Dissecting interactions between EB1, microtubules and APC in cortical clusters at the plasma membrane

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Summary
End-binding protein (EB) 1 binds to the C-terminus of adenomatous polyposis coli (APC) protein and to the plus ends of microtubules (MT) and has been implicated in the regulation of APC accumulation in cortical clusters at the tip of extending membranes. We investigated which APC domains are involved in cluster localization and whether binding to EB1 or MTs is essential for APC cluster localization. Armadillo repeats of APC that lack EB1- and MT-binding domains are necessary and sufficient for APC localization in cortical clusters; an APC fragment lacking the armadillo repeats, but containing MT- and EB1-binding domains, does not localize to the cortical clusters but instead co-aligns with MTs throughout the cell. Significantly, analysis of endogenous proteins reveals that EB1 does not accumulate in the APC clusters. However, overexpressed EB1 does accumulate in APC clusters; the APC-binding domain in EB1 is located in the C-terminal region of EB1 between amino acids 134 and 268. Overexpressed APC- or MT-binding domains of EB1 localize to APC cortical clusters and MT, respectively, without affecting APC cluster formation itself. These results show that localization of APC in cortical clusters is different from that of EB1 at MT plus ends and appears to be independent of EB1.

Key words: Adenomatous polyposis coli, End-binding protein 1, Microtubules

Introduction
Adenomatous polyposis coli (APC) protein is the product of a tumor suppressor gene mutated in colorectal cancer (Groden et al., 1991) and is involved in downregulating β-catenin signaling by mediating β-catenin degradation (for a review, see Polakis, 1999). However, there is increasing evidence for another role of APC in organizing the MT cytoskeleton. The C-terminus of APC has a MT-binding site and, in vitro, APC stimulates MT assembly and bundling (Munemitsu et al., 1994). In epithelial cells, APC protein accumulates in cortical clusters at the tip of actively extending membranes (Näthke et al., 1996; Barth et al., 1997b), suggesting a role for APC in promoting cell extension and cell migration (Barth et al., 1997a; Näthke et al., 1997; Pollack et al., 1997).

Overexpression of APC or C-terminal APC fragments causes APC accumulation at the plus end of MTs, and this accumulation has been attributed to APC binding to EB1 (Askham et al., 2000; Mimori-Kiyosue et al., 2000a). EB1 (Su et al., 1995) binds to distal (plus) ends of interphase MTs (Berrueco et al., 1998; Morrison et al., 1998; Mimori-Kiyosue et al., 2000b) and in vitro can promote MT polymerization in the presence of the C-terminal-binding domain of APC (Nakamura et al., 2001).

The APC-EB1 complex may act as a MT capturing complex in interphase cells that directs MT plus ends to specific cortical sites at the tip of extending membranes. In yeast, the EB1 homologue Bim1p links MT plus ends to the cortical protein Kar9p, which is localized to a defined area at the bud tip (Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000). Alternatively, an APC-EB1 complex at the plus ends of MT may link MT to a yet unknown anchoring complex at the cortex; in this context, Kar9p localization at the bud tip is independent of Bim1p (Miller et al., 1999; Miller et al., 2000).

Little is known about cortical APC cluster formation and the role of EB1 in determining APC localization in these clusters. Therefore, we have investigated which APC domains are involved in APC cluster localization and how this localization is affected by expression of EB1 mutant proteins. Our results show that the localization of APC in cortical clusters is different from that of EB1 at MT plus ends and further indicate that it is independent of EB1.

Materials and Methods
Cell lines and cDNA constructs
Madin-Darby canine kidney (MDCK) type II/G cells and conditions for their growth have been described previously (Mays et al., 1995). MDCK cells were transiently transfected with fluorescent protein vectors described below using lipofectamine reagent as described by the manufacturer (Gibco BRL, Gaithersburg, MD).

