Membrane Localization of Adenomatous Polyposis Coli Protein at Cellular Protrusions

TARGETING SEQUENCES AND REGULATION BY β-CATENIN

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Adenomatous polyposis coli protein (APC) translocates to, and stabilizes, the plus-ends of microtubules. In microtubule-dependent cellular protrusions, APC frequently accumulates in peripheral clusters at the basal membrane. APC targeting to membrane clusters is important for cell migration, but the localization mechanism is poorly understood. In this study, we performed deletion mapping and defined a minimal sequence (amino acids 1–2226) that efficiently targets APC to membrane clusters. This sequence lacks DLG-1 and EB1 binding sites, suggesting that these partners are not absolutely required for APC membrane targeting. A series of APC sequences were transiently expressed in cells and compared for their ability to compete endogenous APC at the membrane; potent inhibition of endogenous APC targeting was elicited by the Armadillo- (binds KAP3A, B56α, and ASEF) and β-catenin-binding domains. The Armadillo domain was predicted to inhibit APC membrane localization through sequestration of the kinesin-KAP3A complex. The role of β-catenin in APC membrane localization was unexpected but affirmed by overexpressing the APC binding sequence of β-catenin, which similarly reduced APC membrane staining. Furthermore, we used RNA interference to show that loss of β-catenin reduced APC at membrane clusters in migrating cells. In addition, we report that transiently expressed APC-yellow fluorescent protein co-localized with β-catenin, KAP3A, EB1, and DLG-1 at membrane clusters, but only β-catenin stimulated APC anchorage at the membrane. Our findings identify β-catenin as a regulator of APC targeting to membrane clusters and link these two proteins to cell migration.

The adenomatous polyposis coli (APC) tumor suppressor is functionally inactivated by a spectrum of APC gene mutations in colon cancer (1). Tumor-associated APC mutations result in truncated APC peptides with altered function, often characterized by a reduced ability to associate with microtubules (2, 3), to bind centrosomes and kinetochores, and to facilitate chromosome segregation (4, 5). Many APC mutations also disrupt APC-dependent β-catenin degradation, causing oncogenic β-catenin to accumulate in the nucleus and activate transcription of genes that promote cell transformation (1, 6). The subcellular localization of APC is regulated by nuclear-cytoplasmic trafficking (7) and by plus-end-directed KAP3A/kinesin-dependent movement along microtubule filaments toward the cell periphery (8–10). APC, like CLIP170 and EB1, is now recognized as a microtubule plus-end-binding protein (11).

Two distinct sites of APC localization at the cell membrane have been linked to cell migration. In polarized Vero fibroblasts, APC was found to accumulate at the actin-rich leading membrane lamellipodia of migrating cells (12). The most frequent concentration of APC occurs at the tips of microtubule-dependent cellular protrusions (8, 13), and this membrane cluster pattern is often linked to the polarity of cell migration (13, 14). The accumulation of APC at lamellipodia was recently found to be mediated through a direct interaction of APC with the Rac1/Cdc42 effector protein, IQGAP1 (12). The proteins that regulate APC localization at distinct cellular protrusions, however, have not yet been defined.

APC forms a stabilizing complex at microtubule plus-ends with EB1 (15) and co-localizes at the basal membrane in membrane clusters with endogenous β-catenin (13), KAP3A (9), and DLG-1 (16). The interaction of APC with KAP3A/kinesin stimulates movement of APC along microtubules toward the membrane (9), whereas phosphorylation of APC by GSK-3β can cause APC to dissociate from microtubules (17) and prevents its accumulation at cell protrusions (14). In this study, we adopted a systematic deletion mapping approach to characterize the APC sequences that mediate accumulation of APC in membrane clusters at the tips of cellular protrusions. We employed different methods including competition assays, co-transfection experiments, and siRNA silencing with the aim of identifying the binding partners that regulate APC membrane localization. Our findings support a role for KAP3A in the translocation of APC to the membrane but strongly implicate a role for β-catenin in the clustering and anchorage of APC at cellular protrusions. We propose that β-catenin mediates the effects of Cdc42-mediated inactivation of GSK-3β on APC aggregation at cell migration-associated protrusions.

