

Characterization of Functional Domains of Human EB1 Family Proteins*

Received for publication, June 12, 2003, and in revised form, September 23, 2003
Published, JBC Papers in Press, September 26, 2003, DOI 10.1074/jbc.M306194200

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EB1 family proteins are evolutionarily conserved proteins that bind microtubule plus-ends and centrosomes and regulate the dynamics and organization of microtubules. Human EB1 family proteins, which include EB1, EBF3, and RP1, also associate with the tumor suppressor protein adenomatous polyposis coli (APC) and p150^{glued}, a component of the dynactin complex. The structural basis for interaction between human EB1 family proteins and their associated proteins has not been defined in detail. EB1 family proteins have a calponin homology (CH) domain at their N terminus and an EB1-like C-terminal motif at their C terminus; the functional importance of these domains has not been determined. To better understand functions of human EB1 family proteins and to reveal functional similarities and differences among these proteins, we performed detailed characterizations of interactions between human EB1 family proteins and their associated proteins. We show that amino acids 1–133 of EB1 and EBF3 and the corresponding region of RP1, which contain a CH domain, are necessary and sufficient for binding microtubules, thus demonstrating for the first time that a CH domain contributes to binding microtubules. EB1 family proteins use overlapping but different regions that contain the EB1-like C-terminal motif to associate with APC and p150^{glued}. Neither APC nor p150^{glued} binding domain is necessary for EB1 or EBF3 to induce microtubule bundling, which requires amino acids 1–181 and 1–185 of EB1 and EBF3, respectively. We also determined that the EB1 family protein-binding regions are amino acids 2781–2820 and 18–111 of APC and p150^{glued}, respectively.

Microtubules are important for many cellular functions, such as intracellular transportation, maintenance of cell shape and cell polarity, and chromosome segregation. Appropriate functioning of microtubules requires appropriate regulation of their dynamics. Although microtubules are intrinsically dynamic, their dynamics can be regulated by many proteins *in vitro* and *in vivo* (1–3). In most cells, the minus-ends of microtubules are anchored at the centrosome, whereas plus-ends are more dy-

namic and are responsible for searching and contacting targets such as cell cortex and chromosomes. Proteins that bind plus-ends of microtubules may have important roles in regulating dynamics and functions of microtubules (4).

Human EB1 was initially identified as a protein that interacts with the C-terminal portion of the adenomatous polyposis coli (APC)¹ tumor suppressor protein (5). EB1 homologs have since been identified in many species (6–10). Whereas there is only one EB1 gene each in *Saccharomyces cerevisiae* (BIM1) and *Schizosaccharomyces pombe* (Mal3), there are three genes in humans encoding EB1 family proteins: EB1, RP1, and EBF3 (5, 11–13). There is a single form of EB1, but there are two forms of RP1 translated from different initiation codons and two forms of EBF3 translated from two alternatively spliced mRNAs (11). All three human EB1 family proteins appear to express ubiquitously (11). Human EB1 family proteins have been shown to bind APC (5, 11, 13), and EB1 also has been shown to associate with p150^{glued} (14–16), a microtubule plus-end-binding protein as well as a component of the dynactin complex that together with the cytoplasmic dynein forms a minus-end-directed microtubule motor (14, 17–19).

EB1 family proteins associate with microtubule plus-ends and centrosomes preferentially at endogenous levels but associate with the entire microtubules when overexpressed (6, 8, 12, 20–24). EB1 family proteins have been shown to regulate the assembly and functions of microtubules. Bim1p regulates microtubule dynamics in interphase *S. cerevisiae* cells and anchors microtubule plus-ends at the bud cortex through the interaction with Kar9p (20, 25–28). Inactivation of *Drosophila* EB1 affects the assembly, dynamics, and positioning of the mitotic spindle (10, 29). In mammalian cells, overexpression of EB1 and EBF3 has been shown to induce bundling of microtubules (24).

All EB1 family proteins have a highly conserved N-terminal region that contains a CH domain (Conserved Domain Database pfam00307) and an EB1-like C-terminal motif (Conserved Domain Database pfam03271), which is unique to EB1 family proteins (Fig. 1). The CH domain has been shown to be important for binding actin in some proteins, but its function in many other proteins is not clear (30). The EB1-like C-terminal motif (amino acids 209–254 of EB1) is partially overlapped with a proposed leucine zipper motif (amino acids 196–224 of EB1) (23), which is also called coiled-coil region (amino acids 191–226 of EB1) (9).

Despite the important functions of the EB1 family proteins, the structural basis for many of these functions is still not

* This work was supported in part by the Gillson Longenbaugh Foundation and NCI, National Institutes of Health, Grant CA 70371 and Cancer Center Core Grant CA 6672. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: APC, adenomatous polyposis coli; CH, calponin homology; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; MAPs, microtubule-associated proteins; PKA, protein kinase A; CAP-Gly domain, cytoskeleton-associated protein-glycine rich domain; EST, expressed sequence tag.

TABLE I
Primers used for generating cDNA fragments by PCR

Name	Upstream primers (5' → 3')	Downstream primers (5' → 3')
EB1d2	GCGGATCCAAGATGGCAGTGAACGTATAC	AGCAACAAGGGAAGGAGC
EB1d3	GCGGATCCAAGATGGCAGTGAACGTATAC	AAGCTTATTGACCTTGTCTGGCAGCCACA
EB1d4	GGATCCAAGATGGCACATGACATGCTGGCC	AAGCTTATTGACCTTGTCTGGCAGCCACA
EB1d6	GGATCCAAGATGGCAAGTCGACATGACATGCTG	AAGCTTAATCGAAAACTTCTTG
EB1d7	GCGGATCCAAGATGGCAGTGAACGTATAC	CTCGAGTTATCGCACCACACCCAGG
EB1d8	GCAGCAGGTCAACGTATTGAAGCTT	AAGCTTTTAATACTCTTCTTGCTCCTCC
EB1d9	GCAGCAGGTCAACGTATTGAAGCTT	GCAAGCTTGGGCCCCCTTCATCAGG
EB1d10	GCAGCAGGTCAACGTATTGAAGCTT	AAGCTTATTTCATCTGTGGCATACAG
EB1d11	GCAGCAGGTCAACGTATTGAAGCTT	GCGTCGACCTCTGCAATACAGGGTCG
EB1d12	GAATTCTTGAAGACTTGGAGAAAGAG	AAGCTTTTAATACTCTTCTTGCTCCTCC
EB1d13	CTACTTCGGAAGCTACGG	AAGCTTATTCATCTGTGGCATACAG
EB1d14	CTACTTCGGAAGCTACGG	AAGCTTTTAACTACTCTTCTTGCTCCTCC
EB1d15	GAATTCTTGAAGACTTGGAGAAAGAG	AAGCTTATTCATCTGTGGCATACAG
EB1d16	GCGAATTCTGATTTGCCAGGAGAACG	AAGCTTTTAACTACTCTTCTTGCTCCTCC
EB1d17	GCAGCAGGTCAACGTATTGAAGCTT	GCAAGCTTCTTGTCTCCTCTGTGG
EB1d18	GCGGATCCAAGATGGCAGTGAACGTATAC	GCGTCGACCTCTGCAATACAGGGTCG
EBF3d3	GAATTCTAATGGCCGTCATGTGTACTC	AAGCTTCACTGGCCCTGCCGCGC
EBF3d4	GGATCCGTATGGCCCATGATATGCTTGCATGG	AAGCTTCACTGGCCCTGCCGCGC
EBF3d5	GAATTCTAATGGCCGTCATGTGTACTC	ATCCTTTCCATCATAGTTTC
EBF3d6	GGATCCGTATGGCCCATGATATGCTTGCATGG	AAGCTTCCCTCAGTACTCGTCTCTGG
EBF3d7	GAATTCTAATGGCCGTCATGTGTACTC	AAGCTTTCAGCCCTGTGGGGGACGTCCTC
EBF3d8	GAATTCTAATGGCCGTCATGTGTACTC	CTCGAGTCACCGGAGAATGCAGGG
EBF3d9	TGGATGGGCTGGAGAAGG	AAGCTTATTCCTCTGTGGCATAGAG
RP1d3	GATATCGGAATGGCGGTCAATGTG	AAGCTTCATTGCCCTTGTCTGTG
RP1d4	CTCGAGCAATGCCTCATGACATCATTTGCA	AAGCTTCATTGCCCTTGTCTGTG
RP1d5	GAATTCTAAGAGCGTGGAAAAGG	AAGCTTATTCCTTCTGAAGCATACAG
APCd7 ^a	GTTTGGGTGAGAATTGAGG	AAGCTTTTAGGTTCCACTGGATTCTGTG
APCd8	CCTGAACCTCTTTATTCAGG	AAGCTTTTAGGTTCCACTGGATTCTGTG
APCd9	GAATTCGCAGCTCAAGCAAAACACAGTTC	AAGCTTTTAGGTTCCACTGGATTCTGTG
APCd10	GAATTCGAGTGACTCCTTTTAATTAC	AAGCTTTTAGGTTCCACTGGATTCTGTG
APCd11	AAGCCCTAGGAAAAGCAGC	AAGCTTTTAGGTTCCACTGGATTCTGTG
APCd12	GAATTCGCAGCTCAAGCAAAACACAGTTC	AAGCTTAAGTTTTTGAATCTCGCTTC
APCd13	GAATTCGCAGCTCAAGCAAAACACAGTTC	AAGCTTAATTAACCTGGAGTTGGGATC
APCd14	GAATTCGAGTGACTCCTTTTAATTAC	AAGCTTAAGTTTTTGAATCTCGCTTC
P150d1	CTCGAGATGAGTGGGAGGCAAGC	AAGCTTAAGAATCAGGTGTCTCTGG
P150d2	CTCGAGATGAGTGGGAGGCAAGC	AAGCTTAGATGCCATGCCCTTCATC

