The Pleckstrin Homology Domain of the Arf6-specific Exchange Factor EFA6 Localizes to the Plasma Membrane by Interacting with Phosphatidylinositol 4,5-Bisphosphate and F-actin*

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The Arf6-specific exchange factor EFA6 coordinates membrane trafficking with actin cytoskeleton remodeling. It localizes to the plasma membrane where it catalyzes Arf6 activation and induces the formation of actin-based membrane ruffles. We have shown previously that the pleckstrin homology (PH) domain of EFA6 was responsible for its membrane localization. In this study we looked for the partners of the PH domain at the plasma membrane. Mutations of the conserved basic residues suspected to be involved in the binding to phosphoinositides redistribute EFA6-PH to the cytosol. In addition, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) breakdown also leads to the solubilization of EFA6-PH. Direct binding measured by surface plasmon resonance gives an apparent affinity of ~0.5 μM EFA6-PH for PI(4,5)P2. Moreover, we observed in vitro that the catalytic activity of EFA6 is strongly increased by PI(4,5)P2. These results indicate that the plasma membrane localization of EFA6-PH is based on its interaction with PI(4,5)P2, and this interaction is necessary for an optimal catalytic activity of EFA6. Furthermore, we demonstrated by fluorescence recovery after photobleaching and Triton X-100 detergent solubility experiments that in addition to the phosphoinositides, EFA6-PH is linked to the actin cytoskeleton. We observed both in vivo and in vitro that EFA6-PH interacts directly with F-actin. Finally, we demonstrated that EFA6 could bind simultaneously filamentous actin and phospholipids vesicles. Our results explain how the exchange factor EFA6 via its PH domain could coordinate at the plasma membrane actin cytoskeleton organization with membrane trafficking.

The ADP-ribosylation factor (Arf) family, which includes six isoforms, plays a key role in the intracellular vesicular transport (for review, see Ref. 1). Arf proteins are known to regulate the membrane recruitment to specific compartments of different protein coats necessary to generate membrane curvature and vesicle formation. In addition, they control the activation of phospholipid modifying enzyme also involved in membrane vesicle formation.

The specific function of each Arf isoform remains largely unknown. At least it results partly from the localization of each Arf isoform to specific intracellular compartment. The signals responsible for the membrane targeting of the soluble and highly diffusing inactive Arf proteins are the objects of intensive search. Honda et al. (2) have elegantly demonstrated that the Golgi localization of Arf1 was due to its interaction with membrin, an endoplasmic reticulum-Golgi SNARE (soluble N-ethyglycine factor attachment receptor) protein. Regarding Arf6, Claing et al. (3) have shown that the GDP-bound form binds to β-arrestin, an AP-2/clathrin adaptor involved in the internalization of G-protein-coupled receptors. This interaction is probably important for the function of Arf6 in G-protein-coupled receptor endocytosis.

Likewise, the binding of GDP-bound Arfs to their guanine nucleotide exchange factors (GEFs)2 is critical to localize the Arf proteins to their proper membrane (for review, see Ref. 4). The Arf specific GEF family, also referred to as the Sec7 family, comprises 15 members divided in two classes, the large and the small GEFs, which are responsible for the activation of 6 Arf isoforms. This larger number of GEFs suggests that each Arf is under the control of several ArfGEFs. Growing evidence indicates that the ArfGEFs are targeted to specific compartments where they will activate a cognate Arf. Little is known about how the large GEFs, GBF1, Big1, and Big2 are targeted to their working compartments, respectively cis-Golgi, endoplasmic reticulum-Golgi intermediate (5), and trans-Golgi network (6). In contrast, the small Arf-GEF family members (Arno/Cytohe-

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2 The abbreviations used are: GEF, guanine nucleotide exchange factors; PH, pleckstrin homology; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; GTPγS, guanosine 5’-3-((thio)triphosphate; GST, glutathione S-transferase; FRAP, fluorescence recovery after photobleaching; BHK, baby hamster kidney; PLC, phospholipase C; GFP, green fluorescent protein; EGFP, enhanced GFP.