MATERIALS AND METHODS

Cell Culture and Transfection—NIH 3T3 mouse fibroblasts and SW480 (APCmut/mut) human colon carcinoma cells were cultured under standard conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and were confirmed free of mycoplasma. For transfection experiments, cells were grown on glass coverslips in 8-well trays (Nuncclone) and at 12 h post-seeding were...
transfected with 1–3 μg of DNA/ml of medium using Lipofectamine 2000 (Invitrogen). The lipid-DNA mix was left on cells for 6 h before replacing the medium and processing cells 40 h later (18). For proteasome inhibition, cells were exposed to 20 μM MG132 (Calbiochem) 9 or 12 h prior to fixation and immunostaining. To examine the influence of disrupting the microtubule or actin cytoskeletons, cells were treated with 33 μM nocodazole (Sigma) or 1 μM latrunculin A (Molecular Probes), respectively, for 1 h.

Cell Scratch-induced Migration Assay—NIH 3T3 or Madin-Darby canine kidney cells were grown to confluence on coverslips. Individual wounds were made with a Gilson P-1000 pipette tip. Cells were washed twice with phosphate-buffered saline, and fresh medium was added. Cells were fixed at 6 h after wounding and immunostained as below. For siRNA experiments, cells were transfected 48 h before wounding.

Immunofluorescence Microscopy and Antibodies—Cells were grown on coverslips at medium density and fixed in 3.7% formalin (tissue culture grade from Sigma)/phosphate-buffered saline for 20 min, followed by permeabilization with 0.2% Triton X-100/phosphate-buffered saline for 10 min at 36 h post-transfection, and incubated with various antibodies as previously described (19). The following primary antibodies or serum were used: anti-APC Mab (Ab57, 1:80 dilution; Oncogene Research Products), anti-APC rabbit polyclonal M-APC (purified used at 1:1000, unpurified used at 1:80 and supplied by Dr. Inke Nathke), anti-Axin rabbit polyclonal (Zymed Laboratories; validated by detection of ectopic Axin in transfected cells), anti-FLAG Mab (M2, 1:1000 dilution; Sigma), anti-hemagglutinin tag rabbit polyclonal (sc-805, 1:250 dilution; Santa Cruz Biotechnology), or EB1 monoclonal (610534/B56). A series of GFP-tagged APC expression plasmids were constructed by PCR amplifying DNA sequences from the pCMV-APC template using primers that contained BamHI and Sall restriction enzyme sites and cloning the purified PCR products in-frame into the corresponding BamHI/Sall sites of pEGFP-N1. The actual sequences for the forward and reverse primers are given in Table 1, and this approach was used to construct the following plasmids: pGFP-tagged APC-(334–900), APC-(2226–2644), APC-(2650–2843), APC-(1941–2226), APC-(1941–2032), and APC-(2033–2060). Construction of pAPC-YFP was described by Henderson et al. (21). Other expression vectors were supplied by colleagues as indicated: p-HA-hGSK-3β, p-FLAG-hAxin (from Trevor Dale; Ref. 22), p-HA-B56α (from D.Virshup; Ref. 23), pDLG-EGFP (from T. Akiyama; Ref. 24), pKAP3A and KAP3A(ΔArm) (from T. Akiyama; Ref. 9), FLAG-tagged β-catenin vectors pFLAG-β-catenin(wild type) and pFLAG-β-catenin S33Y/A218–467 and S33Y/D695 (from E. Fearon; Ref. 25). Full-length EB1 expression vector pCMV-EB1 was supplied by Dr. B. Vogelstein (26). A series of GFP-tagged APC expression plasmids were constructed by PCR amplifying DNA sequences from the pCMV-APC template using primers that contained BamHI and Sall restriction enzyme sites and cloning the purified PCR products in-frame into the corresponding BamHI/Sall sites of pEGFP-N1. The actual sequences for the forward and reverse primers are given in Table 1, and this approach was used to construct the following plasmids: pGFP-tagged APC-(334–900), APC-(2226–2644), APC-(2650–2843), APC-(1941–2226), APC-(1941–2032), and APC-(2033–2061). The same strategy was used to clone pGFP-tagged APC-(1–302) except that the restriction sites used were HindIII and PstI (primers in Table 1). The sequence APC-(1379–2080) is contained in the plasmid pAPC-HC-GFP that was described by Rosin-Arbesfeld et al. (27) and kindly supplied by Dr. Mariann Bienz (Cambridge).