For construction of different APC expression vectors, pCDNA1/ APC0-87 (kindly provided by Paul Polakis, Onyx Pharmaceuticals) was used as a template. This vector encodes full-length human APC with a short additional amino-acid sequence domain, inserted between amino acids 470 and 471 and derived from exon 10A of the APC gene, which is found in some splice variants of APC (Sulekova and Ballhausen, 1995). For easier comparison, amino acid positions for human APC are given without the ‘exon 10A’ domain. Vectors for fluorescent proteins were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Iitalicic sequences in the primers correspond to the respective APC or EB1 sequences. Underlined sequences in the primers mark restriction sites used for cloning. All constructs were...
confirmed by sequencing. For schematic representations of the constructs, see Fig. 2.

GFP-APC (APC amino acids 1-2843): full-length APC was amplified by PCR with the 5' primer GATCCGAGCTCTCAAGGGATGCTGACCCAGCTTAATGCTCAAATGATTGTG and the 3' primer GATCCGAGCTCTCAAGGGATGCTGACCCAGCTTAATGCTCAAATGATTGTG and cloned SacII/BamHI into pEGFP-C1. GFP-APCw0E1 (GFP-APC without the internal EcoRI fragment; deletion of APC amino acids 221-2166): ‘in frame’ ligation of the two internal EcoRI sites of the GFP-APC cDNA. GFP-APCE1X1 (APC amino acids 220-872): the APC internal 5'¢ ligation of the two internal EcoRI sites in the GFP-APC cDNA. GFP-APCE1X1 (APC amino acids 220-872) was amplified by PCR (Invitrogen, Carlsbad, CA) as described by the manufacturer. Full-length C20 cDNA (Clontech) and cloned into pCR4BluntTOPO transferase (GST) fusion protein in bacteria. (Amersham Pharmacia) for the expression of glutathione S-transferase (clone GST Z-5; Santa Cruz Biotechnology, Inc.) were used. APC (clone C20; Santa Cruz Biotechnology, Inc.) and to glutathione GST fusion proteins, rabbit polyclonal antisera to the C-terminus of APC (clone TUB2.1; Sigma) at 1:100. For immunoaffinity purification of GST fusion proteins, rabbit polyclonal antisera to the C-terminus of APC (clone C20; Santa Cruz Biotechnology, Inc.) and to glutathione S-transferase (clone GST Z-5; Santa Cruz Biotechnology, Inc.) were used.

Immunofluorescence microscopy

2×10^5 MDCK cells were seeded onto 22×22 mm collagen-coated coverslips in 35 mm tissue culture dishes and fixed 12 to 16 hours later. Cells were rinsed once in PBS pH 7.4 (2.7 mM KCl, 1.5 mM KH₂PO₄, 1.5 mM MgCl₂, 1 mM EGTA, 137 mM NaCl and 8.1 mM NaHPO₄), fixed for 5 minutes in –20°C methanol and then rinsed once in PBS and blocked for 20 minutes at room temperature in PBS with 1% BSA and 2% goat serum. Cells were labeled for immunofluorescence as described elsewhere (Barth et al., 1997b) and analyzed with a Delta Vision™ full-spectrum optical sectioning microscope system (Applied Precision, Inc., Seattle, WA: Beckman Center Cell Sciences Imaging Facility). The percentage of cortical APC clusters that had total or partial overlap with EB1 was determined in basal sections of 10 MDCK cells, which had a total of 15 cluster areas immunolabelled for endogenous APC and EB1.

