

# Adenomatous Polyposis Coli and Asef Function Downstream of Hepatocyte Growth Factor and Phosphatidylinositol 3-Kinase\*

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Mutations of the tumor suppressor adenomatous polyposis coli (APC) are responsible for sporadic and familial colorectal tumors. APC negatively regulates Wnt signaling by inducing  $\beta$ -catenin degradation. It has also been shown that APC plays a role in the organization of cytoskeletal networks. APC interacts with Asef and Asef2, Rac1- and Cdc42-specific guanine nucleotide exchange factors (GEFs), and stimulates their GEF activity; thereby regulating cell morphology, adhesion, and migration. Truncated mutant APCs present in colorectal tumor cells activate Asef and Asef2 constitutively and contribute to their aberrant migratory properties. We show here that hepatocyte growth factor (HGF), as well as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), induce the accumulation and colocalization of APC and Asef in membrane ruffles and lamellipodia of epithelial cells. Both APC and Asef were found to be required for HGF-induced cell migration. Furthermore, we show that the effects of HGF, bFGF, and EGF on APC and Asef are mediated by the activation of phosphatidylinositol 3-kinase (PI3-kinase) and require the PH domain of Asef. These results suggest that Asef and APC function downstream of HGF and PI3-kinase, and play critical roles in growth factor-mediated regulation of cell morphology and migration.

Mutations of the tumor suppressor gene adenomatous polyposis coli (APC)<sup>2</sup> are responsible for familial adenomatous polyposis (FAP), a dominantly inherited disease characterized by multiple adenomatous polyps in the colon (1, 2). The APC gene is also somatically mutated in the majority of sporadic colorectal tumors. The majority of the somatic mutations in APC is confined to its central region and result in the generation of truncated gene products. It is well known that APC induces degradation of  $\beta$ -catenin, a key Wnt signaling effector (3–6). Furthermore, it has recently been shown that APC also

interacts with various other cellular proteins, including Asef, Asef2, IQGAP1, and kinesin-2, and regulates the organization of cytoskeletal networks, thereby controlling cell adhesion and motility (7–15).

Asef is a guanine-nucleotide exchange factor (GEF) specific for Rac1 and Cdc42 (9–11, 15, 16). APC interacts via its armadillo repeat domain with an APC-binding region (ABR) in the NH<sub>2</sub> terminus of Asef. In addition to this ABR, Asef contains Dbl homology (DH), Pleckstrin homology (PH), and Src homology 3 (SH3) domains. The SH3 domain of Asef inhibits its own GEF activity by intramolecular binding to the DH domain (17, 18). The PH domain of Asef binds to phosphatidylinositol 3,4,5-trisphosphate (PIP3) and is required for its localization to the plasma membrane (19). APC enhances the GEF activity of Asef, presumably by relieving the intramolecular negative regulation and thereby regulates cell morphology, adhesion, and migration. A mutant form of Asef lacking the ABR shows strong GEF activity even in the absence of APC. Furthermore, truncated mutant APCs present in colorectal tumor cells activate Asef constitutively and cause increased aberrant migration. APC also activates Asef2, which has significant structural and functional similarities to Asef (11, 15). Thus, truncated mutant APCs, Asef and Asef2 may be important for adenoma formation as well as tumor progression to invasive malignancy.

HGF is known to be important for embryonic development, wound healing, tissue regeneration, hematopoiesis, and tissue homeostasis (20, 21). The HGF receptor, which is encoded by the proto-oncogene *c-met*, is a tyrosine kinase, and its activation by HGF induces cell motility, invasion, and proliferation. Furthermore, HGF signaling is known to play a crucial role in tumor development and malignant progression, in particular by increasing tumor invasiveness and metastatic potential. Because the effects of APC-activated Asef on MDCK cells appear to be similar to those of HGF, we attempted to examine whether APC and Asef function downstream of HGF. In the present study, we show that APC and Asef indeed function downstream of HGF and that Asef is required for HGF-induced migration.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—HeLa and Caco-2 cells were cultured in MEM supplemented with 10% fetal calf serum (FCS) and 0.1 mM non-essential amino acids. MDA-MB-157 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. H1666 and DLD-1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS.

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<sup>2</sup> The abbreviations used are: APC, adenomatous polyposis coli; GEF, guanine nucleotide exchange factor; ABR, APC-binding region; DH, Dbl homology; PH, Pleckstrin homology; GST, glutathione S-transferase; HA, hemagglutinin; mAb, monoclonal antibody; HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; PI3-kinase, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; shRNA, short hairpin RNA.

Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) or Fugene (Roche Applied Science).

