Amer2 Protein Interacts with EB1 Protein and Adenomatous Polyposis Coli (APC) and Controls Microtubule Stability and Cell Migration*

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Background: Amer2 localizes to the plasma membrane, interacts with adenomatous polyposis coli, and regulates Wnt signaling.

Results: Amer2 recruits the microtubule-associated protein EB1 to the plasma membrane and affects the stabilization of microtubules and cell migration.

Conclusion: Amer2 is a novel regulator of microtubule stability by interacting with EB1.

Significance: A novel membrane-associated regulator of microtubule stabilization at the plasma membrane was identified and shown to affect cell migration.

EB1 is a key factor in the organization of the microtubule cytoskeleton by binding to the plus-ends of microtubules and serving as a platform for a number of interacting proteins (termed +TIPs) that control microtubule dynamics. Together with its direct binding partner adenomatous polyposis coli (APC), EB1 can stabilize microtubules. Here, we show that Amer2 (APC membrane recruitment 2), a previously identified membrane-associated APC-binding protein, is a direct interaction partner of the C-terminal end of the tumor suppressor protein adenomatous polyposis coli (APC) (1). It was then shown to bind preferentially at the plus-ends of growing microtubules and to dissociate rapidly from the more mature microtubule lattice, thereby generating comet-like structures that can be visualized by fluorescence microscopy (2). EB1 recruits a variety of proteins to the microtubule plus-ends that control microtubule dynamics, suggesting that it represents a platform for microtubule regulators. Because of their association with growing microtubule ends, EB1 and its binding partners are collectively termed microtubule plus-end tracking proteins or +TIPs. It was recently shown that +TIPs associate with EB1 by short sequence stretches containing (S/T)xIP amino acid motifs (3). In vitro and in vivo studies have revealed in part opposing effects of EB1 on different parameters of microtubule dynamics, including polymerization, catastrophe frequency, pausing, and rescue (4). In mammalian cells and Xenopus egg extracts, EB1 promotes microtubule growth and stability, at least in part by lowering catastrophe frequencies (5, 6). APC is a +TIP protein that can bind and stabilize microtubules in clusters at the cell cortex (7, 8). APC cooperates with EB1 in the stabilization of microtubules both in vitro and in vivo (9, 10) but may also localize and act independently of EB1 at microtubules (7, 11, 12).

Besides its role in microtubule biology, APC has a well-established function as a negative regulator of the Wnt/β-catenin pathway by promoting degradation of β-catenin (13). Among other interaction partners, it can bind to members of the Amer (APC membrane recruitment) protein family, consisting of Amer1/WTX (Wilms tumor gene on the X chromosome), Amer2, and Amer3, which share conserved domains that interact with the N-terminal armadillo repeats of APC (14, 15). Amer1 is a tumor suppressor and negative regulator of Wnt signaling (15–17). Amer2 is a membrane-associated phosphatidylinositol 4,5-bisphosphate-binding protein that interacts with APC via two conserved APC-binding domains and recruits it to the plasma membrane (15, 18). Amer2 negatively regulates Wnt signaling probably by interfering with β-catenin (18). Here, we show for the first time that Amer2 directly interacts with the microtubule-associated protein EB1 and recruits it to the plasma membrane. Moreover, we reveal a role for Amer2 in regulating microtubule stability presumably by providing a platform for the microtubule-binding proteins APC and EB1 to promote cell migration.

EXPERIMENTAL PROCEDURES

DNA Constructs and siRNAs—The following constructs have been described previously: pcDNA-FLAG-Amer2 and
pcDNA-FLAG-Amer1 (15), EB1-GFP (19), CMV-APC (20), and pcDNA3.1-FLAG (21). Amer2-SKNN, Amer2-TKNN, and Amer2-SKNN/TKNN were generated by PCR mutagenesis, exchanging amino acids IP with NN. For expression of the GST-Amer2(559–671) protein, the cDNA encoding amino acids 559–671 of human Amer2 was amplified by PCR and inserted into pGEX-4T3 (Amersham Biosciences). The sequences of GFP siRNA and Amer2 siRNA (termed siAmer2-1) have been described (18). Other sequences were as follows: luciferase siRNA, 5’-CUUACGCUGAGUACUUCCA-3’; EB1 siRNA, 5’-UGGCCUUGAGAAGAUGUGA-3’ (22); and APC siRNA, 5’-AAGACGUUGCGAGAAGUUGGA-3’. All siRNAs were purchased from Dharmacon.