To construct a plasmid containing the GFP-tagged APC-binding domain of β-catenin, β-catenin-(218–467)-GFP, the pFLAG-β-catenin cDNA was used as template to amplify the sequence aa 218–467 using the forward primer 5′-GTCGACGGTGGATCCCGGGTCCCAGGGTGACAAC-3′ and reverse primer 5′-ACCGGGTGATCCCAAGCGACAGATGGCAGCTCAGTGC-3′ (BamHI site underlined). The PCR fragment was cut with KpnI and BamHI and inserted in-frame into the same sites in pEGFP-N1 (Clontech). Construct integrity was confirmed by restriction analysis and sequencing. The same cloning strategy and vector were used to construct the plasmid pβ-catenin-(1–218)-GFP using the PCR forward primer 5′-GTCGACGGTGGATCCCATGCACTCAAGCTGATTTGATG-3′ and reverse primer 5′-ACCGGTGGATCCCGGGTCCCAGGGTGACAAC-3′ (BamHI site underlined). The PCR fragment was cut with KpnI and BamHI and inserted in-frame into the same sites in pEGFP-N1 (Clontech). Construct integrity was confirmed by restriction analysis and sequencing.

Cell Extract Preparation and Western Blot Analysis—Cells were resuspended in protein extraction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, supplemented with protease inhibitor mix; Roche Diagnostics) and shock frozen in liquid nitrogen. After a quick thaw at 37 °C, cells were refrozen in liquid nitrogen, thawed on ice for 20 min, and cleared of insoluble components by centrifugation at 13,000 rpm at 4 °C for 15 min. The supernatant containing total protein was quantitated using Bio-Rad protein assay. Cell extracts

| Construct | Forward primer | Reverse primer | Cloning site |
|-----------|----------------|----------------|-------------|
| APC-(1–302)-GFP | CCTGACGTCGACGATGGCTGTTGCAG | GATCGACGTCGACGATGGACTCTGAAGATGACCTGTTGCAG | HindIII (fwd) PstI (rev) |
| APC-(334–900)-GFP | GATCGACGTCGACGTTGCTGGATCCCGAACAGATGTCACAAGGTAAGACCCAG | GATCGACGTCGACGATGCCAGACAGAGGGGCAGCAACTGATG | HindIII (fwd) PstI (rev) |
| APC-(2226–2644)-GFP | GATCGACGTCGACGAGATGGCTGTTTCTAAAACAGAGGATGTTTGG | GATCGACGTCGACGATGCGAGGCAGGACAATGATTCATATTC | HindIII (fwd) PstI (rev) |
| APC-(2650–2843)-GFP | GATCGACGTCGACGAGATCTCAGTACTCAAGGTAAGACCCAG | GATCGACGTCGACGATGCCAGACAGAGGGGCAGCAACTGATG | HindIII (fwd) PstI (rev) |
| APC-(1941–2226)-GFP | GATCGACGTCGACGAGATCTCAGTACTCAAGGTAAGACCCAG | GATCGACGTCGACGATGCCAGACAGAGGGGCAGCAACTGATG | HindIII (fwd) PstI (rev) |
| APC-(1941–2032)-GFP | GATCGACGTCGACGAGATCTCAGTACTCAAGGTAAGACCCAG | GATCGACGTCGACGATGCCAGACAGAGGGGCAGCAACTGATG | HindIII (fwd) PstI (rev) |
| APC-(2033–2060)-GFP | GATCGACGTCGACGAGATCTCAGTACTCAAGGTAAGACCCAG | GATCGACGTCGACGATGCCAGACAGAGGGGCAGCAACTGATG | HindIII (fwd) PstI (rev) |

*J. Woodgett, unpublished information.*
Regulation of APC Trafficking to the Membrane

**RESULTS**

**APC Localizes at Membrane Clusters at the Tips of Cellular Protrusions**—APC can associate with microtubules, stimulate their bundling and polymerization in vitro (2, 3, 17), and move along them toward the plus-ends in a kinesin-dependent pathway (9). The APC-dependent bundling of microtubules contributes to extensions of the plasma membrane, referred to as cellular protrusions, and APC accumulates in “clusters” at the actin cortical zone of these membrane protrusions just beyond reach of the microtubules (see Fig. 1A). Using an anti-APC antibody well characterized for detection of APC at these structures (13), we detected APC at microtubule filaments in actively migrating Madin-Darby canine kidney epithelial cells and confirmed that APC accumulates in clusters at the membrane periphery in a region at which the microtubules do not extend (see arrows in supplemental Figs. S1 and S2). Scoring of cells revealed that uncontacted NIH 3T3 cells exhibit 0–5 protrusions, and most commonly 3 protrusions; however, only 1–2 protrusions/cell display APC staining at membrane clusters (see supplemental Fig. S2). APC was previously shown to co-localize at these membrane clusters with the binding partners β-catenin (13), DLG-1 (16), KAP3A (9), and to a lesser extent EB1 (15, 28). We also observed that endogenous APC frequently co-localizes with β-catenin, EB1, and DLG-1 at cortical clusters, and confocal microscopy determined co-localization with β-catenin at the basal membrane (see supplemental Figs. S3 and S4).