^a The APCd7 was generated by digesting the PCR product using BglII, which cut at codon 2763, and HindIII, which cut at the 3' primer.

clear. Although EB1 family proteins associate with microtubules and can induce microtubule bundling, they do not contain any previously characterized microtubule-binding domains. Although there have been some characterizations of the interactions of EB1 with APC and with p150^{glued}, these interactions have not been well defined (15, 23). Moreover, all three human EB1 family proteins express ubiquitously, but most studies so far have focused on EB1. To better understand functions of human EB1 family proteins and to reveal potential similar and different functions of these proteins, we performed detailed characterization of interactions between human EB1 family proteins and their associated proteins.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Nocodazole Treatment—Human colon cancer cell lines HCT116 and DLD-1 were purchased from the American Type Culture Collection (Manassas, VA) and were grown in McCoy's 5A medium and Dulbecco's modified Eagle's medium (high glucose), respectively, containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transfections were performed using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instructions. To depolymerize microtubules, cells were treated with 10 µM nocodazole at 37 °C for 1 h, as previously described (24).

Plasmids—Plasmids containing cDNAs encoding full-length EB1 family proteins and APC have been described (11, 31). Most cDNA fragments for EB1 family proteins or APC were generated by PCR using plasmids containing the corresponding full-length cDNA as the template. The cDNA fragment of p150d1 was generated by reverse transcriptase-PCR using total RNA from HCT116 cells. The p150d2 cDNA fragment was generated by PCR using plasmids containing p150d1 as the template. The cDNA fragments generated by PCR and sequences of primers used in PCR are summarized in Table I. PCR products were first cloned into pCR2.1 vector (Invitrogen) and sequenced. PCR fragments that had correct sequences were then transferred, with the

TABLE II
cDNA fragments obtained by restriction enzyme digestion

Name	Codons	Enzyme ^a
EB1d1	106–268	5'-EcoRI
EB1d5	1–106	3'-EcoRI
EBF3d1	144–281	5'-BglII
EBF3d2	1–145	3'-BglII
EBF3d10	1–236	3'-SacI
RP1d1	187–327	5'-BglII
RP1d2	1–217	3'-BamHI
EBF3/RP1	1–145/188–327	3'-BglII/5'-BglII
RP1/EBF3	1–188/145–281	3'-BglII/5'-BglII
APCd1	2075–2671	5'-BglII, 3'-BglII
APCd2	2167–2843	5'-EcoRI
APCd3	2167–2560	5'-EcoRI, 3'-XhoI
APCd4	2560–2843	5'-XhoI
APCd5	2167–2671	5'-EcoRI, 3'-BglII
APCd6	2673–2843	5'-BglII

^a Only enzymes used to cut within cDNA to generate ends as indicated are listed.

correct reading frame, into an appropriate plasmid of the pEGFP-C vector set (Clontech) for expressing GFP-tagged proteins in mammalian cells or into pGSTag (32) for expressing GST fusion proteins in *Escherichia coli*. For expressing untagged proteins in mammalian cells, PCR fragments were transferred into pCIN (33). Some cDNA fragments were obtained by digestion of plasmids containing longer cDNA fragments using the desired restriction enzymes and then transferred into vectors described above. These cDNA fragments are summarized in Table II.

Antibodies, Immunoblotting, and Immunofluorescent Staining—Primary antibodies against the EB1 family proteins, APC, and α -tubulin, and secondary antibodies conjugated with horseradish peroxidase, fluorescein, or Texas Red have been described previously (11, 24). Rhodamine-conjugated secondary antibodies were purchased from Pierce. The monoclonal antibody against GFP (JL-8) was purchased from

TABLE III
Primers used for site-directed mutagenesis of APC

Mutant	Sequence (5' → 3') ^a
S2789D	CCTTTTAATTACAACCCAGACCCCTAGGAAAAGC
S2830D	CCAGTGGAAACCCAAGATCCTAAGCGCCATTCTGGGTCTTACC
S2789A	CCTTTTAATTACAACCCAGCCCTAGGAAAAGC
S2793,2794D	CCCAAGCCCTAGGAAAGACGACGACGATAGCACTTCAGC
S2789E	CCTTTTAATTACAACCCAGAACCTAGGAAAAGC
S2793,2794E	CCCAAGCCCTAGGAAAGAGAAGCAGATAGCACTTCAGC
S2818E	CAACACAAAGAAGCGAGATGAGAAAACCTGACAGCACAGAATCC
S2793E	CCCAAGCCCTAGGAAAGAGAGCGCAGATAGCACTTCAGC
S2794E	CAAGCCCTAGGAAAAGCGAGGCAGATAGCACTTCAGC
S2799E	GCGCAGATAGCACTGAAGCTCGGCCATCTCAG
T2813E	CCAACCTCAGTGAATAACAACGAAAAGAAGCGAGATTCCAAAAC

^a Mutated codons are underlined.

Clontech, and antibodies against p150^{glued} and hDLG were purchased from Transduction Laboratories (Lexington, KY). Immunoblotting and immunofluorescent staining were performed as previously described (11, 24).

Characterization of Microtubule-binding Domain and Microtubule-bundling Domain of EB1 Family Proteins—HCT116 or DLD-1 cells seeded on sterile cover glass were transfected with plasmids expressing EGFP-tagged (for mapping microtubule-binding domain) or untagged (for mapping microtubule-bundling domain) full-length fragments of (EB1 family proteins). The day after transfection, cells were fixed and stained with α -tubulin antibody as previously described (24). EGFP-tagged proteins were visualized directly using a fluorescein filter.

Mapping of APC- and p150^{glued}-binding Domains of EB1 Family Proteins—An *in vitro* pull-down assay similar to that described previously (11) was used to map the APC- and p150^{glued}-binding domain of EB1 family proteins. Different EGFP-tagged fragments of EB1 family proteins were expressed in HCT116 cells by transient transfection. Lysates were prepared from cells the day after transfection and incubated with purified GST-tagged APC or p150^{glued} fragments immobilized on glutathione-agarose beads (Sigma). Purified protein complexes were then resolved by SDS-PAGE, and EGFP-tagged proteins were detected by immunoblotting using an anti-EGFP antibody.

Some fragments of EB1 were also expressed as GST-tagged proteins in *E. coli*, affinity-purified, and used to bind wild-type APC from HCT116 cell lysate as previously described (11).

Mapping of EB1 Family Protein-binding Domains of APC and p150^{glued}—An *in vitro* pull-down assay similar to that described above was used. Different APC or p150^{glued} fragments were expressed as GST-tagged proteins, purified, and then used to pull down EB1 family proteins from HCT116 cell lysates. EB1 family proteins were detected by immunoblotting.

Site-directed Mutagenesis—Potential phosphorylation sites of APC were predicted using NetPhos 2.0 (available on the World Wide Web at www.cbs.dtu.dk/services/NetPhos). Mutations were introduced into APCd4 using the QuikChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The sequences of primers used for site-directed mutagenesis are listed in Table III. The DNA sequences of all mutants were confirmed by DNA sequencing.