**Expression Vectors and Antibodies**—Asef- $\Delta$ APC (amino acids 127–619) and Asef-ABR (amino acids 73–126) fused to GFP were generated as described previously (9). HA-tagged full-length Asef (Asef-full) and Asef- $\Delta$ APC were subcloned into pcDNA3.1 (Invitrogen), respectively. Mutants of the PH domain, Asef-K444L/R448L and Asef-W526L, were generated using PCR mutagenesis and cloned into pcDNA3.1 and pGEX (Amersham Biosciences). Myc-tagged full-length APC and APC-1309 (amino acids 1–1309) were subcloned into pCS2. Myc-tagged p85DN subcloned in pcDNA3 was obtained from T. Hirano (Osaka University School of Medicine, Osaka, Japan) (22). Mouse monoclonal antibody (mAb) to APC was raised against a peptide containing amino acids 119–250 of APC. Rabbit polyclonal antibodies (pAb) to Asef were prepared by immunizing rabbits with a peptide containing amino acids 1–113 or 485–605 of Asef, and used for immunoblotting, immunostaining, and immunoprecipitation. Antibodies were purified by affinity chromatography using columns to which the antigens used for immunization had been linked. Mouse mAb to Myc tag (9E10) and rabbit pAb to GST were obtained from Santa Cruz Biotechnology. Rabbit pAb to Myc-tag (polyclonal version of 9E10) was obtained from MBL. Rat mAb against HA tag (3F10) and mouse mAb against  $\alpha$ -tubulin were from Roche Applied Science and Oncogene Research, respectively. pAbs to Akt and phospho-Akt (Ser-473) were from Cell Signaling Technology.

**Immunostaining**—Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline. Cells were double stained with anti-APC mAb and anti-Asef pAb, anti-Myc mAb, and anti-Asef pAb or anti-Myc pAb and anti-APC mAb and stained with anti-Myc mAb or anti-HA mAb for 60 min at room temperature. Staining patterns obtained with these antibodies were visualized by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG, anti-rat IgG, or anti-mouse IgG, or Alexa Fluor 594-conjugated goat anti-rabbit IgG or anti-mouse IgG for 60 min at room temperature. Cells were photographed with a Carl Zeiss LSM510 laser microscope.

**Immunoprecipitation, Immunoblotting, and Protein Lipid Overlay Assays**—Immunoprecipitation, immunoblotting, and protein lipid overlay assays were performed as described previously (10, 19).

**Short Hairpin RNA (shRNA)**—DNA oligonucleotides encoding shRNAs were subcloned into the U6 promoter vector pSHAG-1. The sequences of the region in the human APC and Asef cDNA targeted for shRNAs were 5'-AACTGAGGCATCTAATATGAAGGAAGTACTT-3' and 5'-CCAAAAGCCAGCAGCGAATGTTCTTCTCTT-3', respectively. The sequences of mut-shRNA-APC and mut-shRNA-Asef were 5'-AACTAAGGCATATAATATGAAGGAAATACTT-3' and 5'-CCAAAAGCCAGAAGCGACTGTTCTGTCTCTT-3', respectively.

**Cell Migration Assays**—Cell migration assays were performed using Transwell migration chambers (Costar) as described previously (10). In brief, after 24 h of transfection, HeLa cells ( $2.0 \times 10^4$  cells per well) were resuspended in normal medium and added to the upper compartment of the Transwell chamber and allowed to migrate to the underside of the top

chamber for 4 h. The top chamber was coated by spotting fibronectin (100 ng) in 10  $\mu$ l of phosphate-buffered saline onto the underside of the filter membrane, allowed to air-dry, and both sides of the membrane were then coated with 10  $\mu$ g/ml collagen type I (Koken) overnight. HGF was added to the lower chamber at 20 ng/ml. Cells transfected with pSHAG-1 expressing shRNA-APC or shRNA-Asef were cultured for 72 h before use in migration assays. Cell migration was determined by counting the cells that migrated to the lower side of the polycarbonate filters.

**Statistic Analysis**—Statistic analysis was performed using the Student's *t* test. A *p* value of <0.05 was considered statistically significant.

