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PH domain-mediated membrane targeting of Asef

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Abstract

The APC-associated guanine nucleotide exchange factor (GEF) Asef regulates cell morphology and migration. Asef contains a pleckstrin homology (PH) domain in addition to Dbl homology (DH), APC-binding (ABR), and Src homology 3 (SH3) domains. Here we show that the PH domain of Asef binds to phosphatidylinositol 3,4,5-trisphophate [PtdIns(3,4,5)P3] and targets Asef to the cell–cell adhesion sites in MDCK II cells. Furthermore, we demonstrate that overexpression of Asef in MDCK II cells results in increases in the amounts of E-cadherin and the actin filaments at the sites of cell–cell contact. These results suggest that Asef is targeted via its PH domain to the cell–cell adhesion sites and is involved in the regulation of cell adhesion.

Keywords: APC; Asef; PH domain; GEF; G-protein; E-cadherin

Asef is a guanine nucleotide exchange factor (GEF) for the Rho family of small GTPases and contains Dbl homology (DH), Pleckstrin homology (PH), and Src homology 3 (SH3) domains [1]. Asef also possesses a region that binds to the tumor suppressor APC (APC-binding region; ABR). This interaction activates the GEF activity of Asef, and thereby promotes reorganization of the actin cytoskeletal network and affects cell morphology and migration. Furthermore, truncated mutant APCs present in colorectal tumor cells activate Asef constitutively and contribute to their aberrant migratory properties [2,3].

It has been shown that PH domains determine the subcellular localization and/or activity of PH domain-containing proteins by interacting with phosphatidylinositol phosphate [4–10]. For example, the PH domain of Dbl binds to PtdIns(4,5)P2 and PtdIns(3,4,5)P3, and these interactions modulate the GEF activity and subcellular localization of Dbl [11]. In the present study, we have examined the functional significance of the PH domain of Asef. We show here that the PH domain of Asef binds to PtdIns(3,4,5)P3 and targets Asef to the cell–cell adhesion sites in MDCK II cells.

Materials and methods

Expression vectors and antibodies. HA-tagged full-length Asef (Asef-full), Asef-ΔAPC (amino acids 127–619), Asef-PH (amino acids 419–552), and Asef-ΔPH (amino acids 1–459) were subcloned into pcDNA3.1 (Invitrogen), respectively. Rat monoclonal antibody (mAb) to E-cadherin (ECCD-2) and mouse mAb to hemagglutinin (HA)-tag (12CA5) were obtained from Calbiochem and Boehringer, respectively. For immunoblotting, mouse mAb to E-cadherin and α-tubulin were from Transduction Laboratories and Oncogene Research, respectively. Rabbit polyclonal antibody (pAb) to GST was from Sigma.

Protein–lipid overlay assay. Protein–lipid overlay assays were performed using PIP strips™ (Echelon) following the manufacturer's instruction. Briefly, PIP strips™ were blocked in 3% fatty acid-free BSA in TBS-T (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) at room temperature for 1 h. The strips were then incubated with 2.0 μg/ml of GST-Asef-PH (amino acids 423–555) overnight in the dark at 4 °C. Then the strips were washed three times in TBS-T and incubated overnight

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with anti-GST antibody at 4 °C. The strips were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare Biosciences) for 1 h at room temperature. The signals were detected by enhanced chemiluminescence (ECL) (GE healthcare biosciences).

Cell culture and transfection. MDCK II cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Plasmids were transfected into these cells using Lipofect-AMINE 2000 (Invitrogen).

Adenovirus infection. Adenovirus infection was performed as described [2]. In brief, cells were plated in 6-well tissue culture plates (1.2×10^6 cells per well). After 1.5 h of incubation at 37 °C, cells were infected with adenoviruses (MOI = 200–300), cultured for 24 h and then subjected to immunostaining and immunoblotting. Immunofluorescence staining showed that infection efficiency was >90%.