Determination of Fractions of Cells without Microtubule Plus-end-localized p150^{glued} or EBF3—To quantify the fraction of cells without microtubule plus-end localized p150^{glued} and EBF3 caused by the expression of EGFP-tagged EB1 fragments, cells transfected side-by-side with the same plasmid expressing an EGFP-tagged EB1 fragment were stained with primary antibodies against either EBF3 or p150^{glued} and rhodamine-conjugated secondary antibodies. The localization of EBF3 or p150^{glued} in more than 50 randomly selected green cells, indicative of expression of EGFP-tagged EB1 fragments, was examined. The percentage of cells without microtubule plus-end-localized of p150^{glued} or EBF3 among transfected cells was then calculated. Two independent transfections of each EB1 fragment were performed.

RESULTS

Microtubule-binding Domain of EB1 Family Proteins—We mapped the microtubule-binding domain of EB1 family proteins by examining the distribution pattern of EGFP-tagged fragments of EB1 family proteins in transiently transfected cells. Similar approaches have previously been used to identify the microtubule-binding domain of other MAPs (34–36). Our initial characterizations showed that only the N-terminal con-

served region-containing part of EB1 family proteins associated with microtubules *in vivo*, whereas the EB1-like C-terminal motif-containing part did not (Fig. 1). Examination of additional N-terminal fragments of EB1 family proteins showed that EGFP-tagged proteins containing amino acids 1–133 of EB1 and EBF3 and the corresponding region of RP1 (amino acids 44–176) colocalized with microtubules. Furthermore, the filamentous distribution pattern of these EGFP-tagged EB1 family protein fragments disappeared along with microtubules in cells treated with nocodazole (Fig. 1). Deletions from either the N terminus (EB1d4, EBF3d4, and RP1d4) or C terminus (EB1d5 and EBF3d5) of this 133-amino acid fragment abolished its microtubule-binding ability (Fig. 1). These results demonstrated that the N-terminal 133 amino acids of EB1 and EBF3 and the corresponding region of RP1 are sufficient and necessary for association with microtubules.

Microtubule-bundling Domain of EB1 Family Proteins—We then characterized the microtubule-bundling domain of EB1 and EBF3. We had previously shown that overexpression of EB1 and EBF3 induced microtubule bundling in many mammalian cell lines (24). Many MAPs have been shown to induce microtubule bundling when overexpressed, and their microtubule-binding domains are necessary but not sufficient for their microtubule-bundling activity (36–38). To identify the microtubule-bundling domain of EB1 family proteins, we expressed fragments of EB1 family proteins in HCT116 cells and then examined the microtubule structure of transfected cells by immunostaining α -tubulin. Deletion of amino acids 3–17 from EBF3 (EBF3d6) (Fig. 2) abolished the ability of EBF3 to associate with microtubules and to induce microtubule bundling (data not shown). On the other hand, amino acids 1–145 of EBF3 (EBF3d2) clearly associated with microtubules but could not cause microtubules to bundle (Fig. 1 and data not shown). Thus, the microtubule-binding domain was required but not sufficient for EBF3 to induce microtubule bundling. We then examined the ability of additional N-terminal fragments of EBF3 to induce microtubule bundling and found that the EBF3 fragment containing amino acids 1–185 (EBF3d8) was able to induce microtubule bundling, whereas the EBF3 fragment containing amino acids 1–165 (EBF3d7) could not (Fig. 2). An EB1 fragment containing amino acids 1–181 (EB1d7) also was able to induce microtubule bundling (Fig. 2). These results show that the putative coiled-coil region is not required for the microtubule-bundling activity of EB1 and EBF3. Similar to full-length EBF3 and EB1, EBF3d8 and EB1d7 induced microtubule bundling efficiently in DLD-1 cells, which expressed only truncated APC (Fig. 2).

Our previous study showed that overexpression of RP1 did not induce microtubule bundling (24). To determine the region of RP1 that caused it to behave differently from EB1 and EBF3, we examined the ability of two fusion proteins between EBF3 and RP1 (Fig. 2A) to induce microtubule bundling. Overexpres-

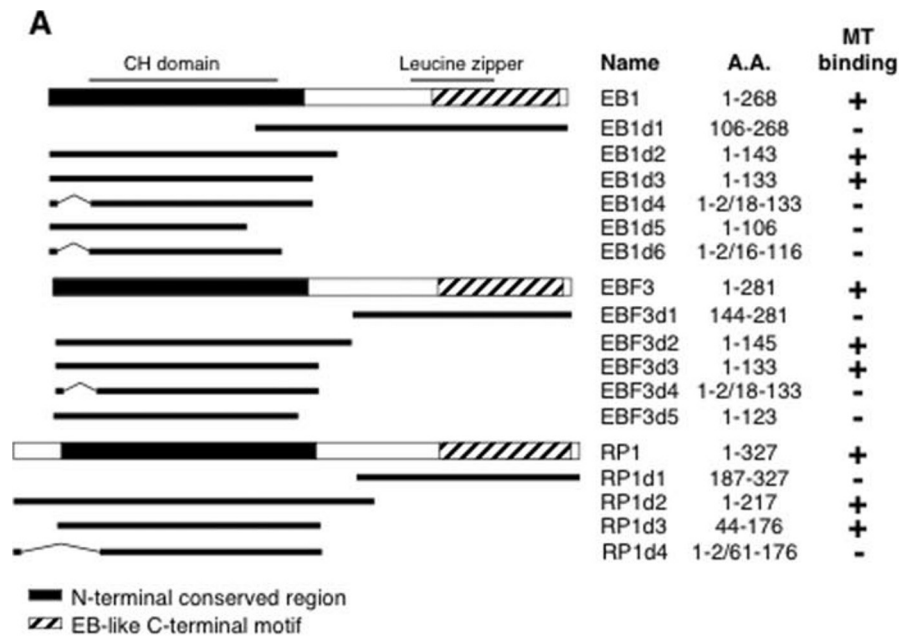
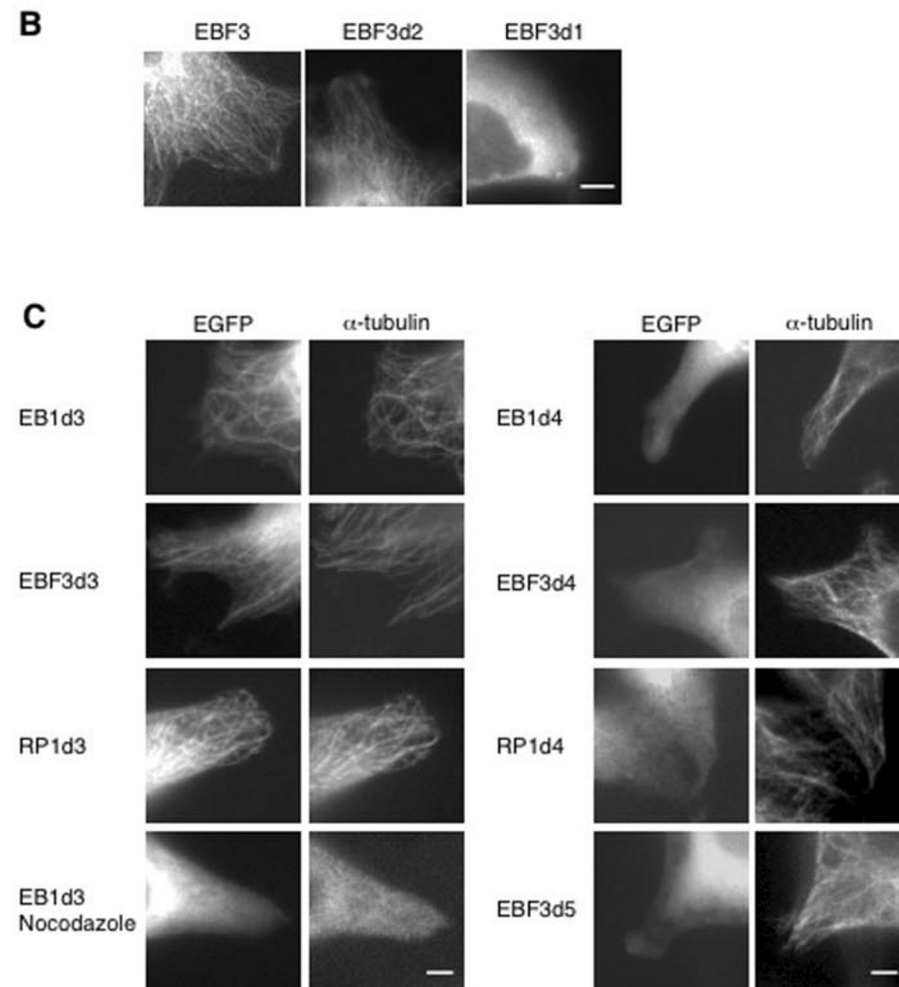


FIG. 1. Identification of the microtubule-binding domain of EB1 family proteins. *A*, schematic summary of the mapping of the microtubule-binding domain of EB1 family proteins. A.A., amino acids; MT, microtubule. Plus and minus signs indicate proteins that do and do not, respectively, bind microtubules. *B*, association of the N-terminal conserved region of human EB1 family proteins with microtubules. *C*, defining the microtubule-binding domain of EB1 family proteins. The bottom row of the left panel shows the result of cells treated with nocodazole. HCT116 cells were transfected with plasmids expressing indicated fragments of human EB1 family proteins tagged with EGFP. Microtubules were revealed by immunofluorescent staining of α -tubulin, whereas EGFP-tagged proteins were visualized directly using a fluorescein filter. Bar, 10 μ m.