## RESULTS

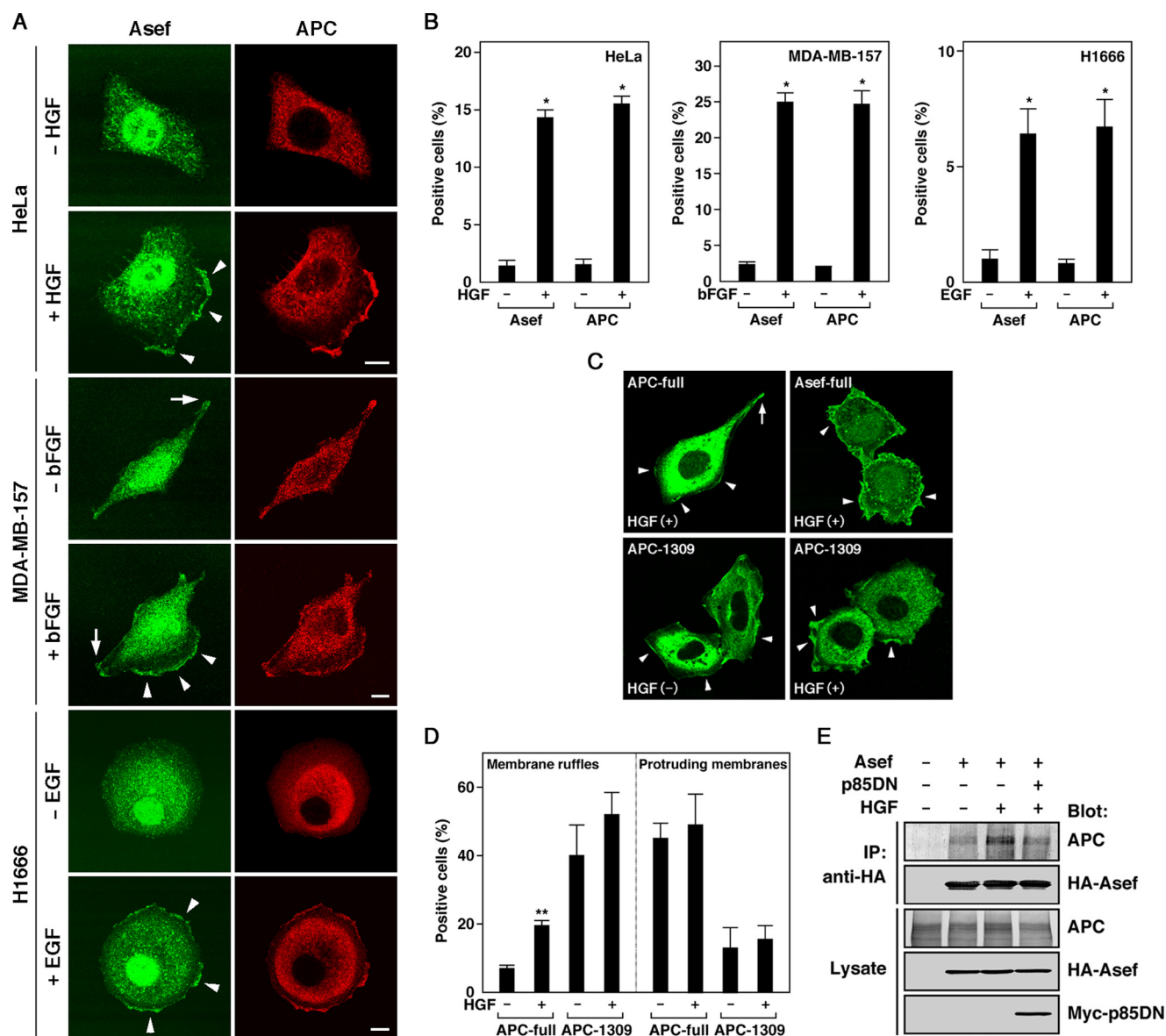
**HGF, bFGF, and EGF Induce the Colocalization of APC and Asef in Membrane Ruffles**—APC has been detected at a number of intracellular sites, but the bulk of APC resides in the cytoplasm (23). The most striking feature of APC localization is its accumulation in clusters near the distal ends of microtubules at the edges of migrating epithelial cells (13). APC is also reported to accumulate at lamellipodia due to an interaction with IQGAP1 in migrating cells (14). To examine the possible regulation of APC and Asef by HGF, we examined whether the distribution of APC and Asef within HeLa cells is altered in response to HGF treatment. Immunostaining of HeLa cells with anti-APC and anti-Asef antibodies revealed that APC and Asef are localized mainly in the cytoplasm in the absence of HGF stimulation (Fig. 1, *A* and *B*). When cells were treated with HGF, APC and Asef were observed to accumulate and colocalize in membrane ruffles and lamellipodia. Consistent with this result, when APC and Asef were exogenously expressed in HeLa cells, both proteins were found to accumulate in membrane ruffles and lamellipodia in the presence of HGF stimulation (Fig. 1, *C* and *D*). Ectopically expressed wild-type APC also accumulated efficiently at the tips of membrane protrusions, but accumulation in these regions was not significantly altered by HGF treatment.

Consistent with these results, *in vivo* pull-down experiments revealed that the amounts of APC co-immunoprecipitating with Asef increased when HeLa cells were treated with HGF (Fig. 1*E*), suggesting that HGF stimulation increases the amounts of APC-Asef complex in the cells.

We also examined whether the subcellular distribution of APC and Asef is changed in response to bFGF or EGF treatment. In these experiments, we used three different cell lines, HeLa, MDA-MB-157, and H1666, as each responds better to different growth factors: HGF, bFGF, and EGF, respectively. We found that bFGF stimulation of MDA-MB-157 cells and EGF stimulation of H1666 cells induced the accumulation and colocalization of APC and Asef in membrane ruffles (Fig. 1, *A* and *B*).

**Subcellular Localization of Truncated APC and Asef in Colorectal Tumor Cells**—It has been reported that more than 80% of the APC mutations in sporadic tumors reside in its central region, termed the mutation cluster region (MCR, codons 1286–1513) (24). These mutations typically result in the generation of truncated APC proteins that lack binding sites for

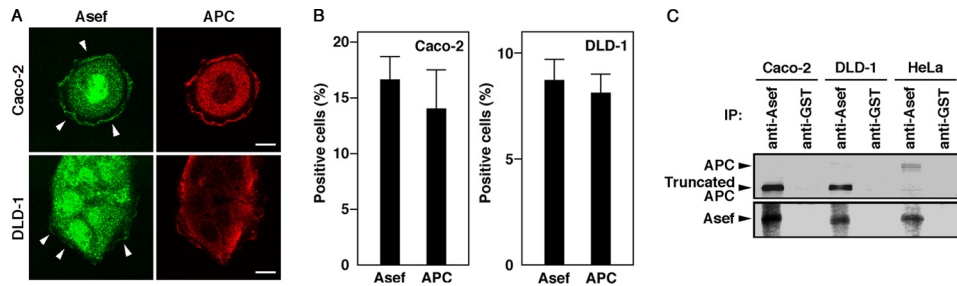
## APC/Asef and HGF-induced Cell Migration