Immunostaining. After 48 h of transfection or 24 h of adenovirus infection, MDCK II cells were fixed with 3.7% formaldehyde in PBS. Fixed cells were double-stained with mouse mAb to HA and trimetylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Molecular Probes) or rat mAb to E-cadherin and TRITC-conjugated phalloidin for 1 h at room temperature. Staining patterns obtained with anti-HA mAb and anti-E-cadherin mAb were visualized with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG or anti-rat IgG for 60 min at room temperature. The cells were photographed with a Carl Zeiss LSM510 laser scanning microscope.

Detergent extraction of E-cadherin. Detergent extraction of E-cadherin from MDCK II cells was performed as described [12]. Briefly, cells were plated in 12-well tissue culture plates (4.8×10^5 per well). After 24–36 h of adenovirus infection, cells were washed with HMF buffer (HCMF [Ca^{2+}/Mg^{2+}-free Hepes-buffered saline, pH 7.4] containing 2 mM $CaCl_2$), and 200 μl of 1.0% NP-40 in HMF buffer was added. Samples were incubated for 20 min on ice, and then centrifuged at 18,000*g* for 20 min. To the supernatant, 100 μl of 3× SDS-sample buffer was added and used as the detergent-soluble fraction. The pellet fraction was dissolved in 300 μl of 1× SDS-sample buffer and used as the detergent-insoluble fraction. E-cadherin and α-tubulin were detected by immunoblotting.

Immunoblotting. Immunoblotting was performed as described previously [2].

Results and discussion

To investigate whether the PH domain of Asef would bind to specific phospholipids, we performed a protein–lipid overlay assay using a recombinant fusion protein consisting of the PH domain of Asef and glutathione-*S*-transferase (GST-Asef-PH). As shown in Fig. 1, GST-Asef-PH strongly interacted with PtdIns(3,4,5)P3, whereas GST alone showed no specific interaction. These results suggest that Asef interacts with PtdIns(3,4,5)P3 via its PH domain in living cells.

We next examined the significance of the PH domain in membrane targeting of Asef. We generated expression constructs encoding HA-tagged Asef-full, Asef-ΔPH, Asef-PH, and Asef-ΔAPC. Asef-ΔPH is a mutant that lacks the carboxy-terminal region including the PH domain, while Asef-PH is a fragment containing the PH domain of Asef. Asef-ΔAPC is a mutant that lacks the amino-terminal APC-binding region ABR. Immunoblotting analysis with anti-HA antibody revealed that each protein of the expected molecular size was expressed when MDCK cells were transfected with each construct (data not shown).

When cells were transfected with Asef-full, exogenously expressed Asef was localized mainly at the lateral membranes and slightly in the cytoplasm (Fig. 2). Likewise, Asef-ΔAPC was also able to localize to the lateral plasma membrane (Fig. 2), suggesting that APC-binding activity is not essential for the membrane localization of Asef. By contrast, Asef-ΔPH did not localize to the sites of cell–cell contact but localized in the cytoplasm and nucleus. On the other hand, Asef-PH was specifically enriched at the sites of cell–cell contact. Thus, the PH domain is responsible for membrane localization of Asef in MDCK II cells.

Rac is known to regulate E-cadherin-mediated cell–cell contacts [12–15]. We therefore investigated the effects of Asef on the localization of E-cadherin and the actin filaments. When MDCK II cells were infected with adenoviruses encoding HA-tagged Asef-ΔAPC (Ad-Asef-ΔAPC) that possesses stronger GEF activity than wild-type Asef, the amounts of E-cadherin localized at the sites of cell–cell contact were prominently increased (Fig. 3A). Furthermore, the amounts of the actin filaments localized at the cell–cell contact sites were also markedly increased (Fig. 3A). By contrast, cells infected with adenoviruses encoding LacZ (Ad-LacZ) or HA-tagged Asef-ΔDH (Ad-Asef-ΔDH) that lacks the DH domain and does not possess GEF activity did not show any changes in the localization