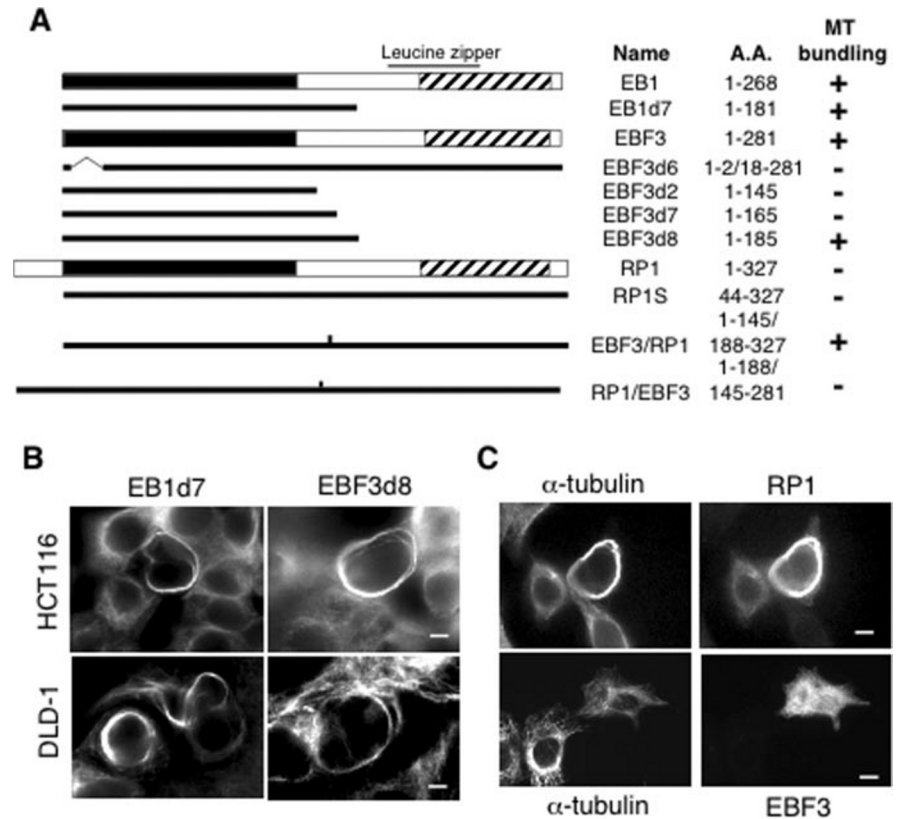


sion of the EBF3/RP1 fusion protein caused microtubules to bundle, and the EBF3/RP1 protein associated with microtubule bundles (Fig. 2C). In contrast, overexpression of the RP1/EBF3 fusion protein did not induce microtubule bundling, although it associated with microtubules (Fig. 2C).

APC-binding Domain of EB1 Family Proteins—Although an

EB1 fragment containing the C-terminal 86 amino acids (amino acids 183–268) has been shown to bind APC (5) and amino acids 219–241 have been speculated to be sufficient for the APC binding (15), the APC-binding domain of EB1 has not been carefully defined. Moreover, the APC-binding region of other members of EB1 family has not been studied. To identify

FIG. 2. Identification of the microtubule-bundling domain of EB1 family proteins. *A*, schematic summary of the mapping of the microtubule-bundling domain of EB1 family proteins. A.A., amino acids; MT, microtubule. *Plus* and *minus* signs indicate proteins that do and do not, respectively, induce microtubule bundling. *B*, induction of microtubule bundling by the microtubule-bundling domain of EB1 and EBF3. The microtubule structure in HCT116 or DLD-1 cells transfected with plasmids expressing the indicated proteins were revealed by immunofluorescent staining using α -tubulin antibody. *C*, induction of microtubule bundling by the EBF3/RP1 fusion protein but not by RP1/EBF3 fusion protein. HCT116 cells transfected with plasmids expressing EBF3/RP1 (*upper panel*) or RP1/EBF3 (*lower panel*) were double-stained with antibodies for α -tubulin (*left column*) and RP1 (*upper right panel*) or EBF3 (*lower right panel*). Bar, 10 μ m.



the APC-binding domain of EB1, we expressed various EB1 fragments (Fig. 3A) as GFP-tagged proteins in HCT116 cells and investigated which could be pulled down by the GSTAPC4 fusion protein. The result showed that the EB1 fragment containing amino acids 208–251 (EB1d15) was the minimum EB1 fragment sufficient for binding APC (Fig. 3B). Further deletions from the C or N terminus of this region (EB1d11, EB1d13, and EB1d14) eliminated the APC binding ability (Fig. 3). Because the APC-binding region of EB1 was contained within the EB1-like C-terminal motif conserved among EB1 family proteins, we investigated whether the corresponding regions of EBF3 and RP1 also could associate with APC. The result showed that the EBF3 fragment containing amino acids 217–260 (EBF3d9) and the RP1 fragment containing amino acids 259–302 (RP1d5) were able to bind APC (Fig. 3B). On the other hand, our results showed that EB1 and EBF3 mutants lacking part of their C-terminal APC-binding domain (EB1d18 and EBF3d10) could not bind APC (Fig. 3). These results show that there is most likely only one APC-binding site in EB1 and EBF3.

Although RP1d5 could associate with GSTAPC4, the efficiency was much lower than that of corresponding regions of EB1 and EBF3 (Fig. 3B). This result suggested that the binding between APC and RP1 was much weaker than that between APC and EB1 or EBF3, which could explain why we were unable to confirm a previously reported association between APC and RP1 (11, 13). To test this hypothesis, we used GSTAPC4 to pull down RP1 from lysates of cells that did or did not overexpress RP1. As shown in Fig. 3C, GSTAPC4 could pull down RP1 only when RP1 was overexpressed, and GSTAPC4 consistently pulled down the long form of RP1 more efficiently than it pulled down the short form of RP1.

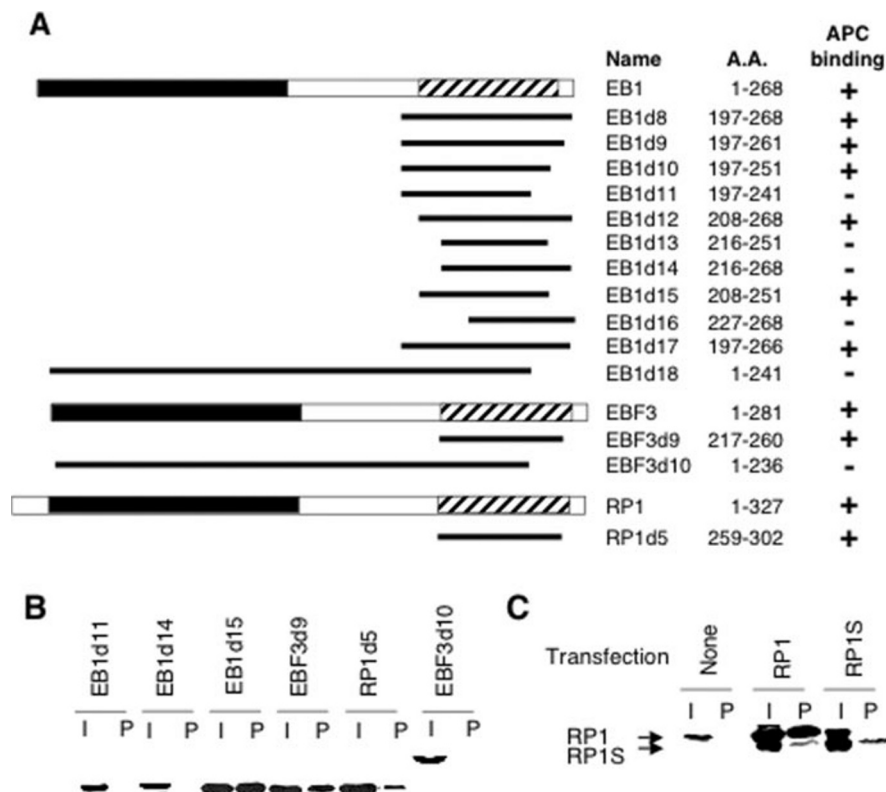
EB1-binding Domain of APC—To better understand the interaction between EB1 family proteins and APC, we also characterized the EB1 family protein-binding domain of APC. The C-terminal 170 amino acids of APC (amino acids 2674–2843)

have been shown to be sufficient for binding EB1 (39). However, amino acids 2537–2544 of APC have been suggested to be the EB1 family protein-binding region (12).