Etienne-Manneville and Hall (14) showed in rat astrocytes that the appearance of APC at membrane clusters correlated with cdc42-dependent inactivation of the kinase GSK-3β. To determine whether APC accumulation at membrane clusters in NIH 3T3 cells is likewise regulated by cdc42, we overexpressed a cdc42 dominant negative mutant in NIH 3T3 cells and observed an ~50% reduction in APC cluster staining, whereas constitutively active cdc42 had no significant effect (data not shown). Therefore, APC targeting and anchorage at membrane clusters in NIH 3T3 fibroblasts display similar characteristics to that reported in other cell types and thus provide a suitable model system to analyze APC at the membrane.

We transfected NIH 3T3 cells with pAPC-YFP and observed staining at both microtubule filaments and at membrane clusters as revealed by co-staining microtubules with α-tubulin antibody (Fig. 1A); YFP alone stained diffusely throughout the cell (data not shown). In addition, association of APC-YFP with microtubules was abolished by the microtubule-disrupting drug nocodazole (data not shown). Three APC sequences have been proposed to mediate microtubule association, either directly via the Basic domain (2, 29) or indirectly through the APC-binding proteins EB1 (15, 30) or KAP3A (9) (see Fig. 1B). The influence of protein interactions on APC microtubule-dependent localization or at membrane clusters is, however, poorly defined.

**Defining the Minimal Sequence Required for Targeting of APC to Membrane Clusters**—We next mapped the sequences that promote APC localization at microtubules or membrane clusters. The sequence

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**FIGURE 1.** Ectopic APC-YFP and endogenous APC accumulate in membrane clusters at the plus-ends of microtubules. A, NIH 3T3 cells were transfected with pAPC-YFP, co-stained with α-tubulin Mab (Molecular Probes) to detect microtubules, and processed for immunofluorescence microscopy. Cell images show that APC-YFP co-localized with bundles of microtubule filaments, accumulating in clusters at the ends of membrane protrusions (see arrows). Staining for endogenous APC in NIH 3T3 cells with rabbit polyclonal APC-antibody M-APC revealed a similar pattern along microtubules and at membrane protrusions (see arrows). B, diagram of APC protein domains showing sites for binding of different partners and microtubule binding regions, which include the Armadillo repeats (9), the “Basic domain” (2, 29), and C-terminal EB1 binding site (15, 30). NES, nuclear export signal.
reported for APC binding to, and stabilization of, microtubules is the so-called Basic domain (2, 8, 17) (see Fig. 2A). There is less agreement regarding targeting of APC at membrane clusters, with different sequences having been proposed including aa 1–2158 (8), 461–777 (ARM domain; Refs. 9, 28) and 932–1866 (9). To help resolve this issue, we performed a systematic deletion mapping to identify the membrane cluster targeting sequence(s) of APC. A series of C-terminal APC truncation mutants (Fig. 2A) were transfected into NIH 3T3 (APCwt/wt) and SW480 (APCmut/mut) cells, and the transfected cells were then compared for APC distribution at microtubule filaments or membrane clusters (Fig. 2, B and C). The distribution profiles were similar in both cell lines, and successive C-terminal deletions caused a progressive reduction in APC at microtubule filaments. The least active mutant, APC-(1–1309), is similar to the endogenous form expressed in SW480 colon cancer cells, which does not locate at cellular protrusions or contribute to their assembly.

FIGURE 2. APC amino acids 1941–2226 are important for targeting APC to MT-dependent membrane clusters. A, diagram of untagged plasmids expressing APC full-length (aa 1–2843) and C-terminal truncations showing deletion of the EB1/DLG binding sites (1–2644), the Basic domain (1–2226), the SAMP3 domain (1–1941), and the remaining β-catenin/Axin binding sites (1–1309). B, images of NIH 3T3 cells transfected and stained with APC Ab7 (ectopic APC is well above background). Some staining was observed at MT filaments and at membrane protrusions (arrows) but rarely for the 1941 and 1309 mutants. C, quantification of the proportion of transfected cells with APC localized along MT filaments or at MT-dependent clusters at membrane protrusions. APC mutants were compared with wild-type APC in 100–200 transfected NIH 3T3 cells or SW480 cells per sample. SW480 cells express only a truncated mutant form of APC-(1–1337). Data are presented as the means ± S.E. of two-three experiments. The region aa 1941–2226 was found to be more important for localizing APC at membrane clusters than its association with microtubules.