Fig. 1. Subcellular distribution of endogenous APC and EB1 in MDCK cells. Basal sections of MDCK cells co-stained for EB1 (green in a-e and a'-e') and APC (red in a'-e' and a''-e''). Arrowheads, cortical APC clusters at the tip of cell extensions; black arrowheads, APC clusters without EB1; white arrowheads, APC clusters partially overlapping with EB1. Bars, 10 μm. Insets, higher magnification of the upper left cell extension in a'-e' co-stained for EB1 (green) and APC (red). Bar, 2.5 μm.
Protein-binding assay
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Protein-binding assay
Bacterial cultures were resuspended in 50 mM Tris pH 8, 2 mM EDTA, 0.1% Triton-X-100, 1mM DTT and complete protease inhibitor cocktail (Roche Molecular Biochemicals), then lysed with a French press (ThermoSpectronic, Rochester, NY). GST or MBP fusion proteins were purified with glutathione-sepharose (Sigma) or amylose-resin (New England Biolabs) according to the manufacturer’s instructions. GST-APCCT was further purified by immunoprecipitation with anti-APC C20 (Santa Cruz Biotechnology, Inc) bound to protein A-sepharose (Amersham Pharmacia), and GST was further purified by immunoprecipitation with anti-GST Z-5 (Santa Cruz Biotechnology, Inc). Equivalent amounts of GST-APCCT or GST bound to the respective immunoaffinity resins and, as a control, the immunoaffinity resins alone were incubated for 2 hours at 4°C with 20 μg MBP as a control or with 7 μg MBP-EB1 or MBP-EB1CT in 10 mM phosphate buffer pH 7.4, 2.7 mM KCl, 137 mM NaCl, 10 mM maltose, 1% Triton-X-100, 1 mM DTT and then washed four times with 30 volumes of the same buffer. Proteins bound to the immunoaffinity resins were analyzed by SDS-PAGE as described previously (Barth et al., 1997b). Polyacrylamide gels were stained with Coomassie Brillant Blue R-250 and scanned with a ScanJet IIc (Hewlett-Packard Co., Palo Alto, CA).

MT pelleting assay
Purified bovine brain tubulin (kindly provided by Eugenio de Hostos, University of California, San Francisco) was polymerized in PEM (80 mM Pipes/Dipotassium Salt pH 6.9, 1 mM MgCl₂, 1 mM EGTA), 0.2 mM GTP at a concentration of 5.5 μg/μl for 10 minutes at 37°C. Tubulin was diluted to 1.1 μg/μl with PEM, 20 μM Taxol and incubated for another 10 minutes at 37°C. MBP fusion proteins in 10 mM phosphate buffer pH 7.4, 2.7 mM KCl, 137 mM NaCl, 10 mM maltose were pre-cleared by a 30 minute centrifugation at 4°C and 200,000 g in a TL100 Ultracentrifuge (Beckman). Taxol and MgCl₂ were added to the fusion proteins to a final concentration of 20 μM and 5 mM, respectively. 100 μl of polymerized tubulin at a concentration of 1.1 μg/μl was mixed with 100 μl of MBP, MBP-EB1 or MBP-EB1CT fusion protein, respectively, and incubated for 10 minutes at RT. Equivalent amounts of MBP-EB1 and MBP-EB1CT (~50 μg) were used in the assay. MBP fusion proteins bound to polymerized tubulin were precipitated by centrifugation through a 30% glycerol cushion in PEM, 5 μM Taxol, 1 mM Pefabloc (Roche Molecular Biochemicals) for 30 minutes, at RT at 160,000 g in a SW60 rotor (Beckman). After centrifugation, the supernatant with the remaining tubulin-MBP fusion protein solution and half of the cushion was removed and the
Centrifugation tubes were washed twice with dH₂O before complete removal of the residual cushion. Pellets were resuspended in SDS-PAGE loading buffer and analyzed by SDS-PAGE (Barth et al., 1997b). Polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 and scanned with a ScanJet IIc (Hewlett-Packard Co., Palo Alto, CA).

Results and Discussion
Endogenous APC and EB1 do not colocalize
Distributions of endogenous APC and EB1 in normal Madin-Darby canine kidney (MDCK) epithelial cells were compared by double immunofluorescence (Fig. 1). EB1 accumulates throughout the cell periphery in small granules (Fig. 1a-e) that have been previously shown to be at the distal (plus) ends of MTs (Berrueta et al., 1998; Morrison et al., 1998; Mimori-Kiyosue et al., 2000b) (Fig. 4a-c). In contrast, endogenous APC localizes in cortical clusters at the tip of cell extensions (Fig. 1a′-e′; arrowheads) and in a filamentous distribution throughout the cytoplasm along MTs (Fig. 1a′-e′; Fig. 2a-c). We note that EB1 does not accumulate in cortical APC clusters (Fig. 1; black arrowheads) but it is often found in close proximity to these clusters (Fig. 1; white arrowheads). 22% of the cortical APC clusters show some overlap with EB1. In general, EB1 granules are located throughout the cell cortex, whereas APC clusters are restricted to the basal surface and to the tip of cell extensions (Fig. 1; arrowheads) (data not shown).