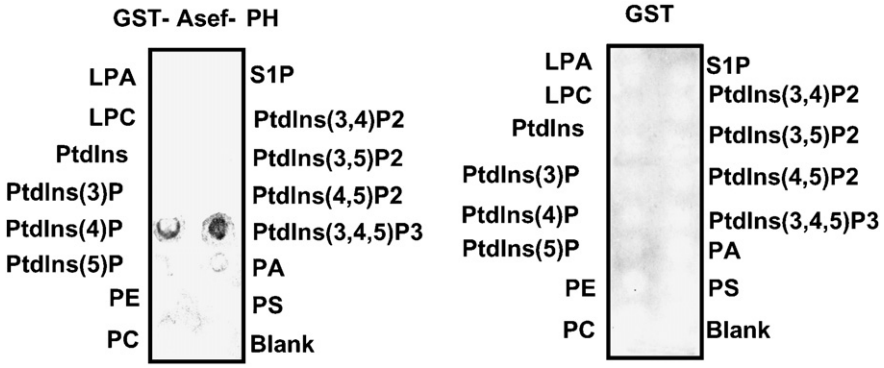

Fig. 1. The PH domain of Asef preferentially interacts with PtdIns(3,4,5)P3. PIP Strips™ were incubated with GST-Asef-PH (2.0 μg/ml) and proteins bound to lipids were detected with anti-GST antibody. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; LPA, lysophosatidic scid; LPC, lysophosphocholine; S1P, sphingosine-1-phosphate. GST protein was used as a control.

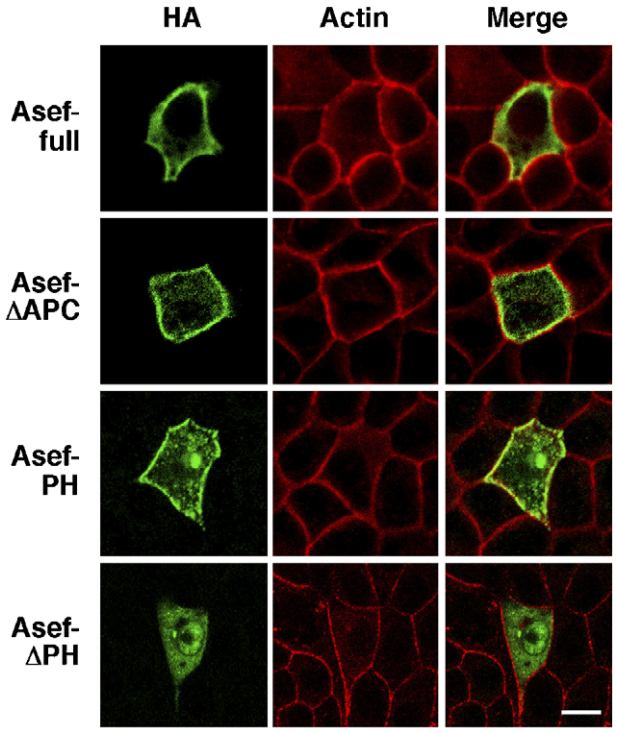

Fig. 2. Subcellular localization of HA-tagged Asef proteins in transfected MDCK II cells. MDCK II cells were transfected with expression plasmids encoding HA-tagged Asef-full, Asef-ΔAPC, Asef-PH or Asef-ΔPH. MDCK II cells were double-stained for Asef (anti-HA antibody) and actin (TRITC-phalloidin). Bar, 10 μm.