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We observed that removal of the C-terminal 200 amino acids of APC caused an ~40–60% reduction in localization at microtubules and at membrane clusters (Fig. 2, B and C). DLG-1 and EB1 bind to the extreme C terminus of APC and may therefore contribute to regulation of APC at microtubules and the membrane. Further deletion of the Basic domain abolished association with microtubule filaments. This is consistent with a role for the Basic domain in the binding and stabilization of microtubules. However, deleting the Basic domain restored APC membrane cluster localization to wild-type levels, suggesting that a domain outside of the Basic domain and the C terminus contributes to APC membrane localization. Of all the deleted sequences tested, APC-(1–2226) was preferentially targeted to the tips of cellular protrusions,
and this was greatly reduced by further removal of the sequence aa 1941–2226. The sequence aa 1941–2226 contains two 20 aa repeat β-catenin binding elements and a SAMP3 Axin-binding domain. To determine whether these or other APC protein interaction domains can facilitate targeting to membrane clusters, we constructed a series of GFP-tagged APC sequences, transfected them into NIH 3T3 cells, and compared their localization profiles. Of the sequences tested, the central β-catenin binding region showed the highest level of cluster staining when compared with GFP alone (see Fig. 3A); however, we emphasize that even this APC sequence displayed relatively poor membrane cluster staining (visible in 6–9% of cells, data not shown) when compared with the APC-(1–2226) sequence (37% of cells, Fig. 2C). This indicates that subdivision of the 1–2226 sequence results in significant loss of membrane targeting. In contrast to a previous report (28), the GFP-ARM domain sequence did not efficiently locate at membrane clusters (Fig. 3A). Our findings are most consistent with those of Mimori-Kiyosue et al. (8) and indicate that the minimal core sequence required to efficiently target ectopic human APC to membrane clusters is aa 1–2226.

Localization of Endogenous APC at Membrane Clusters Is Inhibited by Overexpression of the Armadillo Domain, the β-Catenin Binding Region, and the Basic Domain—The GFP-tagged APC sequences described in Fig. 3, A and B, were transiently overexpressed in NIH 3T3 cells, and the cells were then stained with antibodies to detect endogenous APC. Integrity of GFP-APC fusions was assayed by Western blot and fluorescence microscopy (Fig. 3B and supplemental Fig. S5). Transfected cells (>200 cells/sample) were individually scored for accumulation of endogenous APC at the tips of cellular protrusions (Figs. 3C and 4). Of those fragments tested, two sequences (the N-terminal sequence 1–302 and the C-terminal SAMP3 Axin-binding domain) did not compete for APC membrane localization. The other fragments showed varying levels of competition, with the weakest inhibition by the C-terminal EB1/DLG binding sequence (2650–2843), indicating that disrupted binding of DLG-1 and EB1 does not significantly affect clustering of APC at protrusions even though they can colocalize in the same vicinity. Of the other sequences tested, three displayed significantly affect clustering of APC at protrusions even though they can colocalize in the same vicinity. Of the other sequences tested, three displayed significant difference with p value of 0.013.

The overexpression of GFP-APC-(1379–2080) was sufficient to block APC cluster staining in up to 55% of transfected cells (Figs. 3C and 4). This APC region comprises both β-catenin and Axin binding motifs; however, a comparison of subfragments that bind only to Axin-(2033–2061) or to β-catenin-(1941–2032) revealed that the competition for β-catenin binding specifically diminished APC at membrane clusters. In contrast, competing out Axin with the sequence 2033–2061 caused a modest increase in APC at clusters; this may correlate with the stabilization of β-catenin that results from loss of Axin. These findings are consistent with the deletion analysis of Fig. 2 and suggest that the combination of KAP3A and β-catenin contributes to localization of APC at membrane clusters. To further validate this hypothesis, we constructed two short GFP-tagged β-catenin sequences, one corresponding to the APC-binding domain of β-catenin (aa 218–467) and a control N-terminal fragment (aa 1–217). When transfected into NIH 3T3
cells, staining for endogenous APC revealed that the APC-binding domain of β-catenin caused a specific inhibition of APC accumulation at membrane clusters, and this was similar to the degree of inhibition observed for the APC β-catenin binding sequence (up to 55% of cells) (see Fig. 5). The β-catenin-(1–217) peptide had no effect on membrane targeting of APC.