The N-terminal Armadillo repeat domain of APC is necessary and sufficient for APC localization in cortical clusters
To define the APC domain required for APC localization in cortical clusters, full-length APC and different domains of APC fused to GFP were expressed in MDCK cells (Fig. 2). Full-length GFP-APC localized in cortical clusters into which MTs terminated (Fig. 2a-c; black arrowheads) and along MTs (Fig. 2a-c; white arrowheads). An APC mutant lacking a large central part of the protein (GFP-APCwoE1) did not colocalize in cortical clusters containing endogenous APC (Fig. 2d-f) even though GFP-APCwoE1 retains the N-terminal APC-APC dimerization domain (Joslyn et al., 1993). However, GFP-APCwoE1 did localize along MTs (Fig. 2d-f) probably through its C-terminal MT- and/or EB1-binding domains.

Fig. 3. Binding of EB1 and the C-terminal EB1 domain to MTs or APC in vitro. (a) MT pelleting assay. Lanes 1-3 show 1/20 of the respective MBP fusion proteins after pre-clearance by centrifugation and before incubation with polymerized tubulin. Lanes 4-6 show 2/3 of the MT pellets after incubation with the respective MBP fusion proteins and centrifugation through a glycerol cushion. Lane 7, 2.5 μg purified bovine brain tubulin. (b) Binding of MBP-EB1 fusion proteins to the EB1-binding domain in GST-APCCT. Precipitation of the respective MBP fusion proteins by GST-APCCT bound to Protein A (APC AB+GST-APCCT) (lane 7-9). GST-APCCT appears as a double band of which the faster migrating one is probably caused by partial degradation. In control assays, MBP fusion proteins were incubated with anti-GST antibody (GST AB) bound to Protein A (lane 1-3); with GST bound to GST AB/Protein A (GST AB+GST) (lane 4-6) and with anti-APC antibody (APC AB) bound to Protein A (lane 10-12). Approximately 1/15 of the total MBP fusion proteins used for each binding assay are shown in lane 13-15. M, Molecular weight standards.
Next, we examined the distribution of APC mutants lacking C-terminal MT- and EB1-binding domains. We made GFP-APC E1X1, which contains the highly conserved APC domain and armadillo repeats 1-9 of 10 APC armadillo repeats as defined by Huber and Weis (Huber and Weis, 2001) including the ‘exon 10A’ domain and APCARM-GFP, which contains armadillo repeats 3-9. Both mutant proteins prominently colocalize with endogenous cortical APC clusters, but neither protein localizes along MTs (Fig. 2g-q). Expression of APCARM-1.Rep-GFP, which contains armadillo repeats 4-9, strongly diminished, but did not completely eliminate mutant protein localization to cortical clusters of endogenous APC (Fig. 2r-t).

In summary, armadillo repeats 3-9 are necessary and sufficient to mediate localization of APC to cortical clusters. In this context, it is interesting to note that the cortical localization of Drosophila APC2 (E-APC) is disrupted by a single point mutation in its armadillo region (Townesley and Bienz, 2000). At present, we do not know whether these armadillo repeats are sufficient for de novo assembly of cortical clusters of APC. Armadillo repeat domains in other proteins have been shown to mediate protein-protein interactions between diverse binding partners and, generally, more than one repeat is involved in mediating a particular interaction (Hülsken et al., 1994; Huber and Weis, 2001). Accordingly, a binding partner to the APC armadillo repeat domain may specify a cortical targeting site for APC. As the armadillo repeat domain of APC is sufficient to localize to APC clusters, it is possible that an unknown binding partner of this domain is involved in APC cluster formation. One possibility is the APC-stimulated Rac-specific guanine nucleotide exchange factor (Asef), which is a binding partner for the APC armadillo repeat domain (Kawasaki et al., 2000). However, overexpression of Asef causes dissociation of the cortical APC clusters in MDCK cells (Kawasaki et al., 2000), perhaps by competing with another endogenous binding partner and thereby disrupting APC cluster formation.