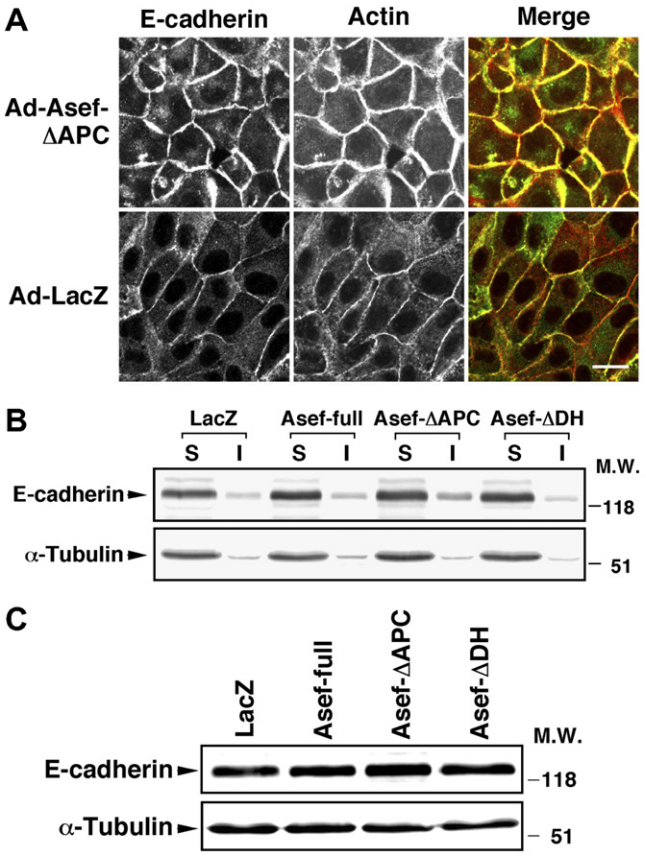

Fig. 3. Subcellular localization of actin and E-cadherin in MDCK II cells (A) MDCK II cells infected with the adenovirus encoding HA-tagged Asef-ΔAPC (Ad-Asef-ΔAPC) were double-stained for E-cadherin and actin (TRITC-phalloidin). As a negative control, LacZ adenovirus (Ad-LacZ) was used. Bar, 10 μm. (B) Detergent solubility of E-cadherin from MDCK II cells infected with the adenoviruses encoding HA-tagged Asef-full, Asef-ΔAPC, Asef-ΔDH or LacZ. MDCK II cells infected with indicated adenoviruses were lysed in a buffer containing 1.0% NP-40, and the amounts of E-cadherin in the detergent-soluble (S) and -insoluble (I) fractions were measured by immunoblotting with anti-E-cadherin mAb. Anti-α-tubulin antibody was used as a control. (C) Expression levels of E-cadherin in MDCK II cells infected with indicated adenoviruses. Anti-α-tubulin antibody was used as a control.

of E-cadherin and the actin filaments (Fig. 3A and data not shown). These results suggest that Asef may be involved in the formation of the E-cadherin-based cell–cell contacts and that the GEF activity of Asef is required for this function.

To confirm the results of immunostaining analysis, we examined the effects of Asef on the detergent solubility of E-cadherin. MDCK II cells infected with adenoviruses encoding wild-type or mutant Asefs were incubated with non-ionic detergent Nonidet P-40 (NP-40) and fractionated into NP-40-soluble and -insoluble fractions. Immunoblotting analysis revealed that the amounts of E-cadherin in the NP-40-insoluble fraction were increased in Ad-Asef-full or Ad-Asef-ΔAPC-infected MDCK II cells compared to Ad-Asef-ΔDH- or Ad-LacZ-infected MDCK II cells (Fig. 3B). Because the total amounts of E-cadherin expressed in these cells were almost similar (Fig. 3C), the increase in the amounts of E-cadherin at the cell–cell contact sites is not likely to be due to the Asef-mediated increase in E-cadherin expression levels. These results suggest that Asef increases the amounts of the actin cytoskeleton-associated E-cadherin in MDCK II cells.

We have shown here that the PH domain of Asef binds to PtdIns(3,4,5)P3 and targets Asef to the cell–cell adhesion sites in MDCK II cells. This finding is consistent with previous reports showing that PtdIns(3,4,5)P3 is mainly localized at the plasma membrane [16]. Furthermore, we showed that overexpression of Asef in MDCK II cells results in increases in the amounts of E-cadherin and the actin filaments at the sites of cell–cell contact. Thus, Asef may be involved in the regulation of cell adhesion. Because localization of Asef is regulated by the lipid product of PI3-kinase, it is interesting to speculate that growth factor-induced activation of PI3-kinase may cause PH domain-mediated membrane targeting of Asef. It is also possible that the GEF activity of Asef is regulated by PtdIns(3,4,5)P3 binding to its PH domain, like other Dbl family of GEF proteins such as Dbl and Vav [11,17]. More detailed analysis of the function of Asef PH domain is underway in our laboratory.

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