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sin, EFA6, and BRAG/GEP100 subfamilies) contain a pleckstrin homology (PH) domain adjacent to the catalytic Sec7 domain that is required for their cellular localization. PH domains are structural modules of about 110 amino acids important for membrane association of proteins involved in cellular signaling, membrane trafficking, phospholipid modification, and cytoskeleton organization. Their structural architecture is particularly well conserved. They have a core of seven-stranded β-sandwich structure closed off at one end by a C-terminal α-helix. Several PH domains recognize particular phosphoinositides with high affinity. For example, the PH domain of PLC8 has been shown to interact strongly and specifically with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2). In addition to phosphoinositides, there exists evidence that some PH domains can mediate protein–protein interactions (7). For example, the PH domain of GRK2 interacts with both PI(4,5)P2 and Gβγ subunit of the heterotrimeric G-protein complex (8, 9). Regarding Brutons tyrosine kinase family proteins, their PH domains bind actin in addition to PI(3,4,5)P3 (10–13). In the case of Arno/Cytohesin isoforms, their PH domain has been shown to specifically interact with PI(4,5)P2 or PI(3,4,5)P3 according to the presence or not of a third glycerine in the B1/B2 loop (14). In addition to phosphoinositides, it has recently been demonstrated that the PH domain of Arno interacts also with the GTP-bound form of Arf6 and Arl4 proteins (15, 16).

EFA6 is an Arf6-specific exchange factor that coordinates endocytotic events with actin cytoskeleton reorganization (17). We have previously shown that the protein localizes essentially to the plasma membrane because of the presence of a PH domain; however, the nature of the targeting signals at the plasma membrane is unknown. In the present study we have determined how the cellular localization of EFA6 is regulated. We show that the EFA6-PH domain directly binds the F-actin and targets EFA6 at the plasma membrane into F-actin-enriched structures. Finally, we demonstrate that EFA6 could coordinate membrane trafficking with actin cytoskeleton organization.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Antibodies—Baby hamster kidney cells (BHK) were grown in BHK–21 medium (Invitrogen) containing 5% fetal calf serum, 10% tryptose phosphate broth, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. The following antibodies were used: mouse monoclonal antibody (mAb) against VSV-G epitope (clone P5D4, Roche Diagnostics), rabbit antiserum against GFP (Clontech, Takara Bio Europe), anti-actin mAb AC40 (Sigma), and anti-Arf6 mAb 8A6.2 (provided by S. Bourgoin, Sainte-Foy, Canada), Texas Red-conjugated phalloidin was from Molecular Probes, and fluorescein isothiocyanate and Texas Red-conjugated antibodies were from Jackson ImmunoResearch. Azolecin, ionomycin, unlabeled nucleotides, cytochalasin D, and egg phosphatidylcholine were from Sigma. Liver phosphatidylethanolamine, brain phosphatidylserine, liver phosphatidylinositol (PI), brain phosphatidylinositol 4-phosphate, PI(4,5)P2, and phosphatidylinositol 3,4,5-bisphosphate (PI(3,4,5)P3) were from Avanti Polar Lipids (Birmingham, AL), [35S]GTPγS was from PerkinElmer Life Sciences.

Preparation of Phospholipid Vesicles—Large unilamellar vesicles of azolecin were prepared as described elsewhere (18) and extruded through a 0.4 μm pore size polycarbonate filter (Isopore, Millipore). Vesicles of defined composition were prepared as previously described (19).

Expression and Purification of Recombinant Proteins—Myristoylated Arf6 wild-type (Myr-Arf6) was expressed and purified as previously described (20, 21). Recombinant EFA6.A (referred in the study as EFA6) with an N-terminal hexahistidine (His-EFA6) tag was prepared according to the manufacturer’s instructions (Qiagen). The isolated PH (residues 351–522) and PH + C (351–645) protein domains of EFA6.A fused to GST were purified from bacteria according to manufacturer’s instructions (GE Healthcare). Except for the GST–PH protein, which was kept at 4 °C and used within 2 days after purification, all the other proteins were dialyzed against 20 mm Tris-HCl, pH 7.5, 1 mm dithiothreitol, 100 mm NaCl, 1 mm MgCl2, and 20% glycerol, frozen on liquid nitrogen, and stored at −80 °C.