Antibodies—The rabbit anti-Amer2 polyclonal antibody was produced by immunizing rabbits with a recombinant GST-Amer2 fusion protein containing amino acids 559–671 of human Amer2 (Pineda, Berlin, Germany). The serum was affinity-purified using CNBr-activated Sepharose 4B beads (GE Healthcare) coupled to the antigen. Commercial antibodies were purchased from Sigma (rabbit anti-FLAG; mouse anti-FLAG; mouse anti-acetylated tubulin, clone 6-11B-1; and rabbit anti-pan cadherin), Roche Applied Science (mouse anti-
GFP, mixture of clones 7.1 and 13.1), Epitomics (rabbit anti-GFP), BD Transduction Laboratories (mouse anti-EB1, clone 5), Serotec (rat anti-/H9251-tubulin, clone YL1/2), Abcam (mouse anti-APC, ALI(12–28)), and Cell Signaling (rabbit anti-GAPDH, clone 14C10). Secondary antibodies (Jackson Immunoresearch Laboratories) were either Cy2 and Cy3 conjugates for immunofluorescence or HRP conjugates for Western blotting.

**Yeast Two-hybrid Screen**

— Yeast two-hybrid and 

/galactosidase assays were performed in the yeast L40 strain using pBTM116 as a bait vector and a mouse embryonic day 10.5 library in pVP16 as described previously (21).

**Cell Culture and Transfections**

— Cells were cultured in 10% 

/CO2 at 37 °C in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin (PAA Laboratories). Plasmid transfections were performed using polyethylenimine for HEK293T cells, TransIT-TKO reagent (Mirus, Madison, WI) for MCF-7 cells, and Lipofectamine 2000 (Invitrogen) for U2OS cells. siRNAs were transfected using Oligofectamine (Invitrogen) for 48–72 h according to the manufacturer’s instructions.

**Preparation of Protein Lysates, Subcellular Fractionation, Co-immunoprecipitation, and Western Blotting**

— Cells were washed with PBS and lysed in Triton X-100 buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, and 1 mM PMSF) at 4 °C for 10 min. Lysates were cleared at 13,000 rpm for 10 min at 4 °C. For co-immunoprecipitation, lysates were incubated overnight with anti-FLAG M2 affinity gel beads (Sigma) or mouse anti-GFP antibodies plus protein A/G-Sepharose beads (Santa Cruz Biotechnology). Immunoprecipitates were collected, washed four times with low salt buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100), and eluted with SDS sample buffer. Subcellular fractionation of cells was carried out using a ProteoJET membrane protein extraction kit (Fermentas) according to the manufacturer’s instructions. Western blotting was performed as described (23). Proteins were visualized using Enhanced chemiluminescent reagent (PerkinElmer Life Sciences) and a Fujifilm LAS-3000 LuminoImager.

**RT-PCR Analysis**

— This was performed as described (18). The sequences of the APC primers were 5’-AAGTTGCCGCGCTGTGAACCAAGGGTGAATTGGTGGTG-3’ and 5’-AAGTCCGGCGCAATTTAAGCAGGAGGTTGCAATG.

**FIGURE 2.** Amer2 directly interacts with EB1 via SKIP and TKIP motifs. A, protein sequence alignment of human Amer2 and its mouse, rat, and frog orthologs. The EB1-binding motifs SKIP and TKIP are conserved in all analyzed species and are boxed; identical residues are indicated by asterisks. Numbers below the sequences indicate the amino acid residue positions of human Amer2. B, mutation of both EB1-binding motifs (IP to NN) in the human Amer2 bait abolishes interaction with EB1 prey (cf. Fig. 1B) as shown by plate growth and quantitative 