To identify the EB1 family protein-binding domain(s) of APC, we expressed various APC fragments (Fig. 4A) as GST fusion proteins and examined their ability to pull down endogenous EB1 and EBF3 from cell lysate. As shown in Fig. 4B, only APC fragments containing the C-terminal 170 amino acids (APCd2, APCd4, and APCd6) could bind EB1 and EBF3. In contrast, APC fragments containing amino acids 2537–2544 but not the C-terminal 170 amino acids (APCd1, APCd3, and APCd5) could not bind EB1 or EBF3. Further investigations showed that an APC fragment containing only amino acids 2781–2820 (APCd14) was sufficient to bind EB1 and EBF3 (Figs. 4B). Deletions of either 8 N-terminal or 10 C-terminal amino acids from this APC fragment (APCd11 and APCd13) abolished its ability to bind EB1 and EBF3 (Fig. 4B). Because GSTAPC4 could only pull down RP1 from lysates of cells overexpressing RP1 (Fig. 3C), we did not investigate the RP1-binding region of APC in detail. However, both APCd4 and APCd14 could pull down RP1 from lysate of cells that overexpressed RP1 (Fig. 3C and data not shown). Thus, APC uses the same domain to interact with all three EB1 family proteins.

We carried out preliminary identification of amino acids critical for APC to interact with EB1 family proteins by investigating the ability of phosphorylation-mimicking mutants of APC to interact with EB1 and EBF3. There were several potential phosphorylation sites within the 40 amino acids of the EB1 family protein-binding domain of APC based on low stringent criteria: one for CDC2 (Ser²⁷⁸⁹), three for PKA (Ser²⁷⁹³, Ser²⁷⁹⁴, and Ser²⁸¹⁸), two for casein kinase II (Ser²⁷⁹³ and Ser²⁸¹⁸), and two for protein kinase C (Ser²⁷⁹⁹ and Thr²⁸¹³). As shown in Fig. 4C, either mutation of the CDC2 phosphorylation site Ser²⁷⁸⁹ to Asp or simultaneous mutation of PKA phosphorylation sites Ser²⁷⁹³ and Ser²⁷⁹⁴ to Glu reduced the ability of APCd4 to interact with EB1 and EBF3. Mutations of any other

FIG. 3. Identification of the APC-binding domain of EB1 family proteins. A, schematic summary of the mapping of the APC-binding domain of EB1 family proteins. A.A., amino acids. Plus and minus signs indicate proteins that are and are not, respectively, pulled down by GSTAPC4. B, association of APC with fragments of EB1 family proteins. EGFP-tagged fragments of EB1 family proteins were expressed in HCT116 by transient transfection, and the presence of these proteins in total lysate (I) or in the protein complex pulled down by GSTAPC4 (P) were detected by immunoblotting using an EGFP antibody. C, association of APC with overexpressed RP1. Lysates were prepared from HCT116 cells that were not transfected (None) or transfected with a plasmid expressing both forms of RP1 proteins (RP1) or a plasmid expressing only the short form of RP1 (RP1S) (11), and RP1 in total lysate (I) or in the protein complex pulled down by GSTAPC4 (P) was detected by immunoblotting using an RP1 antibody (11).



potential phosphorylation sites did not have a detectable effect on the interaction between APCd4 and EB1 or EBF3 (Fig. 4C).

Association of APC with EB1 and hDLG—We investigated whether EB1 and hDLG could bind APC simultaneously. A previous study showed that a peptide containing only the C-terminal 15 amino acids of APC could bind hDLG, but it did not rule out the possibility that APC had additional hDLG binding sites (40). As shown in Fig. 5, APCd6, which contained the C-terminal 170 amino acids of APC, could bind hDLG very efficiently, whereas APCd7, which was identical to APCd6 except without the C-terminal 15 amino acids, could not bind hDLG. These results demonstrated that the EB1 family protein- and the hDLG-binding domains of APC were separated and suggested that there was no additional hDLG-binding domain in the EB1-binding region of APC. However, when we pulled down endogenous wild-type APC from cell lysate using GSTEB1, there was no detectable hDLG in the protein complex (Fig. 5B). Similarly, although APC was detected in the anti-hDLG immunocomplex, EB1 was not (data not shown). These results show that EB1 and hDLG are unlikely to bind APC simultaneously.

Interaction between EB1 Family Proteins and p150^{glued}—The association between human EB1 and rat p150^{glued} had previously been shown to require the N-terminal 330 amino acids of p150^{glued} and the C-terminal 84 amino acids of EB1 (15, 16). We sought to define the interaction between EB1 and p150^{glued} in more detail and to investigate whether other members of human EB1 family proteins interact with p150^{glued}. As shown in Fig. 6A, a human p150^{glued} fragment containing amino acids 18–111 (p150d1) could pull down EB1 and EBF3 but not RP1. Deletion from either C terminus (p150d2, amino acids 18–87) or N terminus (p150d3, amino acids 42–111) of p150d1 abolished its interaction with EB1 (Fig. 6B and data not shown). To define the p150^{glued}-binding domain of EB1 in more detail, we expressed different EB1 fragments (Fig. 6C) as EGFP-tagged proteins in HCT116 cells and investigated which could be pulled down by GSTp150d1. As shown in Fig. 6D, the C-termi-

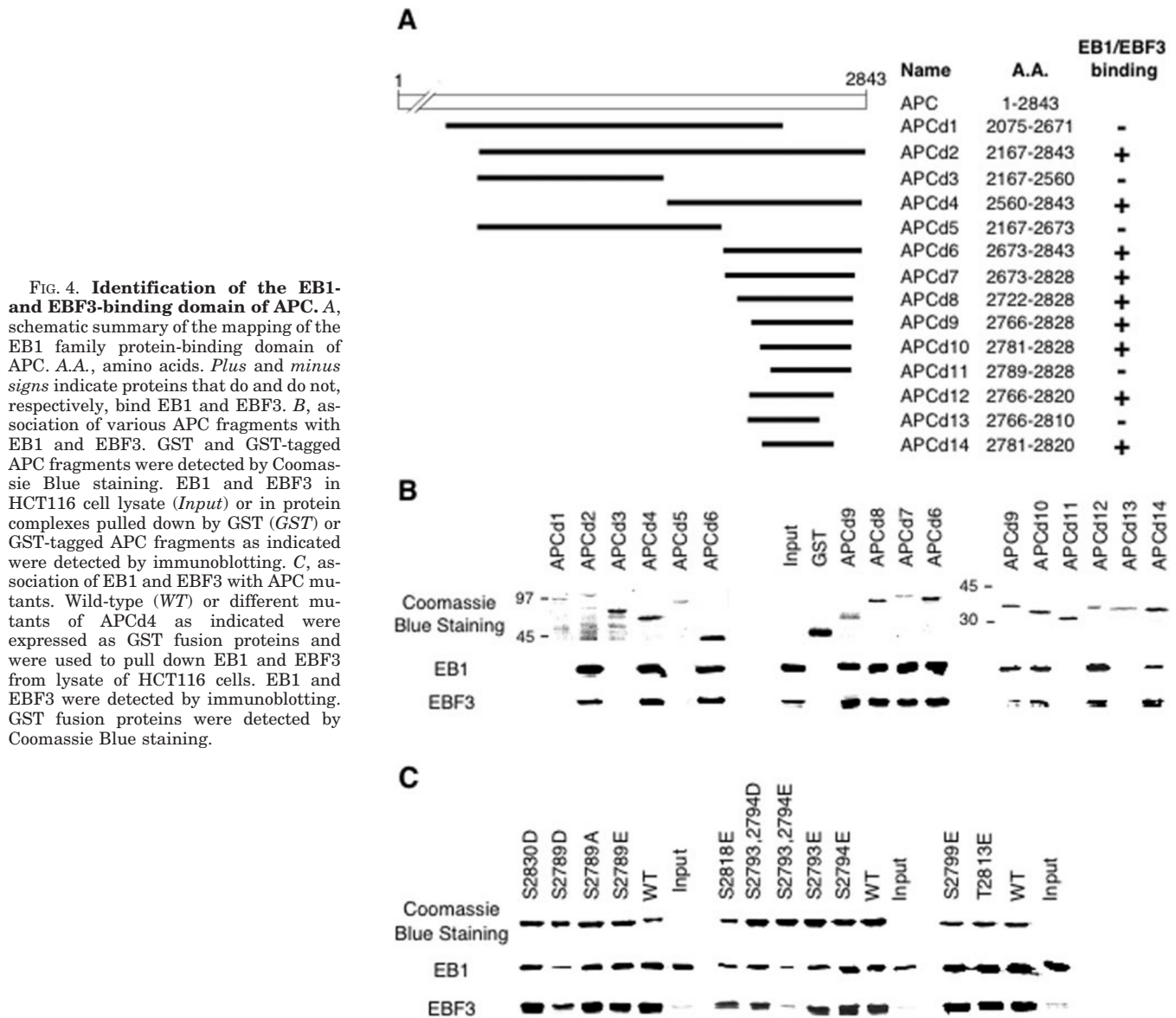
nal 61 amino acids of EB1 (EB1d12) interacted with p150^{glued} very efficiently. Deletions from the N terminus of EB1d12 gradually reduced the association of EB1 with p150^{glued} (EB1d14 and EB1d16), whereas deletion of only 2 amino acids from the C terminus (EB1d17) abolished the ability of EB1 to interact with p150^{glued} (Fig. 6D). Our results thus show that APC and p150^{glued} associate with overlapping but different regions of EB1.