**FIGURE 1. Subcellular localization of APC and Asef.** *A*, colocalization of endogenous APC and Asef in HeLa, MDA-MB-157, and H1666 cells treated with HGF, bFGF, and EGF, respectively. After stimulation with HGF (20 ng/ml for 30 min), bFGF (50 ng/ml for 15 min), or EGF (100 ng/ml for 30 min), cells were double stained with anti-APC and anti-Asef antibodies. *Arrowheads* indicate the areas of colocalization of APC and Asef in membrane ruffles. *Arrows* point to the clusters of APC and Asef at the tips of the membrane protrusions. *Scale bar*, 20  $\mu$ m. *B*, cells with APC or Asef concentrated in membrane ruffles were scored as a percentage of the total number of HeLa, MDA-MB-157, and H1666 cells ( $n > 100$ ). Results are expressed as the mean  $\pm$  S.E. of three independent experiments. \*,  $p < 0.05$ . *C*, comparison of subcellular localization of exogenously expressed APC, APC-1309, and Asef in HeLa cells. HeLa cells were transfected with expression plasmids encoding Myc-tagged APC, APC-1309, or HA-tagged Asef, and were treated with HGF. Cells were stained with antibodies against Myc or HA. *Arrowheads* indicate ruffling membranes. The *arrow* points to the cluster of APC at the tip of the membrane protrusion. *D*, cells with APC or APC-1309 concentrated in membrane ruffles or protruding membranes were scored as a percentage of the total number of transfected cells ( $n > 120$ ). Results are expressed as the mean  $\pm$  S.E. of three independent experiments. \*\*,  $p < 0.01$ . *E*, HGF-stimulated interaction of Asef with APC. HeLa cells were transfected with the indicated plasmids. *Upper two panels*, lysates prepared from HGF-stimulated and unstimulated cells were immunoprecipitated with anti-HA antibody, then were fractionated by SDS-PAGE, and immunoblotted with the indicated antibodies. *Lower three panels*, lysates used in the *upper two panels* were subjected to immunoblotting with the indicated antibodies. p85DN, the dominant-negative mutant of PI3-kinase.

EB1, microtubules, and hDLG, and at least some of the sites for  $\beta$ -catenin and Axin/Conductin. In contrast, the region of APC responsible for binding to Asef, the armadillo repeat domain, is retained in most APC mutants. Therefore, we next examined the subcellular distribution of truncated mutant APCs and Asef expressed in colorectal tumor cells. When the colorectal tumor Caco-2 and DLD-1 cell lines were immunostained with anti-APC and anti-Asef antibodies, APC and Asef were found to accumulate and colocalize in membrane ruffles and lamellipodia as well as the cytoplasm (Fig. 2, *A* and *B*). Consistent with

these results, *in vivo* pull-down assays revealed that truncated mutant APCs found in Caco-2 and DLD-1 cells co-immunoprecipitated with Asef (Fig. 2*C*), similar to wild-type APC expressed in HeLa cells. We next created a plasmid vector expressing a truncated mutant APC typically found in colorectal tumors, APC-1309, and exogenously expressed this in HeLa cells. We found that APC-1309 accumulated in membrane ruffles and lamellipodia of HeLa cells. Accumulation of APC-1309 in HeLa cells not treated with HGF was greater than that of wild-type APC in HGF-treated cells (Fig. 1, *C* and *D*). On the



**FIGURE 2. Subcellular localization of truncated APC and Asef in colorectal tumor cells.** *A*, colocalization of endogenous truncated APC and Asef in Caco-2 and DLD-1 cells. Cells were double stained with anti-APC and anti-Asef antibodies. Arrowheads indicate the areas of colocalization of truncated APC and Asef in membrane ruffles. Scale bar, 20  $\mu$ m. *B*, cells with APC or Asef concentrated in membrane ruffles were scored as a percentage of the total number of colorectal tumor cells, respectively ( $n > 100$ ). Results are expressed as the mean  $\pm$  S.E. of three independent experiments. *C*, association of Asef with truncated APC mutants in Caco-2 and DLD-1 cells. Lysates prepared from Caco-2, DLD-1 and HeLa cells were immunoprecipitated with anti-Asef or anti-GST antibody, separated by 6% SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies. Anti-GST antibody was used as a negative control. In Caco-2 and DLD-1 cells, APC is mutated at codons 1,416 and 1,367, respectively.

other hand, APC-1309 accumulated at the tips of membrane protrusions less efficiently than wild-type APC.

**APC and Asef Are Required for HGF-induced Cell Migration**—It is known that HGF controls cell migration in embryogenesis and induces the invasion and metastasis of tumor cells (20, 21). We therefore examined whether APC and Asef are involved in HGF-induced cell migration. Assays using Transwell migration chambers showed that treatment of HeLa cells with HGF significantly stimulated cell migration (Fig. 3A). To investigate the significance of the interaction between Asef and APC in cell migration, we used plasmid constructs expressing the APC-binding region of Asef (Asef-ABR). Asef-ABR acts as a dominant-negative mutant competing with Asef for binding to APC (10). When Asef-ABR was expressed in HeLa cells, the effects of HGF on cell motility decreased to about 50% of that of vector-transfected cells (Fig. 3A). Thus, the interaction between APC and Asef may be required for HGF-induced cell migration.