/galactosidase assays in yeast two-hybrid experiments. The results of representative experiments are shown. WT, wild-type Amer2 sequence (SKIP/TKIP). E, effect of mutating the SKIP and TKIP motifs of full-length Amer2 on the EB1 interaction. Shown are the results from co-immunoprecipitation of FLAG-Amer2 mutants and EB1-GFP after transient transfection of HEK293T cells. Immunoprecipitation (IP) was performed using 

/Flag-Sepharose, and Western blots were detected by anti-GFP and anti-FLAG antibodies. Numbers indicate kilodaltons. F, FLAG-Amer2-SKNN/TKNN does not recruit endogenous (endo) EB1 to the plasma membrane. Shown are the results of fluorescence staining of MCF-7 cells transiently transfected with FLAG-Amer2 mutants as indicated above the panels. Cells were transfected with anti-FLAG and anti-EB1 antibodies. Dashed lines indicate transfected cells. Scale bar = 10 μm.

**FIGURE 3.** APC links EB1 to Amer2. A, expression of APC promotes co-immunoprecipitation of EB1-GFP with FLAG-Amer2 after transient transfection of HEK293T cells. FLAG-Amer1 served as a negative control. Co-immunoprecipitation was performed using FLAG-Sepharose, and Western blots were detected by anti-tag and anti-APC antibodies. Numbers indicate kilodaltons. **IP,** immunoprecipitation. B, schematic representation of the Amer2-EB1-APC complex.
**Immunofluorescence Staining and Microscopy**—For immunofluorescence staining, cells were grown on glass coverslips, fixed with ice-cold methanol, permeabilized with 0.5% Triton X-100, blocked with DMEM/FCS, and stained with the indicated antibodies. To disrupt microtubules, transiently transfected cells were treated with nocodazole (2 μg/ml) for 1 h in an incubator (10% CO₂ at 37 °C). Photographs were taken with a CCD camera (Visitron, Munich, Germany) on a Zeiss Axioplan 2 microscope using MetaMorph software (Molecular Devices). Images were processed using Adobe Photoshop CS software.

**Cell Migration Assay**—U2OS cells on coverslips in 6-well plates were transfected with siRNAs and allowed to reach confluence. Three wounds of defined size (~850 μm) were made for each coverslip, and cells were allowed to migrate for 12 h.
Methanol-fixed cells were processed for α-tubulin immunofluorescence staining. Measurements at two positions along each of the three wounds were taken, and percentage closure was calculated, followed by statistical analysis (unpaired Student’s t test).

**Xenopus Experiments**—Embryos were injected in both dorsal blastomeres at the four-cell stage with 100 pg of LacZ DNA plus 0.8 pmol of Amer2 morpholino or control morpholino as described previously (18). At stage 12.5, embryos were stained for LacZ and probed by *in situ* hybridization for expression of XPAPC (Xenopus paraxial protocadherin) (24).

**RESULTS**

**Amer2 Interacts with EB1 via (S/T)xIP Motifs**—In a yeast two-hybrid screen using a C-terminal fragment of Amer2 as bait, we isolated several interacting clones covering the C-terminal part of EB1 (Fig. 1, A and B) (3). The Amer2-EB1 interaction was confirmed by co-immunoprecipitation of the transiently expressed full-length proteins in HEK293T cells (Fig. 1C). Endogenous EB1 was also co-immunoprecipitated with transfected Amer2 (Fig. 1D). Used as a control, EB1 only weakly co-immunoprecipitated with the related Amer1/WTX protein (cf. Fig. 3A).

Amer2 is linked to the plasma membrane through its interaction with phosphatidylinositol phosphate lipids (18). Accordingly, Amer2 exhibited membrane and cytoplasmic staining when expressed in MCF-7 cells. Amer2 staining was most prominent at the cell-cell contact areas but was also occasionally observed at the periphery of cells, where there was no contact with neighboring cells. EB1 decorated microtubules (Fig. 1E), as reported previously (2, 15). Importantly, when both proteins were coexpressed, a large fraction of EB1 was recruited to the plasma membrane, colocaling with Amer2 (Fig. 1E). Endogenous Amer2 and EB1 was present at filamentous structures possibly representing microtubules, which would point to recruitment of Amer2 to microtubules by EB1 (Fig. 1F). Transiently expressed Amer2 also recruited endogenous EB1 from microtubule comets to the plasma membrane (Fig. 1G). These data show that Amer2 is a novel interaction partner of EB1 and is able to recruit EB1 to the plasma membrane.