The EB1-binding domain of p150^{glued} we mapped overlapped with the CAP-Gly domain that was important for p150^{glued} to bind microtubules (41). We therefore investigated whether expression of the C-terminal EB1 fragments affected the localization of p150^{glued} at microtubule plus-ends. Expression of EGFPEB1d8 at levels that did not obviously affect the structure of microtubules and the microtubule plus-end localization of EBF3 clearly reduced the microtubule plus-end localization of p150^{glued} (Fig. 7). Unexpectedly, expression of EGFPEB1d9 or EGFPEB1d17, which could not bind p150^{glued}, also reduced the microtubule plus-end localization of p150^{glued} (Fig. 7 and data not shown). In contrast, expression of a N-terminal fragment of EB1 (EGFPEB1d6) did not affect the localization of p150^{glued} (Fig. 7). Fig. 7B shows the fractions of cells without microtubule plus-end-localized EBF3 and p150^{glued} when overexpressing these different EB1 fragments.

DISCUSSION

We describe in this paper important information for interactions between human EB1 family proteins and their known associated proteins. All interaction domains contain 40 or more amino acids. These results suggest that the interaction between EB1 family proteins and their associated proteins involved complex three-dimensional structure rather than short linear peptides of both partners. Determination of the three-dimensional structure of interacting regions of these proteins would greatly facilitate our understanding of these interactions.

The CH Domain and Microtubule Binding—It has been sug-



gested that amino acids 123–175 of RP1 and the corresponding regions of EB1 and EBF3 are the microtubule-binding domain of EB1 family proteins (23). On the other hand, Askham *et al.* (15) reported that the microtubule-binding domain of EB1 was amino acids 1–168. Our results show that the N-terminal 133 amino acids of EB1 and EBF3 and the corresponding amino acids 44–176 of RP1 are sufficient and necessary for these proteins to associate with microtubules *in vivo*. Although it has been suggested that the coiled-coil region is required for EB1 family proteins to interact with microtubules (9), our results show clearly that the coiled-coil region is not necessary for human EB1 family proteins to bind microtubules.

The microtubule-binding domain of EB1 family proteins does not have microtubule-binding motifs of well characterized MAPs such as tau, MAP1A, MAP1B, MAP-2, MAP-4, CLIP-170, and CLIP-115 (34, 36, 42–46). Instead, the microtubule-binding domain of EB1 family proteins contains a CH domain (30). Although the CH domain of EB1 family proteins by itself is not sufficient for microtubule binding, it is necessary for EB1 family proteins to interact with microtubules (Fig. 1). Our findings demonstrate for the first time that a CH domain is involved in binding microtubules.

While this manuscript was being reviewed, Hayashi and

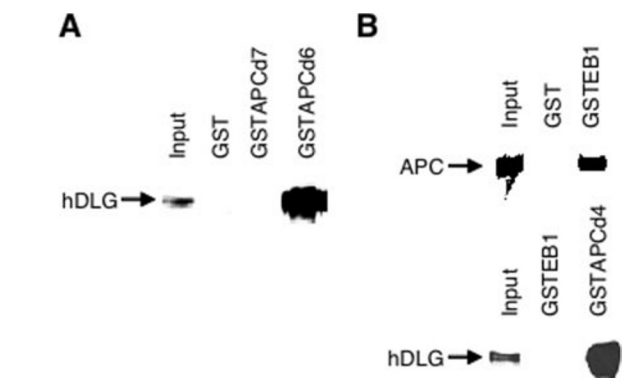
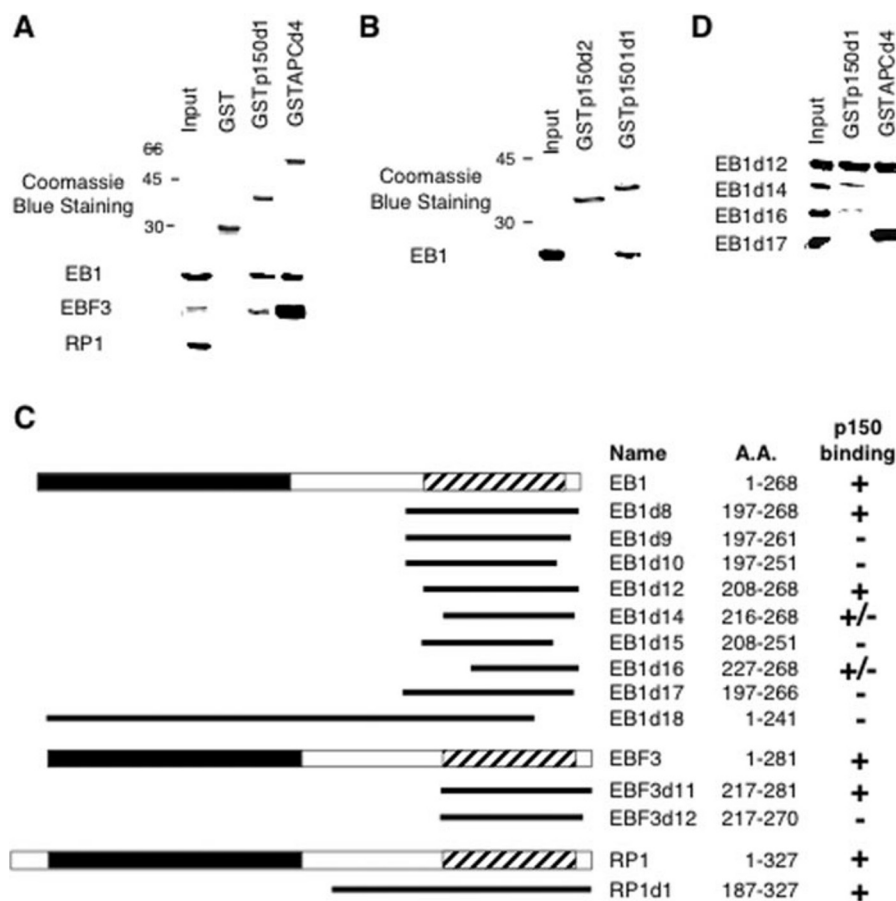


FIG. 5. Interactions among APC, EB1, and hDLG. A, separate hDLG- and EB1 family protein-binding regions of APC. hDLG in total cell lysate (*Input*) or in protein complexes pulled down by GST, GSTAPCd7, or GSTAPCd6 was detected by immunoblotting. B, lack of simultaneous association of EB1 and hDLG with APC. APC (*upper panel*) and hDLG (*lower panel*) in total cell lysate (*Input*) or in protein complexes pulled down by the indicated GST fusion proteins were detected by immunoblotting. Equal aliquots of the same protein complex pulled down by GSTEB1 were resolved on SDS-agarose gel for detecting APC (*upper panel*) and on SDS-PAGE gel for detecting hDLG (*lower panel*).

FIG. 6. Identification of interaction domains of p150^{glued} and EB1 family proteins.