To further confirm these results, we expressed the shRNAs, shRNA-APC and shRNA-Asef, designed to suppress the expression of APC and Asef, respectively, and examined their effects on HGF-induced migration of HeLa cells. As controls, we used shRNAs containing point mutations in these sequences: mut-shRNA-APC and mut-shRNA-Asef. Immunoblotting analysis revealed that expression of APC and Asef was almost completely inhibited in shRNA-APC and -Asef-transfected cells but not in mut-shRNA-APC and -Asef-transfected cells, respectively (Fig. 3B). As we found previously (10), cells expressing either shRNA-APC or -Asef showed decreased motility compared with cells expressing mut-shRNA-APC or -Asef (Fig. 3C). When cultured in the presence of HGF, the migratory activity of cells expressing shRNA-APC or -Asef was not stimulated as efficiently as cells expressing mut-shRNA-APC or -Asef. These results suggest that APC and Asef play important roles in HGF-induced cell migration.

In addition, we examined the effect of HGF on cells transfected with Asef- $\Delta$ APC, a mutant that lacks the NH<sub>2</sub>-terminal ABR and possesses stronger migration stimulatory activity than wild-type Asef (10). HGF was much more effective in stimulating the migration of cells expressing Asef- $\Delta$ APC compared with control cells (Fig. 3A).

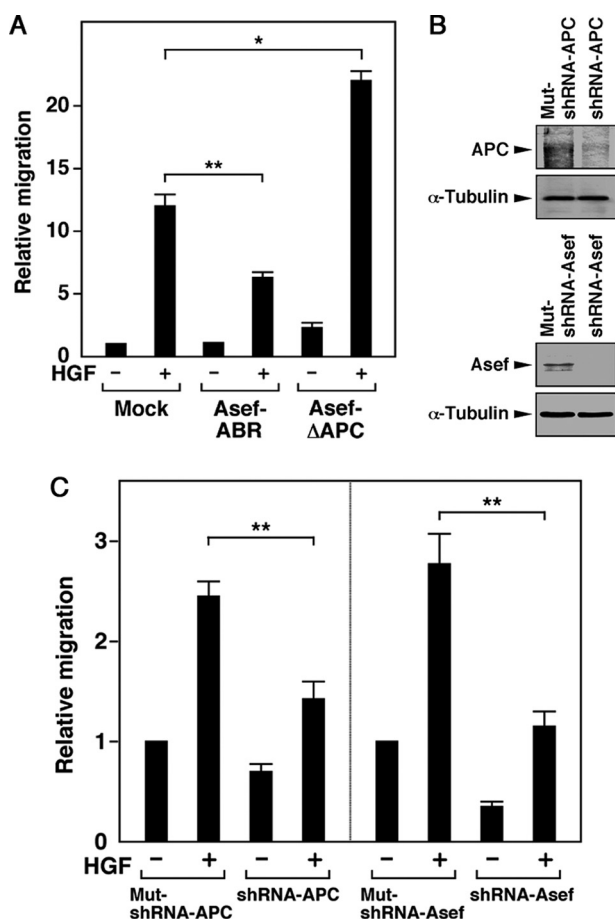
**PI3-kinase Functions Upstream of APC and Asef in HGF Signaling**—It is well known that PI3-kinase is essential to signaling initiated by growth factors that act through receptor-tyrosine kinases (20, 21, 25). Indeed, when a dominant-negative PI3-kinase mutant (p85DN) was overexpressed in HeLa cells, HGF-induced cell migration was significantly inhibited (Fig. 4A). Under these conditions, HGF-induced phosphorylation of Akt was almost completely inhibited (Fig. 4B). However, p85DN did not show any inhibitory effect on cells expressing Asef- $\Delta$ APC (Fig. 4A), suggesting that Asef may function

downstream of PI3-kinase. *In vivo* pull-down assays showed that the amount of the APC-Asef complex is not increased significantly in response to HGF treatment in cells expressing p85DN (Fig. 1E). This suggests that PI3-kinase is required for HGF-induced formation of the APC-Asef complex. Furthermore, cells expressing p85DN did not exhibit HGF-induced accumulation of APC and Asef in membrane ruffles and lamellipodia (Fig. 4C). Also, treatment of HeLa cells with a PI3-kinase inhibitor, LY294002, resulted in an inhibition of HGF-induced cell migration and accumulation of APC and Asef in membrane ruffles (data not shown). These results suggest that PI3-kinase functions upstream of APC and Asef in the HGF signaling pathway. In addition, MDA-MB-157 and H1666 cells expressing p85DN did not show any bFGF- or EGF-induced accumulation of APC and Asef in membrane ruffles, respectively (Fig. 4, D and E). Thus, PI3-kinase may also function upstream of APC and Asef in the bFGF and EGF signaling pathways.