[35S]GTPγS Binding Assay—Myr-Arf6 (0.8 μM) was incubated at 30 °C with [35S]GTPγS (10 μM, ~2000 cpm/pmol) in 50 mm Tris/HC1, pH 8.0, 1 mm MgCl2, 1 mm dithiothreitol, 120 mm NaCl with or without His-tagged EFA6 (20 nm), and 0.5 mg/ml vesicles made of equal proportions of neutral phospholipids phosphatidylincholine and phosphatidylethanolamine, 30% phosphatidylserine, and supplemented or not with 2% of various phosphoinositides. At the indicated times aliquots of 25 μl were pipetted out and filtered, and the radioactivity was measured as described previously (20, 21).

To measure the effect of F-actin on EFA6 nucleotide exchange activity (Fig. 7), myr-Arfl6 (0.8 μM) was incubated at 37 °C with [35S]GTPγS (10 μM, ~2000 cpm/pmol) in 50 mm Tris-HCl, pH 8.0, 1 mm MgCl2, 1 mm dithiothreitol, 20 mm NaCl, 80 mm KCl, 0.1 mm EGTA, 50 μM CaCl2, 0.5 mg/ml azolecin vesicles with or without His-EFA6 (20 nm), and F-actin (3 μM).

Fluorescence Recovery after Photobleaching—Lateral diffusion coefficients and the mobile fraction were measured by FRAP using the Zeiss LSM Meta 510 confocal microscope as previously described (22). FRAP recoveries were acquired at room temperature on GFP-EFA6-PH or GFP-PLC81–PH-expressing BHK cells plated on glass coverslips. For cytochalasin D experiments, cells were incubated for 1 h 30 min at 37 °C in the presence of 2 μM cytochalasin D in BHK–21 medium then replaced by fresh medium for FRAP experiments. The 488-nm line of the Ar+ laser was used for excitation of the GFP. Cells were observed using a 63× water immersion objective (NA 1.2) with a fully opened pinhole. After 20 prebleach scans (one scan every 115 ms), a region of interest with w = 1 μm (w = 2 μm for cytochalasin D experiments) was bleached, and fluorescence recovery was then sampled for 20 s at the same scan frequency. Bleaching time length was carefully adjusted to be less than
EFA6 Localization

one-tenth of the \(t_0\) to achieve correct determination of diffusion coefficients. Experimental recoveries were normalized and corrected for cell z-position fluctuation using another region of interest as an internal standard. Half recovery times (\(t_0\)) and mobile fractions were determined by Levenberg-Marquardt fit of the normalized recovery curves using a 20th-order limited development of a modified equation given in Axelrod (23). Diffusion coefficients (D) were then calculated using the equation \(D = \beta \mu^2/4t_0\). Here, \(\beta\) takes into account the depth of the bleaching (24). Our method was validated by performing FRAP experiments on 1-\(\alpha\)-dimyristoylphosphatidylcholine:12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-phosphatidylcholine (99:1, mol:mol) multilamellar lipid vesicles at 25 °C, giving values for diffusion coefficients of 2.0 \(\pm\) 0.2 \(\mu^2\) s\(^{-1}\) and for mobile fraction of 0.97 \(\pm\) 0.05 (5 experiments), in total agreement with previously published values (for review, see Ref. 25).

**Surface Plasmon Resonance Experiments**—Surface plasmon resonance was measured using a Biacore 2000 instrument. The experiments were performed essentially as described elsewhere (26). The L1 chip was cleaned by a 4-min injection with 1% octylglycoside and by a 4-min injection with 0.5% SDS at a flow rate of 5 \(\mu\)l/min. Before injection of the lip vesicles, each flow cell was conditioned with 1% octylglycoside for 1 min at a flow rate of 10 \(\mu\)l/min. The L1 chip was coated with lipid vesicles at a concentration of 1.5 \(\mu\)g/ml for 11 min at a flow rate of 5 \(\mu\)l/min. The immobilized vesicles were washed with 10 mM NaOH at 100 \(\mu\)l/min flow rate for 12 s to remove unattached vesicles. Purified proteins were diluted in Hepes-buffered saline running buffer to the concentration indicated and perfused at a flow rate of 30 \(\mu\)l/min. The proteins were allowed to associate for 4 min and then allowed to dissociate for at least 4 min. The sensor surface was regenerated by short pulses of 10 mM NaOH. The lipid layers were removed by injecting 1% octylglycoside.