β-Catenin Silencing by RNA Interference Confirms a Role for β-Catenin in APC Localization at Membrane Clusters—We next compared the staining patterns of endogenous APC and β-catenin at membrane clusters in migrating NIH 3T3 cells. In a scratch-induced migration assay, NIH 3T3 cells were assessed 6 h after wounding (see “Materials and Methods” for details) and analyzed by immunofluorescence microscopy for endogenous β-catenin (green) or APC (red) in actively migrating cells 6 h after the scratch (arrow shows direction of migration into the wound). In control siRNA-treated cells at the wound edge, β-catenin and APC frequently co-located at the tips of membrane protrusions. In cells treated with β-catenin siRNA, fewer cells revealed β-catenin at the membrane and there was a decrease in APC staining at membrane clusters. The β-catenin siRNA efficiently reduced β-catenin protein expression by ~85% relative to control siRNA as shown by Western blot. The expression of actin protein was not affected. A consistent correlation was observed between APC staining at membrane clusters and the presence of β-catenin in both control and β-catenin siRNA-treated cells. Data shown are mean ± S.E. from three experiments. Differences between β-catenin-positive and -negative samples were found to be statistically significant where indicated (*, p < 0.5; **, p < 0.01; ***, p < 0.001).

FIGURE 6. siRNA-mediated silencing of β-catenin alters APC accumulation at MT-dependent membrane clusters. A, NIH 3T3 cells were grown to confluence and subjected to the scratch-induced migration assay. Cells were treated with control or β-catenin-specific siRNA (see “Materials and Methods” for details) and analyzed by immunofluorescence microscopy for endogenous β-catenin (green) or APC (red) in actively migrating cells 6 h after the scratch (arrow shows direction of migration into the wound). In control siRNA-treated cells at the wound edge, β-catenin and APC frequently co-located at the tips of membrane protrusions. In cells treated with β-catenin siRNA, fewer cells revealed β-catenin at the membrane and there was a decrease in APC staining at membrane clusters. B, the β-catenin siRNA efficiently reduced β-catenin protein expression by ~85% relative to control siRNA as shown by Western blot. The expression of actin protein was not affected. C, a consistent correlation was observed between APC staining at membrane clusters and the presence of β-catenin in both control and β-catenin siRNA-treated cells. Data shown are mean ± S.E. from three experiments. Differences between β-catenin-positive and -negative samples were found to be statistically significant where indicated (*, p < 0.5; **, p < 0.01; ***, p < 0.001).
the hypothesis that β-catenin contributes to APC accumulation at these migration-associated structures.

Regulation of Ectopic APC-YFP at Cellular Protrusions by β-Catenin and KAP3A—Transiently expressed APC-YFP localizes in the cell along microtubule filaments and accumulates at the ends of cellular protrusions at membrane clusters (e.g. Fig. 1A). We next investigated whether the co-expression of APC-YFP and β-catenin affected their localization patterns at microtubules or at cellular protrusions (Fig. 7). Following transfection into NIH 3T3 cells, FLAG-tagged β-catenin was detected by immunofluorescence microscopy and found to display very poor staining at microtubules or at membrane clusters on its own (Fig. 7, A and C). The co-transfection of APC-YFP induced accumulation of ectopic β-catenin at the microtubule cytoskeleton in 58% of cells and at membrane clusters in 41% of cells (see Fig. 7C). A similar result was obtained for endogenous β-catenin in SW480 cells (supplemental Fig. S9). To confirm the specificity of this regulation, we showed that only β-catenin fragments containing the APC-binding domain were recruited to microtubules or to membrane clusters by APC (Fig. 7A). Conversely, the co-expression of β-catenin significantly increased the proportion of cells in which APC-YFP located at membrane clusters (Fig. 7C). These results indicate that transiently expressed APC and β-catenin can positively affect one another’s microtubule-dependent membrane localization and are quite consistent with our competition and siRNA experiments for endogenous APC.

The endogenous forms of KAP3A, EB1, and DLG-1 have been reported to co-localize at membrane clusters with APC (9, 15,16) (supplemental Fig. S3). In contrast to β-catenin, however, the ectopic forms of KAP3A, EB1, and DLG-1 did not enhance APC-YFP localization at cellular protrusions (Fig. 7C). We next tested whether APC could recruit these binding partners to microtubule structures. In transfected 3T3 cells, there was no microtubule association observed for full-length forms of untagged EB1, GFP-DLG, or GFP-KAP3A when expressed individually. However, the co-transfection of APC-YFP stimulated localization of these proteins to microtubule filaments in 20–45% of transfected cells and to membrane clusters in 17–26% of transfected cells (Fig. 7, B and C). These results indicate that in a transient expression system, APC-YFP can co-localize with β-catenin, EB1, KAP3A, and DLG-1 at membrane clusters and along microtubules, but of these cofactors only β-catenin stimulated APC accumulation at membrane clusters.