**The PH Domain of Asef Is Required for HGF-induced Cell Migration**—PH domains are known to mediate membrane localization of many proteins by binding to membrane lipids, in particular the products of PI3-kinase, phosphatidylinositol 3,4-bisphosphate (PIP<sub>2</sub>), and phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) (26, 27). We previously showed that the PH domain of Asef binds to PIP<sub>3</sub> and targets Asef to the cell-cell adhesion sites in MDCK II cells (19). To further investigate the role of the Asef PH domain, we created expression vectors encoding Asef-K444L/R448L or Asef-W526L. Asef-K444L/R448L is a mutant in which Lys-444 and Arg-448 of Asef are replaced with Leu, while Asef-W526L is a mutant in which Trp-526 is replaced with Leu. Studies of the three-dimensional structures of several PH domains suggest that Lys-444 and Arg-448 of Asef interact directly with PIPs (28, 29). Alteration of Trp-526 in Asef is expected to have a global effect on PH domain folding and function. Consistent with these expectations, lipid dot-blot assays revealed that the mutant PH domains, expressed as GST fusion proteins, were defective in PIP<sub>3</sub> binding activity (Fig. 5).

As described above (Fig. 1C), when Asef was overexpressed in HeLa cells, Asef accumulated in membrane ruffles and lamellipodia regardless of the presence or absence of HGF (Fig. 6A).

## APC/Asef and HGF-induced Cell Migration



**FIGURE 3. Involvement of APC and Asef in HGF-induced cell migration.** *A*, HeLa cells were transfected with expression plasmids indicated in the figure. Cells were added to the upper compartment of a Transwell chamber and allowed to migrate to the underside of the top chamber for 4 h in the presence or absence of HGF. Cell migration was determined by counting the cells that had migrated to the lower side of the polycarbonate filters. Transfection efficiency was routinely > 80%. Results are expressed as the mean  $\pm$  S.E. of four independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . *B*, suppression of APC and Asef expression by shRNAs. HeLa cells were transfected with expression vectors containing oligonucleotides encoding shRNA-Asef or -APC, or mut-shRNA-Asef or -APC, respectively. Lysates prepared from transfected cells were subjected to immunoblot analysis with anti-APC or -Asef antibodies. Anti- $\alpha$ -tubulin antibody was used as a control. *C*, migration of HeLa cells expressing shRNAs. Cells were transfected with expression vectors containing oligonucleotides encoding shRNA-Asef or -APC, or mut-shRNA-Asef or -APC, respectively, and subjected to migration assays using Transwell migration chambers. Cells were allowed to migrate for 4 h in the presence or absence of HGF. Results are expressed as the mean  $\pm$  S.E. of six independent experiments. \*\*,  $p < 0.01$ .

However, Asef-K444L/R448L, when overexpressed in HeLa cells, did not localize to membrane ruffles or lamellipodia, even in the presence of HGF (Fig. 6A). Furthermore, cells transfected with Asef-W526L did not show any obvious morphological change in the presence of HGF, and Asef-W526L displayed a granular distribution in the cytoplasm. These results suggest that the PH domain of Asef is critical for HGF-induced membrane ruffles and lamellipodia formation.

We next examined the migratory behavior of HeLa cells transfected with these mutant Asefs. When wild-type Asef was overexpressed, cells showed a statistically significant increase in their migratory activities, irrespective of HGF treatment (Fig. 6B). In contrast, overexpression of Asef-K444L/R448L and Asef-W526L did not induce cell migration (Fig. 6B). Also, Asef-

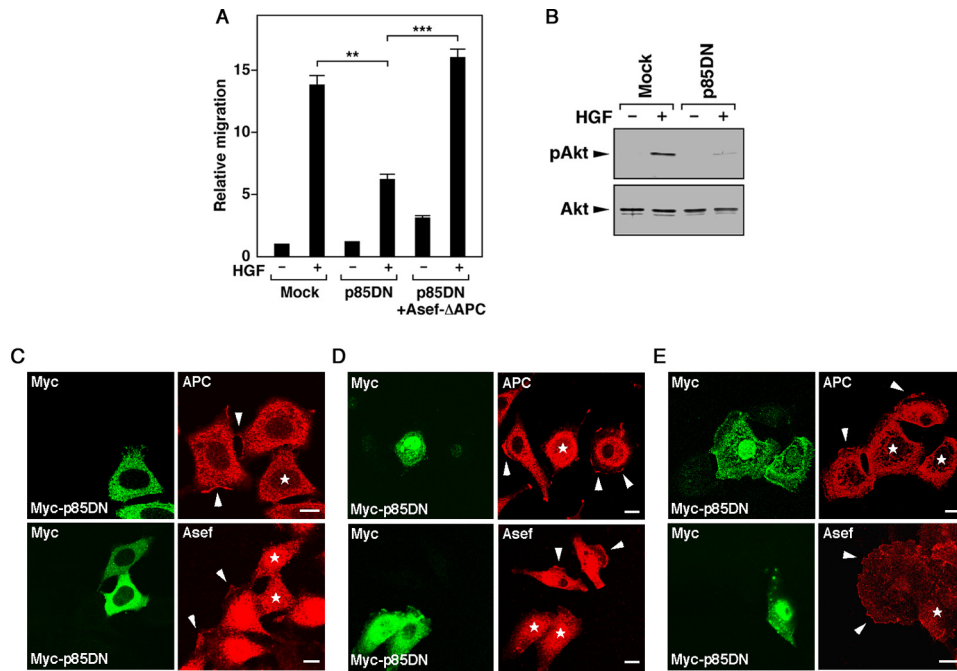
K444L/R448L did not further enhance the migratory activity of HGF-treated cells. Furthermore, cells expressing Asef-W526L exhibited decreased motility compared with control cells, suggesting that Asef-W526L may act as a dominant-negative mutant of endogenous Asef. In these cells, Asef-K444L/R448L and Asef-W526L were expressed at levels comparable with that of wild-type Asef (data not shown). Thus, the PH domain of Asef may be important for HGF-induced cell migration.

