDS PAGE. F-actin sedimented as expected, and neither EB1_{FL}, nor APC-C1, nor a mixture of EB1_{FL} and APC-C1 sedimented by themselves. In addition, neither EB1_{FL} nor APC-C1 alone co-sedimented with F-actin. However, when EB1_{FL} was incubated with APC-C1 to relieve autoinhibition and the mixture was incubated with F-actin, significant amounts of both EB1_{FL} and APC-C1 were found in the pellet fractions. These results provide a foundation for characterizing the effects of mutations on EB1:F-actin binding. (b) Summary of F-actin and MT binding assays performed with the EB1 mutants. Solid bars depict the normalized fraction of the indicated EB1_{FL} construct (2 μ M) that co-sedimented with F-actin (7 μ M), while shaded bars show the normalized fraction of the same EB1_{FL} construct (2 μ M) that bound to MTs (5 μ M). Bars are colored as indicated in the key according to the binding behavior. (c-f) Position of different classes of mutants as determined in (b), mapped onto the EB1 CH domain crystal structure (1PA7). As in (b), the amino acid residues are colored according to their binding behavior (see key in (b)). Each panel is a 90° rotation about the y-axis. (g-j) Summary of previously defined EB1:MT interfaces mapped on to the EB1 CH domain crystal structure (1PA7). Amino acid residues found to be important for MT interactions in

multiple studies are colored purple.[18,24,27] Residues identified only by Maurer et al. are colored blue.[18] Amino acid residues shown to unimportant for MT tracking are yellow[24] (Figure 3g-j). Zhang et al. noted that part of the EB N-terminus differed between their cryo-EM structure and a previous crystal structure [28], suggesting that some subset of the amino acids in this region might be involved in MT binding; this region has been colored dark gray.

Figure 4. Bioinformatic analysis of more divergent EB1 proteins. EB sequences from 8 basal animals and several model unicellular eukaryotes were added to the alignment from Figure S1 and realigned (black names: vertebrates; orange names: basal animals; purple names: unicellular eukaryotes). Amino acids that were mutated in this study are boxed. Yellow boxes indicate the positions of mutations that showed increased polymer binding (either MT or F-actin); red boxes indicate mutations that showed decreased MT binding; black boxes indicate mutations that decreased both MT and F-actin binding.

Figure 5. EB1–F-actin and MT binding is mutually exclusive. (a) Representative fluorescence microscopy images of EB1 incubated with Rhodamine MTs, Alexa-488 phalloidin labeled F-actin, or both as indicated; each image in a given column is adjusted to the same levels, except in row 3, where the main image was adjusted to allow visualization of the bundle, and the inset is normalized the same as the rest of the column. (b-c) Quantification of the microscopy data. The number of F-actin bundles per field of view (b) or MT bundles per field of view (c) counted in n=3 observer blind experiments. These data show that little bundling is observed when MTs and F-actin are incubated without EB1, but when EB1₂₄₈ is incubated with F-actin or MTs, many large bundles are observed. When MTs, F-actin, and EB1₂₄₈ are incubated together, the number of F-actin bundles decreased significantly, while the number of MT bundles was similar to experiments without actin. When EB1_{FL} is incubated with MTs and F-actin, bundling of neither filament is seen, consistent with the notion that EB1_{FL} is autoinhibited. Note that in the fluorescence images, the MT bundles rarely co-localize with the F-actin, suggesting that they are not cross-linked together.

Figure 6. Proposed model of EB1 cytoskeletal regulation. EB1 binds to and tracks the plus ends of MTs in the interior of the cell, where the MTs grow persistently, in part because of polymerization-promoting factors recruited by EB1.[9,33] As the MTs encounter the actin-rich cell periphery, the EB1 is competed off the MTs, promoting MT depolymerization.

Figure1

Figure 1

Figure 2

Figure3

Figure 3

Figure4

Figure 4

Figure 5

Figure 6