The C-terminal half of EB1 binds APC but not MTs

Although endogenous EB1 does not accumulate in cortical APC clusters and we did not observe a concentration of endogenous APC at distal MTs ends (Fig. 1), EB1 could facilitate APC transport to cortical sites. In that case, expression of an EB1 mutant protein that can bind to APC but is unable to bind to MTs should inhibit APC cluster formation. The N-terminal half of EB1 is highly conserved and contains the MT-binding site defined in the EB1 homologue RP1
The APC-binding domain in EB1 has not been defined, but Kar9p binding to the yeast EB1 homologue Bim1p is mediated by the C-terminal half of Bim1p (Miller et al., 2000).

To identify a domain in EB1 that binds to APC but not MTs, the C-terminal half of EB1 (MBP-EB1CT) and, as a control, full-length EB1 (MBP-EB1) were purified as MBP fusion proteins from bacteria (Fig. 3a; lanes 1-3; Fig. 3b; lanes 13-15). Another EB1 mutant fusion protein containing the N-terminal half of EB1 (MBP-EB1NT) was also expressed but it could not be purified from bacteria. MBP-EB1 co-pelleted with polymerized bovine brain tubulin; only trace amounts of MBP-EB1CT and MBP were detected in the pellet (Fig. 3a, lanes 4-6).

To analyze interactions of MBP-EB1 and MBP-EB1CT with APC, a C-terminal domain of APC containing the EB1-binding region (Askham et al., 2000) was purified as a glutathione S-transferase (GST) fusion protein from bacteria and further purified by immunoaffinity precipitation with an antiserum to the C-terminus of APC (Fig. 3b; lanes 7-9; GST-APCCT). Both MBP-EB1 and MBP-EB1CT, but not MBP, co-pelleted with GST-APCCT (Fig. 3b; lanes 7-9). The MBP fusion proteins did not co-pellet with GST (Fig. 3b; lanes 4-6) or with the respective immunoaffinity resins alone (Fig. 3b; lanes 1-3 and 10-12). In summary, amino acids 134 to 268 in EB1CT bind to APC but not MTs. This region in EB1 contains a domain with homology to the Kar9-binding site in the yeast EB1 homologue Bim1p (Miller et al., 2000).

Subcellular localization of EB1 mutant proteins in MDCK cells

Full-length EB1 and EB1 mutant proteins were expressed in MDCK cells as fusions to DsRed fluorescent protein (Figs 4,5). Similar to endogenous EB1, DsRed-EB1 localized to MT distal (plus) ends (arrows in Fig. 4a-c). However, unlike endogenous EB1, DsRed-EB1 accumulated in cortical APC clusters (Fig. 5a-c; white arrowheads), probably because of an excess of EB1, which can then interact with additional binding sites. In 44% of cells transiently transfected with DsRed-EB1, all cortical APC clusters contained DsRed-EB1; in 19% of those cells some APC clusters contained DsRed-EB1 and others did not; in 37% of those cells DsRed-EB1 did not co-localize with cortical APC clusters (number of cells analyzed n=111).

DsRed-EB1CT, which contains the APC-binding domain (Fig. 3), dominantly colocalized with endogenous APC in cortical clusters (Fig. 4d-f; Fig. 5g-i; white arrowheads). In all cells transiently transfected with DsRed-EB1CT, all cortical APC clusters contained DsRed-EB1CT (number of cells analyzed n=109). Note that expression of DsRed-EB1CT does not appear to disrupt APC cluster formation. Furthermore, DsRed-EB1CT had a diffuse cytoplasmic distribution and co-aligned only occasionally with a few MT plus ends (Fig. 4d-f; compare DsRed-EB1 in 4b with DsRed-EB1CT in 4e), consistent with the lack of EB1CT binding to MTs in vitro (Fig. 3a). The weak residual localization of DsRed-EB1CT at MT plus ends may be caused by low amounts of APC in these areas (Fig. 4d-f; arrows).