Various known +TIPs share a specific four-amino-acid motif, (S/T)xIP, which makes direct contact with the C-terminal part of EB1 (3). We found two perfect matches of the (S/T)xIP motif in our Amer2 bait, SKIP at amino acid 606 and TKIP at amino acid 637, which are conserved in Amer2 from different species (Fig. 2A). SKIP and TKIP motifs of Amer2 were mutated to SKNN and TKNN, respectively, and mutants were analyzed for EB1 interaction. In yeast two-hybrid assays, mutations of either motif alone did not affect interaction with the EB1 prey; however, mutation of both motifs completely abrogated the interaction (Fig. 2B). In co-immunoprecipitation experiments, which provide more stringent conditions for interactions, mutation of SKIP alone already strongly reduced the binding of full-length Amer2 to EB1, whereas mutation of TKIP had a minor effect. Binding to EB1 was completely abolished in the double mutant Amer2-SKNN/TKNN (Fig. 2C). In line with this, plasma membrane recruitment of endogenous EB1 by Amer2 was reduced but not completely abolished by the SKIP mutation, whereas mutation of TKIP had only a minor effect. Recruitment of EB1 was completely abrogated in the double mutant (Fig. 2D). These data show that Amer2 binds directly to EB1 via the (S/T)xIP consensus motifs and that two of these motifs are functional, albeit with different affinities for EB1.

**Amer2 Forms a Scaffold for APC and EB1 Complex Formation**—APC interacts with Amer2 via its N-terminal armadillo domain (15, 18) and with EB1 via the C-terminal EB1-binding domain (1). It might therefore link EB1 to Amer2. Indeed, the amount of EB1 co-immunoprecipitated with Amer2 was greatly increased when APC was coexpressed (Fig. 3A). Our data suggest that Amer2 acts as a scaffold for a multiprotein complex containing the microtubule-interacting proteins EB1 and APC (Fig. 3B).

**Amer2 Stabilizes Microtubules Together with EB1**—Next, we analyzed whether microtubule stability is affected by Amer2 using the occurrence of acetylated tubulin as a marker for stable microtubules (25). In MCF-7 cells, coexpression of Amer2 and EB1 led to a marked increase in and concomitant enrichment of stabilized microtubules at the cell cortex close to the plasma membrane, in line with the preferential membrane localization of Amer2 and EB1 in these cells (Fig. 4A, panels a and b). In contrast, coexpression of EB1 with the Amer2-SKNN/TKNN mutant (Fig. 4A, panels c and d) or transfection of Amer2 alone (panels e and f) did not significantly alter the intensity and distribution of acetylated microtubules, indicating that direct interaction of both proteins is required for microtubule stabilization. Coexpression of Amer2 and EB1 did not alter the general pattern of the microtubule network as revealed by staining with an antibody to α-tubulin (data not shown).

To analyze whether Amer2 is required for microtubule stability, we performed loss-of-function experiments. Indeed,
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Amer2 knockdown of Amer2 in HEK293T and HeLa cells led to a marked decrease in acetylated tubulin as determined by Western blotting (Fig. 4B). In line with this, immunofluorescence staining revealed that Amer2 knockdown strongly reduced the number of stable microtubules in HeLa cells (Fig. 4C). Acetylated tubulin levels were also reduced after knockdown of APC or EB1 (Fig. 4D). To address the point of microtubule stabilization by Amer2-EB1 in a different experimental setup, Amer2-EB1 cotransfectants of U2OS cells were treated with low doses of nocodazole to disrupt microtubules and then stained with anti-α-tubulin antibodies. This revealed focal retention of microtubules at areas of EB1 membrane localization, suggesting that microtubules associated with Amer2-EB1 are resistant against nocodazole treatment. In contrast, no such stabilization was observed in cells expressing EB1 together with the Amer2-SKNN/TKNN mutants deficient for EB1 binding (Fig. 4E). Together, our data suggest that Amer2 stabilizes microtubules in conjunction with EB1 and APC.