A, association of EB1 family proteins with the N-terminal 111 amino acids of p150^{glued}. EB1 family proteins in total cell lysate (*Input*) or in protein complexes pulled down by the indicated GST fusion proteins were detected by immunoblotting. **B**, defining the EB1 binding domain of p150^{glued}. EB1 in total cell lysate (*Input*) or in protein complexes pulled down by the indicated GST fusion proteins were detected by immunoblotting. **C**, identification of the P150^{glued} binding domain of EB1. Different EGFP-tagged fragments of EB1 were individually expressed in HCT116 cells by transient transfection, and the presence of these proteins in the total cell lysate (*Input*) or in protein complexes pulled down by the indicated GST fusion proteins were detected by immunoblotting using an EGFP antibody. Equal amounts of GSTp150d1 and GSTAPC4 were used in each pull-down experiment. **D**, schematic summary of the mapping of the p150^{glued}-binding domain of EB1 family proteins. A.A., amino acids. Plus and minus signs indicate proteins that are and are not, respectively, pulled down by GSTp150d1.



Ikura (47) published the crystal structure of the N-terminal 130 amino acids of EB1 and showed that the core structure of this EB1 fragment was most similar to that of the CH domain of calponin, spectrin, and fimbrin. They also showed that this EB1 fragment was sufficient for association with microtubules *in vitro*. These observations are very similar to our results (47). However, our results provide additional information that this fragment appears to be the minimal region of EB1 for binding microtubules, because fragments with deletions of either amino acids 3–17 or amino acids 117–133 cannot interact with microtubules. Intriguingly, the structures of amino acids 8–13 and 123–126, both of which may be important for the interaction with microtubules, could not be determined. Additional studies will be necessary to understand why the CH domain of EB1 family proteins associates with microtubules, whereas the similar CH domains, in tandem repeat, of calponin, spectrin, and fimbrin interact with actin.

Microtubule-bundling Domain—Overexpression of many MAPs has been shown to cause microtubules to bundle. Microtubule bundles induced by MAP-2 and tau have been shown to be caused by MAP-2 and tau forming cross-bridges between microtubules and are mostly separate bundles emanating from central to peripheral areas of cells (37, 38). In contrast, microtubule bundles induced by CLIP-170, another microtubule plus-end-binding protein, are usually circumferential rings around the perimeter of cells and are proposed to be a consequence of the overgrowth of microtubule plus-ends (34). The microtubule bundles induced by EB1 family proteins are circumferential rings similar to those induced by CLIP-170 (24). Because EB1 family proteins bind microtubule plus-ends and EB1 increases the rescues and decreases pauses and catastrophes of microtubules in *Xenopus* egg extracts (48), it is likely that the microtubule bundling induced by EB1 family proteins

is also a consequence of overgrowth of microtubule resulting from stabilization of microtubule plus-ends.

A recent study suggested that both EB1 and APC are required to promote the polymerization of tubulin *in vitro* (49), and another study suggested that the full-length EB1 was required for EB1 to induce microtubule bundling *in vivo* (15). We have previously shown that overexpression of wild-type EBF3 or EB1 causes microtubules to bundle not only in cells that expressed wild-type APC but also in those that expressed only mutant APC that could not interact with EB1 family proteins (24). Results presented in this study show that EB1 and EBF3 mutants without APC- and p150^{glued}-binding domains induce microtubule bundling in HCT116 cells, which expressed wild-type APC, and DLD-1 cells, which expressed only mutant APC that could not associate with EB1 family proteins (Fig. 5). Therefore, our results consistently show that interaction with APC is not necessary for EB1 and EBF3 to stabilize microtubules and to cause microtubule bundling *in vivo*.

The difference between our *in vivo* results and those of *in vitro* studies (49) could simply be due to difficulties in identifying an *in vitro* condition, such as types and concentrations of salts and proteins, truly mimicking that of *in vivo*. A possible reason for the difference in defining the microtubule-bundling domain of EB1 between our study and that of Askham *et al.* (15) is that we used full-length EB1 family proteins or fragments of EB1 family proteins without any heterologous amino acids attached, whereas Askham *et al.* (15) used green fluorescent protein-tagged EB1. Our experience has been that untagged full-length and fragments of EB1 and EBF3 are more efficient than their EGFP-tagged counterparts in causing microtubule bundling. This could also explain why microtubule

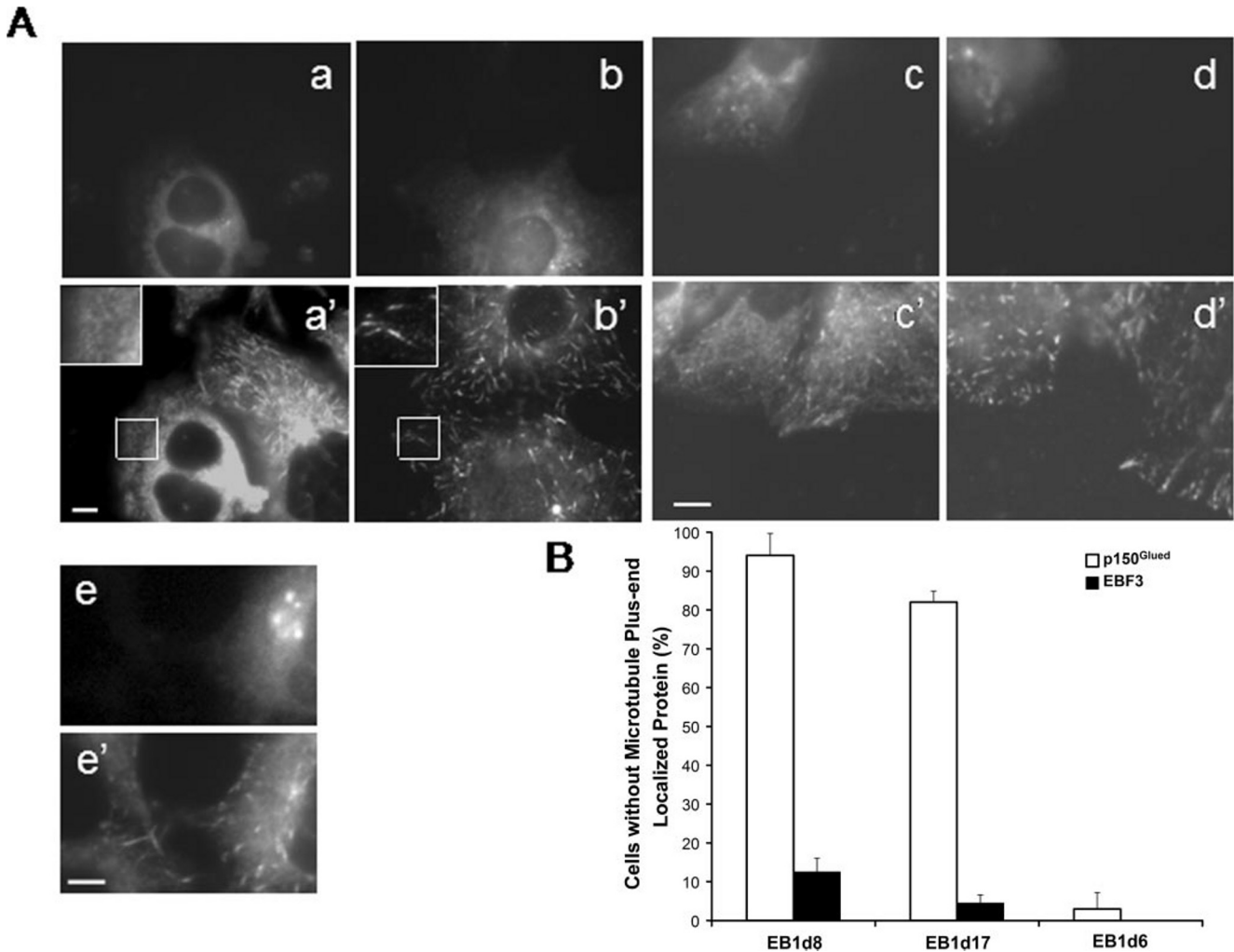


FIG. 7. Reduction of microtubule plus-end localized p150^{glued} in cells expressing C-terminal EB1 fragments. A, HCT116 cells (a, a', b, b', e, and e') or DLD-1 cells (c, c', d, and d') were transfected with plasmids expressing EGFPEB1d8 (a, a', b, and b'), EGFPEB1d17 (c, c', d, and d'), or EGFPEB1d6 (e and e'). p150^{glued} (a', c', and e') and EBF3 (b' and d') were revealed by immunofluorescent staining. The transfected cells were detected by EGFP signal (a–e) using a fluorescein filter, and the exposure times are identical between a and b and between c and d. The insets in a' and b' show an enlarged view of a region in the absence of the plus-end localized p150^{glued} and presence of the plus-end localized EBF3, respectively, of cells expressing EGFPEB1d8. Bar, 10 μ m. B, quantification of the loss of microtubule plus-end localization of p150^{glued}. The percentage of cells transfected with each indicated EB1 fragment (x axis) that have reduced microtubule plus-end localization of p150^{glued} or EBF3 (y axis) is shown.

bundles shown by Askham *et al.* (15) are different from what we have observed (24).