Fig. 5. Colocalization of DsRed-EB1 and DsRed-EB1CT but not of DsRed-EB1NT in cortical APC clusters. (a-i) Basal sections of cell extensions of cells expressing full-length DsRed-EB1 (a-c), DsRed-EB1NT (d-f) or DsRed-EB1CT (g-i). Cells were co-stained with APC antiserum (green). DsRed-EB1 predominantly localizes to the distal (plus) ends of MTs (arrows in b, c) (Fig. 4a-c) and colocalizes with APC in cortical clusters at the tip of the extension (white arrowheads in a-c). DsRed-EB1NT shows filamentous staining at MT distal (plus) ends and along MTs (arrows in e, f) (Fig. 4g-i) but does not colocalize with APC in cortical clusters (black arrowheads in d-f). DsRed-EB1CT colocalizes with APC at the tip of the extension (white arrowheads in g-i). Bar, 10 μm.
DsRed-EB1NT, which contains the MT-binding domain, localized to MTs but was not as restricted to MT distal (plus) ends as endogenous EB1 (arrows in Fig. 4g-i) or DsRed-EB1 in cells expressing approximately the same levels of these fusion proteins (compare Fig. 4h with 4b) (data not shown). Significantly, DsRed-EB1NT did not colocalize with endogenous APC in cortical clusters (Fig. 5d-f; black arrowheads). The transfection efficiency for DsRed-EB1NT was lower than that of other EB1 proteins; only one third of the cells transfected with DsRed-EB1NT showed extensive filamentous DsRed-EB1NT localization that overlapped with MT plus ends marked by EB1 (Fig. 4g-i), whereas in two thirds of the transfected cells, DsRed-EB1NT distributed more diffusely throughout the cytoplasm. Therefore, this EB1 fragment may be less stable or less efficient in its binding properties than full-length EB1. However, DsRed-EB1NT did not localize to cortical APC clusters in any of the transfected cells (number of cells analyzed n=80; 29 of these cells showed filamentous DsRedEB1NT distribution).

Since overexpression of EB1 mutant proteins did not have a disruptive (dominant-negative) effect on the localization of endogenous APC to cortical clusters, binding to EB1 does not appear to be essential for assembly of APC into cortical clusters. The subcellular distributions of EB1-CT and -NT mutants reflected their binding properties to APC and MTs, respectively. These results show that cortical APC clusters and cortical EB1-containing MT plus ends are spatially different structures (see also Fig. 1 for endogenous APC and EB1). Note, however, that we show in Fig. 1 and 2 that the APC clusters are closely associated with EB1-containing MT plus ends and that we have previously demonstrated that these clusters dissociate after nocodazole-induced disassembly of MTs (Näthke et al., 1996). Although the existence of cortical APC clusters is ultimately dependent on an intact MT cytoskeleton (Näthke et al., 1996), the results shown here emphasize that these clusters are not identical to a bundle of free cortical MT plus ends, but they seem to contain additional organizing components that assemble them at particular sites of the cortex. This is consistent with previously published results from Mimori-Kiyosue et al. (Mimori-Kiyosue et al., 2000a), which show that after disruption of MTs, cortical APC clusters do not disassemble immediately but redistribute along actin stress fibers at the basal plasma membrane (Mimori-Kiyosue et al., 2000a).

Cortical APC clusters at the tip of cell extensions have been correlated with a role of APC in promoting cell extension and directed cell migration by affecting MT dynamics in these areas (Barth et al., 1997a; Näthke et al., 1997; Pollack et al., 1997). Similar to the Kar9p-EB1 complex in mitotic yeast cells, the APC-EB1 complex may act as a MT-capturing complex that redirects MT plus ends to specific sites at the tip of extending membranes. However, the order of assembly of the APC-EB1 complex is different from previous predictions (Askham et al., 2000; Mimori-Kiyosue et al., 2000) and has similarity to the process in budding yeast. In yeast, Kar9p assembly at the bud tip is independent of EB1 (Miller et al., 1999; Miller et al., 2000). Similarly, our results indicate that APC assembly into cortical clusters is independent of EB1; our data indicate a role of the APC armadillo domain in targeting APC to cortical clusters. Thus, EB1-containing MT plus ends may be captured by pre-existing cortical APC clusters, thereby causing the re-orientation of a subset of MTs towards that region of the membrane and the formation/stabilization of a polarized membrane extension.

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