**Cell Fractionation, Triton X-100 Solubilization, and Immunoblotting**—10^6 BHK cells were transfected with EGFP-EFA6-PH or EGFP-PCLδ-PH-expressing vectors and washed in phosphate buffer 40 h later. For cytosol/membrane fractionation, the cells were then homogenized in 250 mM sucrose, 3 mM imidazole, pH 7.4, with proteases inhibitors. The post-nuclear supernatant was obtained by centrifugation of the cell lysate at 1000 \(\times\) g for 5 min at 4 °C. The post-nuclear supernatant was further centrifuged for 45 min at 100,000 \(\times\) g to separate membrane and cytosolic fractions. For Triton X-100 solubilization, the cells were lysed in 20 mM Tris-HCl, pH 7.5, 1\% Triton X-100, 120 mM NaCl, 1 mM MgCl\(_2\), with proteases inhibitors for 10 min at 4 °C, and centrifuged 30 min at 15,000 \(\times\) g to separate the Triton X-100-soluble and -insoluble fractions. Aliquots of the pellet and supernatant (% of the pellet for Triton X-100 solubilization experiment) fractions were loaded on 15% SDS-PAGE and analyzed by immunoblotting using an anti-GFP antibody.

Co-immunoprecipitation—BHK-21 cells (2 x 10^6 cells in 10-cm tissue culture dishes) were transfected with different EGFP-EFA6 constructs or EGFP alone. 48 h after transfection, the cells were lysed in 0.5 ml of lysis buffer (20 mM Hapes, pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 1 mM MgCl\(_2\), 0.25 mM phenylmethylsulfonyl fluoride and a tablet of proteases inhibitors (Roche Applied Science). The lysates were clarified by centrifugation at 13,000 \(\times\) g for 10 min, and aliquots of each supernatant were saved for later analysis. Then 5 \(\mu\)g of anti-GFP antibody and 20 \(\mu\)l of protein A-Sepharose CL4B were added and further incubated for 4 h at 4 °C. The beads were then washed in lysis buffer, and the immunoprecipitated proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

**Confocal Immunofluorescence Microscopy**—BHK cells plated on 11-mm round glass coverslips were transiently transfected with pCDNA3 or pEGFP-N1/C1 constructs using the FuGENE 6 transfection reagent as described by the manufacturer (Roche Applied Science). Unless otherwise stated, the cells were washed twice in phosphate-buffered saline 24 h after transfection, then fixed in 3% paraformaldehyde and processed for immunofluorescence analysis as described previously (27). Confocal microscopy analysis was carried out with a Leica TCS-SP5 microscope (Leica Microsystems).

**Actin Sedimentation**—Monomeric G-actin purified from rabbit muscle was stored at 4 °C in G buffer (5 mM Tris/Cl, 0.2 mM ATP, 1 mM dithiothreitol, 0.1 mM CaCl\(_2\), 0.01% NaN\(_3\), pH 7.5). Actin polymerization was performed as described (28). Briefly, actin was polymerized for 5 h at room temperature by the addition of 0.1 M KCl, 1 mM MgCl\(_2\), and 0.2 mM EGTA (KME buffer) to G-actin. For the sedimentation assay, EFA6 constructs at the indicated concentrations were incubated with F-actin for 10 min at room temperature (final volume, 70 \(\mu\)l in G + KME buffer) and then centrifuged for 10 min at 4 °C and 70,000 rpm in TL100.1. Pellets were resuspended in the same buffer and analyzed by SDS-PAGE. For the co-sedimentation assay the procedure was the same as above except than F-actin and GST-EFA6 were incubated with azolecin-cholate micelles (29) supplemented with 0.1% 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-phosphatidylethanolamine. Pellets aliquots were visualized and analyzed in a Fuji LAS-3000 fluorescence imaging system.

**Electron Microscopy**—Samples containing F-actin (5 \(\mu\)M) were incubated for 5 min at room temperature with azolecin vesicles (1 g/liter) in G-KME buffer with or without GST-EFA6 (2 \(\mu\)M). Samples were deposited on glow discharge carbon-coated grids and negatively stained with 1% aqueous uranyl acetate. They were observed with a Philips CM12 TEM (Eindhoven). Acquisitions were made with a Morada digital camera (Olympus SIS).