Consistent with a regulatory translocation role of KAP3A (a kinesin adaptor protein), the co-expression of KAP3A mutant ΔARM5 reduced APC at microtubules and, in particular, at cellular protrusions (Fig. 7C). KAP3A-ΔARM5 acts as a KAP3A dominant negative inhibitor by disrupting kinesin association of APC (9), supporting the conjecture that APC localization at cellular protrusions is at least partly dependent on kinesin transport (9).

We also tested the binding partners GSK-3β, B56α, and Axin and found that these partners did not co-stain with ectopic APC at membrane clusters but, in fact, reduced membrane localization and microtubule association of APC (see supplemental Fig. S10). The inhibitory influence of GSK-3β is consistent with previous reports (14, 17). Given that each of these cofactors promotes β-catenin degradation (1), it is possible that their effects on APC at membrane clusters are in part an indirect consequence of β-catenin turnover.

DISCUSSION

The subcellular localization of APC remains controversial (32); however, it is generally agreed that APC can localize at specific microtubule-dependent structures such as the centrosome (33), kinetochores (4, 5), and the tips of microtubule-dependent cellular protrusions where it accumulates in membrane clusters. This latter localization of APC may contribute to the polarity of cell migration, for instance in epithelial cells during wound healing or the directed movement of colonic epithelial cells out of the intestinal crypt. In this study, we adopted multiple approaches to investigate the mechanism by which APC becomes concentrated at membrane clusters. We have shown that a minimal core sequence comprising amino acids 1–2226 determines optimal targeting of APC to membrane clusters. Shorter APC sequences displayed poor localization to membrane clusters individually, but certain sequences effectively competed for APC binding partners and thereby disrupted membrane targeting of endogenous APC. We employed a combination of competition and RNA interference experiments and, for the first time, have identified β-catenin as a regulator of APC at membrane clusters. The interaction of APC and β-catenin is normally associated with β-catenin degradation (1, 6). Our findings reveal that APC-β-catenin complexes, including possible pre-degradation assemblies of either protein, may be integrally linked to other processes in the cell, such as directed cell movement.

APC can stabilize microtubule polymers in vitro (2, 3, 17), and together with other proteins, including CLIP170 and EB1, it accumulates at the plus-ends of microtubules (8, 13). At the tips of microtubule polymers in cellular protrusions, APC becomes deposited in aggregates or clusters that localize in the vicinity of the basal membrane (see supplemental Fig. S4) and is likely to be associated with cortical actin (8). APC is known to translocate as granules or puncta along microtubule filaments in a plus-end-directed and ATP-dependent fashion (8). This same protractive movement is potentially facilitated by the interaction of APC with the KAP3A-kinesin motor protein complex (9). Once it has arrived at the plus-ends of those microtubule polymers that stretch out to the peripheral membrane, APC somehow must disengage from the microtubule cap complex and be captured by a protein complex associated with cortical actin. Recently, Watanabe et al. (12) showed that the Rac1 effector protein IQGAP1 could mediate association of APC with cortical actin that formed part of the leading edge of a migrating cell; however, they indicated that IQGAP1 is not involved with accumulation of APC at the membrane clusters studied here. Other binding partners, including DLG-1 (12) and EB1 (15), have been postulated to mediate the anchorage of APC at the actin cortex; DLG-1 in particular was a prime candidate because it can bind both to APC and, through its PDZ domains, also to actin. Although it is possible that DLG-1 and EB1 may contribute to regulation of APC at membrane clusters, our findings demonstrate that these proteins are not essential for membrane targeting of APC (Figs. 2, 3, 7).

The microtubule-dependent translocation of APC to the membrane may involve its interaction with the kinesin heavy chain adaptor protein KAP3A, which binds to the Armadillo domain of APC (9). Jimbo et al. (9) showed that endogenous APC and KAP3A can co-localize at membrane clusters, which is consistent with our finding that the co-expression of ectopic APC and KAP3A results in their co-localization at membrane clusters (Fig. 7). When APC-YFP was co-expressed with a dominant negative form of KAP3A that can bind APC but not kinesin, a diminution in APC membrane staining was observed (Fig. 7C). Combined with our observation that the APC Armadillo domain competed for membrane cluster staining of endogenous APC, we speculate that our results support a role for KAP3A in the translocation of APC to the membrane. The fact that overexpressed KAP3A did not stimulate APC accrual at membrane clusters may be due to its singular role in translocation, rather than in anchoring APC at the membrane cortical region.