Overexpression of RP1 does not induce microtubule bundling (24). Our finding that overexpression of the EBF3/RP1 but not the RP1/EBF3 fusion protein induces microtubule bundling suggests that amino acids downstream of 188 of RP1 are unlikely to be the reason that RP1 cannot induce microtubule bundling. The N-terminal 43 amino acids of RP1, which are absent in the short form of RP1 (RP1S) and do not have a corresponding region in EB1 and EBF3 (11), also are unlikely to be the reason that RP1 fails to induce microtubule bundling, because RP1S cannot induce microtubule bundling either (24). Differences between amino acids 44–188 of RP1 and amino acids 1–145 of EBF3 most likely cause the inability of RP1 to induce microtubule bundling. Additional studies will be necessary to understand why RP1 cannot induce microtubule bundling.

The EB1-like C-terminal Motif as a Protein Interaction Domain—Our studies demonstrate that the EB1-like C-terminal motif is important for human EB1 family proteins to associate with APC and with p150^{glued}. However, our results suggest that the N terminus of this domain is more critical for EB1 to

interact with APC than with p150^{glued}, because deletions from the N terminus of this domain abolish the association of EB1 with APC but only reduce the interaction between EB1 and p150^{glued}. On the other hand, the C terminus of EB1 is critical for EB1 to bind p150^{glued} but not for EB1 to associate with APC.

It has been reported that a GST-tagged EB1 fragment containing either amino acids 185–241 or amino acids 219–268 was sufficient to pull down β -catenin, presumably mediated by APC (15). These results thus indicate that neither the N- nor C-terminal highly conserved region of the EB1-like C-terminal motif is necessary for the EB1-APC interaction. However, our study showed that the entire EB1-like C-terminal motif is necessary for the APC binding, and neither amino acids 197–241 nor amino acids 216–268 of EB1 could bind APC. We investigated this by examining the ability of GSTAPC4 to pull down EGFP-tagged EB1 fragments expressed in HCT116 cells (Fig. 3) and the ability of GST-tagged EB1 fragments to pull down wild-type APC from cell lysate (data not shown). In addition, it was reported that the last 50 amino acids of EB1 can bind to APC but not p150^{glued} (15). This is in contrast with our finding that the last 50 amino acids of EB1 binds p150^{glued} weakly but do not have detectable interaction with APC. The

reason for the difference between our results and that of Askham *et al.* (15) is not clear.

The EB1-like C-terminal motif is defined by the amino acid sequence similarity among EB1 family proteins from different species. However, the EB1-like C-terminal motifs of different EB1 family proteins do not function identically. For example, we showed that human EB1 and EBF3 associate with APC and p150^{glued} more efficiently than does RP1. We also have not been able to demonstrate that Bim1p, the EB1 protein of *S. cerevisiae*, interacts with human APC *in vitro*. Additional studies will be necessary to elucidate how the EB1-like C-terminal motifs of different EB1 family proteins function differently.

Association of APC with EB1 Family Proteins and hDLG—Our results show that amino acids 2781–2820 are necessary and sufficient for APC to bind EB1 family proteins and exclude amino acids 2537–2544 as a second EB1 family protein-binding domain as previously suggested (12). Mutagenesis analysis indicates that Ser²⁷⁸⁹, Ser²⁷⁹³, and Ser²⁷⁹⁴ of APC are important for APC to associate with EB1 family proteins. Our results support a previous suggestion that phosphorylation by CDC2 and PKA may regulate the interaction between APC and EB1 family proteins (39) and suggest that Ser²⁷⁸⁹, Ser²⁷⁹³, and Ser²⁷⁹⁴ are the critical phosphorylation targets. Our observations that the S2793E, S2794E mutation strongly reduces the ability of APCd4 to bind EB1 and EBF3, whereas the S2793D, S2794D mutation does not suggest that it is the conformation change rather than the charge change that affects the interaction between APC and EB1 family proteins. On the other hand, we cannot explain why the S2789D mutation affects the interaction between APC and EB1 family proteins more dramatically than does the S2789E mutation.

hDLG is a member of the membrane-associated guanylate kinase family that is commonly found at the cell-cell contact of polarized epithelial cells (50). Although our results show that binding domains for EB1 family proteins and for hDLG of APC are separated, we have not been able to demonstrate that EB1 and hDLG bind APC simultaneously. Therefore, it is unlikely that APC serves as a linker between EB1 and hDLG to anchor microtubules to the cell cortex.

Association of p150^{glued} with EB1 Family Proteins and Microtubules—Previously, EB1 was the only member of the human EB1 family proteins shown to interact with p150^{glued}. Our results show that, *in vitro*, EBF3 also associates with p150^{glued} efficiently, whereas RP1 interacts with p150^{glued} very weakly, similar to their interactions with APC.

Our data show that amino acids 18–111, but neither amino acids 18–87 nor 42–111, of human p150^{glued} bind EB1 (Fig. 6). Thus, the CAP-Gly domain (amino acids 48–90) of p150^{glued}, which is essential for p150^{glued} to associate with microtubules (41), is necessary but not sufficient for human p150^{glued} to associate with EB1. However, competition with microtubules for binding to p150^{glued} is not the only reason that overexpression of C-terminal EB1 fragments reduced the association of p150^{glued} with microtubule plus-ends, because overexpression of C-terminal EB1 fragments that do (EB1d8) and do not (EB1d9 and EB1d17) bind p150^{glued} have similar effects. It has been reported that overexpression of C-terminal EB1 altered microtubule structure (15). However, we observed clear reduction of microtubule plus-end-associated p150^{glued} in cells expressing levels of C-terminal EB1 fragments that did not have obvious effects on the structure of microtubules and the microtubule plus-end localization of EBF3. It is possible that the C-terminal EB1 fragment affects the microtubule plus-end localization of p150^{glued} by sequestering proteins that are important for p150^{glued} to associate with microtubule plus-ends. Because p150^{glued} associates with microtubule plus-ends in

DLD-1 cells, which express only a truncated APC mutant that does not interact with EB1 family proteins, and overexpression of C-terminal EB1 fragments causes the reduction of the microtubule plus-end localization of p150^{glued} in DLD-1 cells, APC is unlikely to be the important protein. It is thus likely that yet unidentified EB1 C terminus-binding proteins contribute to the microtubule plus-end localization of p150^{glued}.

Studies of p150^{glued} involving cDNA constructs mostly used the rat p150^{glued} (51). For our characterization of the interaction between p150^{glued} and EB1 family proteins, we cloned the amino acids 18–111 of human p150^{glued} by reverse transcriptase-PCR using RNA of HCT116 cells based on the sequence of full-length human p150^{glued} (GenBankTM accession number NM_004082). However, our similar attempts to clone a cDNA fragment containing codons 1–111 of human p150^{glued} failed. It had been reported that attempts to isolate human p150^{glued} cDNA containing an initiation codon were unsuccessful (52). The full-length sequence of human p150^{glued} was deduced from cloned partial human p150^{glued} cDNA sequence and the human p150^{glued} genomic sequence, the first exon of which was deduced from mouse and rat p150^{glued} cDNA sequences (52–54). Our query of the human EST data base using the sequence of NM_004082 identified 27 ESTs that contained a new sequence upstream of the exon 2, which encoded amino acids 12–93, and only one EST that contained the exon 1. A search of the human genome sequence showed that the new sequence was encoded by a new exon upstream of the previously reported exon 1 of p150^{glued}, which is tentatively named exon 1A. A similar query of the mouse EST data base did not identify any EST containing the exon 1 but identified more than 20 ESTs that contained the exon 2 and a new sequence. Because the sequence of the human exon 1A does not contain an in-frame translation initiation codon and there is very low similarity between sequences of human and mouse exon 1A, the N terminus of the majority of human and mouse p150^{glued} is likely to be encoded by codon 18. It has been reported that the Ser¹⁹ of rat p150^{glued} is a PKA phosphorylation site and that phosphorylation of this residue diminished the microtubule binding of p150^{glued} (55). However, the Ser¹⁹ is not a phosphorylation site for PKA in the majority of human and probably mouse p150^{glued}, because it is the second amino acid of these proteins.

Acknowledgments—We thank Wei-Ping Lee, Yi Qi, and Weizhe Yao for making some plasmids used in this study.

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