## DISCUSSION

APC has been characterized most extensively in the context of the Wnt signaling pathway and has been shown to function as a negative Wnt regulator in the absence of Wnt signaling (2–6). In the present study, we have shown that APC and Asef function downstream of HGF. We found that HGF induces the accumulation and colocalization of APC and Asef in membrane ruffles and lamellipodia of epithelial cells. In addition, we found that bFGF and EGF induce changes similar to HGF in the localization of APC and Asef. Furthermore, we found that APC and Asef are involved in HGF-mediated cell migration. It is known that multiple GEFs such as Vav, Tiam1, and Sos function as downstream components of signaling pathways that link growth factors to Rac1 and Cdc42 activation (30–32). It would therefore be intriguing to examine whether Asef and other GEFs function in the same pathway or function independent of one another.

PI3-kinase is known to play critical roles in signaling pathways initiated by HGF, bFGF, and EGF (20, 21, 25). Consistent with this, cells expressing p85DN did not show any HGF-, bFGF-, or EGF-induced accumulation of APC and Asef in membrane ruffles. Furthermore, expression of p85DN resulted in the inhibition of HGF-induced cell migration. LY294002 also inhibited HGF-induced cell migration and accumulation of APC and Asef in membrane ruffles. In addition, p85DN did not show any inhibitory effect on cells expressing Asef- $\Delta$ APC. Thus, PI3-kinase may play a critical role upstream of APC and Asef in the HGF signaling pathway.

Consistent with this notion, we previously found that the PH domain of Asef binds to PIP3, a product of PI3-kinase, and targets Asef to the cell-cell adhesion sites in MDCK II cells (19). This finding suggests that Asef localizes to the membrane, where its GTPase substrates are located, by binding to PIP3 via its PH domain. In this study, we examined Asef mutants that are unable to interact with PIP3 and showed that the PIP3 binding activity of the PH domain is important for HGF-induced membrane ruffles and lamellipodia formation. We further demonstrated that this activity is required for HGF-induced cell migration. It has been reported that the PH domain of some GEFs interacts not only with lipids but also with other proteins. For example, Dbl binds via its PH domain to ezrin, a protein involved in cell migration, morphogenesis, and adhesion, and this binding is required for its proper subcellular localization (33). Trio interacts with the actin filament cross-linking protein filamin through its PH domain, and this interaction is required for membrane ruffle formation (34). It is therefore possible that the PH domain of Asef binds not only to PIP3 but also to other proteins that are critical for its function. In fact, the motility of cells expressing Asef-W526L, a mutant whose PH domain



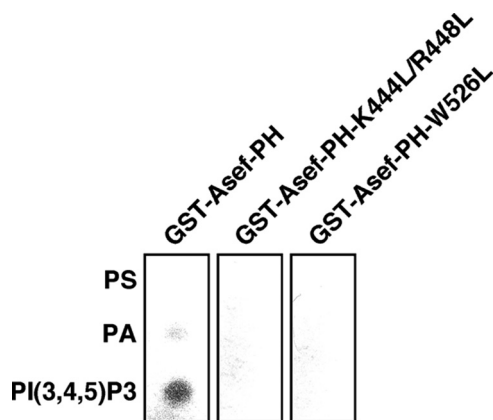
**FIGURE 4. Involvement of PI3-kinase in APC/Asef-mediated cell migration.** *A*, effects of p85DN on HGF-induced cell motility. Cells were transfected with the expression plasmids indicated in the figure. Cells were added to the upper compartment of the Transwell chamber and allowed to migrate to the underside of the top chamber for 4 h in the presence or absence of HGF. Results are expressed as the mean  $\pm$  S.E. of four independent experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . *B*, suppression of HGF signaling by p85DN. HeLa cells transfected with p85DN were stimulated with HGF for 10 min. Cell lysates were separated by SDS-PAGE and immunoblotted with anti-phospho-Akt (Ser-473) antibody. Equal loading of Akt protein was confirmed by immunoblotting analysis using anti-Akt antibody. *C–E*, p85DN inhibits the growth factor-induced accumulation of APC and Asef in membrane ruffles. HeLa (*C*), MDA-MB-157 (*D*), and H16666 (*E*) cells were transfected with expression plasmids encoding Myc-tagged p85DN. After stimulation with HGF, bFGF, or EGF for 30 min, cells were stained with antibodies against Myc and APC or Asef. p85DN-expressing cells are indicated with asterisks. Arrowheads indicate APC and Asef localization in membrane ruffles in non-transfected cells. Scale bar, 20  $\mu$ m.