Amer2, EB1, and APC Are Required for Directed Cell Migration—Microtubules are required for cell migration by providing a basis for cell polarity (26). Knockdown of Amer2 in U2OS cells clearly abrogated cell migration as determined by wound assays (Fig. 5A). Knockdown of either EB1 or APC also reduced cell migration, confirming previous publications (10, 27) and suggesting similar roles for Amer2, EB1, and APC in cell migration. To confirm a role for Amer2 in cell migration in vivo, we knocked down its expression in Xenopus using morpholino oligonucleotides (18). We observed defects in convergent extension movements indicated by reduction of the XPAPC-expressing paraxial mesoderm as well as increased length and width of the negatively stained notochord in Xenopus Amer2-depleted embryos (Fig. 5B).

DISCUSSION

In this study, we have identified Amer2 as a novel key factor in the control of the microtubule cytoskeleton. Amer2 binds to APC and EB1 via distinct binding domains and seems to cooperate with EB1 in microtubule stabilization. Whereas expression of Amer2 and EB1 alone had no or only minor effects on the stability of microtubules, coexpression of both proteins generated stable bundles of microtubules at the cell cortex close to the plasma membrane. Conversely, reduction of Amer2 levels similar to reduction of EB1 and APC strongly reduced the number of stabilized microtubules. Of note, APC increased the amounts of EB1 associated with Amer2 by linking it to the complex (Fig. 3). We propose a model in which Amer2 acts as a scaffold for EB1 and APC to coordinate their functional interaction with microtubules. Both EB1 and APC were shown to be able to stabilize microtubules by acting either separately or together (9, 10). It was suggested that endogenous APC and EB1 colocalize only transiently at microtubule tips (7, 11). Bridging of both factors by Amer2 might foster their cooperation in microtubule stabilization, for instance by increasing the local concentration of these factors at microtubule ends. Interestingly, a similar triple complex of EB1 and APC with the Rho GTPase effector mDia was shown to stabilize microtubules downstream of Rho signaling (10).

Based on its specific interaction with EB1 via (S/T)IP motifs, Amer2 resembles classical +TIPs. Similar to these, Amer2 might track microtubule plus-ends via its association with EB1, e.g. during transport to the plasma membrane. However, we have seen Amer2 mainly at the plasma membrane and sometimes also at fibers together with EB1 (Fig. 1F), but we never detected it at microtubule ends, suggesting that the Amer2-
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EB1-APC complex forms predominantly at the plasma membrane and not at growing microtubules. Membrane association of Amer2 is mediated by phosphatidylinositol 4,5-bisphosphate, suggesting that occurrence of this lipid determines the differential localization of Amer2-EB1 (18). Wnt signaling was shown to stimulate synthesis of phosphatidylinositol 4,5-bisphosphate, and we recently showed that the related protein Amer1 becomes membrane-associated in a phosphatidylinositol 4,5-bisphosphate-dependent manner after stimulation with Wnt3A (28). In similar experiments, we noticed that Amer2 shifted to the plasma membrane fraction after Wnt3A treatment (data not shown). Thus, Wnt signaling might induce membrane association of Amer2, which in turn might recruit EB1 and APC. The consequences of such a mechanism for cortical association of microtubules needs to be further explored. Amer2 resembles the membrane-associated LL5 protein, which interacts with CLASP + TIPs and is required for cortical attachment of microtubules. Similar to Amer2, LL5 requires binding to phospholipids, specifically phosphatidylinositol 3,4,5-trisphosphate, for membrane recruitment, and PI3K signaling was suggested to regulate membrane association of LL5 (29).

Microtubule organization is a prerequisite for directed cell migration, probably because it imposes cell polarity on the migrating cells (26). In line with this, microtubules are more stable at the leading edge than at the trailing edge of migrating cells (30). Our knockdown experiments showed that Amer2, APC, and EB1 are similarly required for cell migration, probably due to their effects on microtubule stability. Moreover, morpholinoo-mediated down-regulation of Xenopus Amer2 resulted in defects in convergent extension cell movements during gastrulation. Thus, Amer2 appears to be a hub for cellular activities, linking the microtubule-based cytoskeleton to cell migration.

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