In this study we co-expressed full-length forms of seven different
FIGURE 7. The effect of co-expressing β-catenin, EB1, DLG, and KAP3A on APC localization. A, APC can recruit β-catenin to microtubules, and this requires the APC binding region of β-catenin. β-catenin constructs (pFLAG-β-catenin wild-type, S33Y/D218–467, and S33Y/D695) (25) were transiently expressed in NIH 3T3 cells, and none of the transiently expressed β-catenin stained at microtubules or at microtubule-dependent membrane clusters. The co-expression of APC-YFP stimulated β-catenin staining at microtubules and at membrane clusters for all constructs except D218–467, which lacks the APC binding region. FLAG-tagged peptides were detected with M2 Mab (Sigma). Note that the β-catenin-(1–695) and -D218–467 constructs contain an S33Y mutation that did not influence microtubule localization (not shown).

B, cells were transfected with pCMV-EB1 (untagged) (26), pDLG-GFP (24), or pKAP3A-GFP (wt or ΔArm mutant) (9) expression vectors in the absence or presence of pAPC-YFP or pCMV-APC. Transiently expressed EB1, DLG, or KAP3A showed no staining at microtubules or at membrane clusters; however, when co-expressed with APC (detected by YFP or with Ab7 Mab; Oncogene Research Products) their staining at these structures was detected as seen in cell images (arrows highlight staining at clusters).

C, cells were immunostained and >100 transfected cells/slide were scored for protein localization at microtubules or at membrane clusters. Values shown are mean ± S.D. from three-five experiments. Statistical analysis of APC localization patterns (lower graphs) revealed that co-expression of KAP3A mutant and β-catenin had negative and positive effects on APC localization, respectively (*, p < 0.5; ***, p < 0.001).
APC binding partners of these, only one protein, β-catenin, was found to consistently stimulate APC accumulation at membrane clusters. β-catenin is usually viewed not as a regulator of APC but is itself regulated by the APC/Axin-dependent degradation process (reviewed in Refs. 6, 34). It has been known for some time that β-catenin frequently co-localizes with APC at membrane clusters (9, 13, 19, 35). Here, we have provided the first direct evidence that both ectopic and endogenous forms of full-length β-catenin contribute to the accumulation of APC at membrane clusters. Because the absence of β-catenin at cellular protrusions did not always prevent APC clustering at these structures but did reduce it, we postulate that β-catenin acts in conjunction with another factor(s) as a regulator of APC anchorage in the membrane cluster region. β-catenin does not bind directly to actin filaments but indirectly associates with actin via an interaction with other proteins (e.g. α-catenin, Axin, DLG-1, IQGAP1). It will be of interest in regard to APC subcellular localization, and its roles in microtubule capture at the actin cortex and in cell migration, to identify the additional factor(s) involved in APC membrane cluster localization and to determine whether this other factor acts in co-operation with β-catenin.

In transient expression assays, we discovered a negative influence of the binding partners GSK-3β, B56α, and Axin on APC localization at microtubules and membrane clusters (supplemental Fig. 55). These proteins all contribute to β-catenin degradation and their individual overexpression is sufficient to cause loss of β-catenin (23, 36), suggesting that this might partly contribute to the decline of APC at the membrane. Conversely, the competition of Axin by APC (2033–2061) peptide resulted in a modest enhancement of APC at membrane clusters (Fig. 3C). GSK-3β kinase activity has elsewhere been reported to promote dissociation of APC from microtubules (17) and to prevent APC accumulation at membrane clusters (14). In particular, Etienne-Manneville and Hall (14) claimed that spatially localized inhibition of GSK-3β (via cdc42 and Par6-atypical protein kinase) at membrane clusters was required to stimulate APC localization at the membrane and to establish cell polarity. Our identification of β-catenin provides a plausible explanation for the effects of cdc42 on APC localization, in that cdc42-dependent inactivation of GSK-3β would result in the stabilization and increased expression of β-catenin at cellular protrusions, leading to increased APC anchorage and clustering at this structure. In view of recent studies that link APC-dependent degradation of β-catenin to the tips of neurite extensions (37) or at cytosolic locations exclusive of microtubules (38), it will be interesting to determine how and when stabilized forms of β-catenin modulate APC at the membrane.

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