It is well known that APC is transported along microtubules in a kinesin-2-dependent manner (8, 12, 13). Thus, it is possible that kinesin-2-dependent transport of APC along microtubules may be involved in HGF-induced accumulation of APC and Asef in membrane ruffles. On the other hand, Asef appears not to be transported along microtubules.<sup>3</sup> It is therefore tempting to speculate that APC transported to membrane ruffles may form a complex with Asef and activate its GEF activity, and thereby activate cell migration. Consistent with this, we found that the amount of the APC-Asef complex is increased in a PI3-kinase-dependent manner when cells were treated with HGF. Because APC is known to be a substrate of several protein kinases such as GSK-3 $\beta$  and casein kinase I (35, 36), it is possible that phosphorylation of APC may be involved in these processes. Although Asef is also known to be phosphorylated by Src-family kinases (37), Asef tyrosine phosphorylation is not required for EGF-induced accumulation in membrane ruffles and lamellipodia, association

with APC, or enhancement of its GEF activity. Further elucidation of the molecular mechanisms by which APC and Asef accumulate in membrane ruffles and activate cell migration is underway in our laboratory.

We found that HGF elicited a much stronger migration stimulatory effect on cells expressing Asef- $\Delta$ APC than on control cells. This finding suggests that at least a part of the effect of HGF may be mediated through Asef, independent of APC. However, this notion appears to be contradicted by results showing that shRNA-APC and shRNA-Asef inhibit migration of HGF-treated cells to a similar extent. We speculate that APC may contribute to HGF-induced migration via both Asef-dependent and -independent mechanisms. In this regard, it is interesting to note that APC could bind directly to and bundle actin filaments (38) and can interact with IQGAP1, which is necessary for actin accumulation at leading edges and for directional migration (14). This issue needs to be investigated in future studies.

In conclusion, we have shown that APC and Asef function downstream of HGF and PI3-kinase. In addition, we have shown that bFGF and EGF also induce the accumulation and colocalization of APC and Asef in membrane ruffles and lamellipodia of epithelial cells. The receptors for HGF, bFGF, and

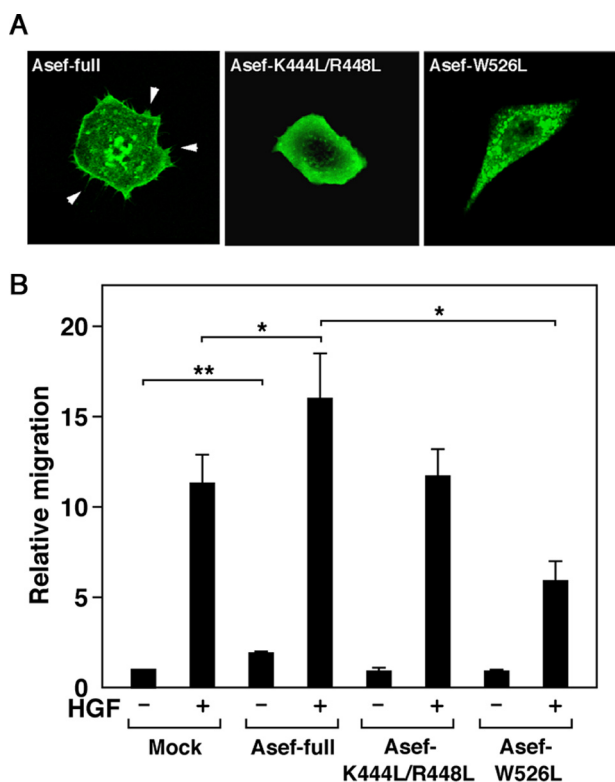


**FIGURE 5. PIP3 binding activity of mutant PH domains.** PIP Strips were incubated with GST-Asef-PH, GST-Asef-PH-K444L/R448L, or GST-Asef-PH-W526L, and proteins bound to lipids were detected with anti-GST antibody. PS, phosphatidylserine; PA, phosphatidic acid.

structure is disrupted, was suppressed to a greater extent than that of cells expressing Asef-K444L/R448L, a mutant in which amino acid residues involved in lipid binding are mutated. In addition, it has also been shown that the PH domain and the products of PI3-kinase are necessary to activate the catalytic activity of the DH domains of some GEFs. It remains to be investigated whether intramolecular interactions between the DH and PH domains regulate the GEF activity of Asef.

<sup>3</sup> T. Akiyama, unpublished observation.

## APC/Asef and HGF-induced Cell Migration



**FIGURE 6. The PH domain of Asef is important for HGF-induced cell migration.** *A*, morphology of HeLa cells expressing mutant Asefs that are unable to bind to PIP3. HeLa cells were transfected with expression plasmids encoding HA-tagged full-length Asef (amino acids 1–619), Asef-K444L/R448L (amino acids 1–619), or Asef-W526L (amino acids 1–619) and treated with HGF. Cells were stained with antibody against HA. Arrowheads indicate filopodia. *B*, migration of HeLa cells expressing Asef mutants. Cells were transfected with the expression plasmids indicated and assessed by migration assays. Results are expressed as the mean  $\pm$  S.E. of five independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

EGF are known to be overexpressed or mutated in various tumors (20, 39, 40). Furthermore, it has recently been reported that PI3-kinase is mutated in colon cancer, breast cancer and glioblastoma at relatively high frequencies (41–44). These findings raise the possibility that APC and Asef contribute to tumorigenesis, invasion and metastasis. Consistent with this notion, Asef has been reported to be mutated in breast cancers (45). It would be interesting to examine whether the mutations identified in breast cancers activate Asef function, and thereby contribute to tumorigenesis.

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