**RESULTS**

The PH Domain Is Responsible for the Plasma Membrane Localization of EFA6—EFA6 is an Arf6-specific nucleotide exchange factor that is constituted of several structural domains. Adjacent to the catalytic Sec7 domain, EFA6 contains a PH domain and in its C terminus part a coiled-coil domain and two proline-rich regions (Fig. 1). We have previously shown that EFA6 localizes to the plasma membrane. We also observed that the deletion of the C terminus part including the PH domain and the coiled-coil motif led to a protein that was mostly detected in the cytosolic fraction (17). To determine precisely which domain was involved in the plasma membrane localization, we expressed different mutants of EFA6 in BHK cells and analyzed their localization by confocal immunofluo-
rescence microscopy (Fig. 1). Deletion of the 124 C-terminal amino acids (ΔC) that does not remove the PH domain does not affect the plasma membrane localization of EFA6. In contrast, deletion of the 241 last residues that contain both the PH and the C terminus domains led to the solubilization of the protein. It indicated that the PH domain is responsible for the plasma membrane localization. This was confirmed by expressing the isolated PH domain fused to GFP, which localized to the plasma membrane similarly to the full-length EFA6.

The PI(4,5)P2 Is Responsible for the Plasma Membrane Localization of EFA6—Next, we looked for partners of the PH domain at the plasma membrane. Several PH domains recognize particular phosphoinositides with high affinity and specificity. This binding results at least for a part from the presence of basic residues in the β1/β2 loop region (30). The alignment of the primary sequences of several PH domains revealed that EFA6 bears some of the conserved basic residues involved in the binding of the phosphoinositides (Fig. 2A). Independent mutation of two of these few basic residues in aspartic acid (R386E and K387E) led to a PH domain that was no longer localized at the plasma membrane as observed by confocal microscopy analysis (Fig. 2B). This result suggested that EFA6-PH localizes at the plasma membrane by interacting with phosphoinositides. Given that EFA6 acts on endocytosis and actin reorganization (17, 31) and that the PI(4,5)P2 plays a key role in both processes (32), we asked whether EFA6-PH could bind specifically to the PI(4,5)P2. We reasoned that if the PI(4,5)P2 recruited the EFA6-PH domain to the plasma membrane, a PI(4,5)P2 breakdown would lead to the appearance of the pro-
EFA6 Localization

...tein in the cytosol. It had been reported that treatment of the cells with ionomycin in the presence of millimolar external calcium results in the activation of PLC isozymes (33). This PLC activation leads to a massive hydrolysis of the PI(4,5)P$_2$. We observed that ionomycin treatment of the cells solubilized the PLC$\delta$-PH used as a model for PI(4,5)P$_2$-specific binding PH domain (34) (Fig. 2B). Interestingly, a very similar effect was observed on EFA6-PH localization, indicating that EFA6-PH binds to PI(4,5)P$_2$ (Fig. 2B). We then tested if the PI(4,5)P$_2$ breakdown could also affect the localization of the full-length EFA6. Indeed, a few minutes after the addition of ionomycin, EFA6 fused to GFP was mostly found in the cytosol, indicating that the PI(4,5)P$_2$ was necessary to localize EFA6 at the plasma membrane (Fig. 2C). This process was reversible as subsequent chelation of external Ca$^{2+}$ by EGTA led to the reappearance of the protein at the plasma membrane (not shown). These results suggest that the interaction between the PH domain and the PI(4,5)P$_2$ is critical for the plasma membrane localization of EFA6.

The EFA6-PH Domain Interacts Strongly and Specifically with the PI(4,5)P$_2$.—To confirm a specific and direct interaction between EFA6 and PI(4,5)P$_2$, we tested in vitro the effect of the addition of phospholipid vesicles of different compositions on the nucleotide exchange activity of EFA6 on Arf6. As shown in Fig. 3A, we observed that the addition of 2% PI(4,5)P$_2$ into the lipid vesicles strongly increased the EFA6-catalyzed activation of Arf6. EFA6 was about three times less active when PI(4,5)P$_2$ was replaced by phosphatidylinositol 4-phosphate or PI (Fig. 3A). Then we tested the effect of the addition of PI(3,4,5)P$_3$ and measured about the same EFA6 activity as that in the presence of PI(4,5)P$_2$. This suggests that in addition to PI(4,5)P$_2$, EFA6 could be activated by the phosphatidylinositol 3-kinase pathway. However, we observed that in quiescent (serum-starved) BHK cells, the PI(3,4,5)P$_3$-specific PH domains of Akt and of the two glycine version of Arno are cytosolic in contrast to EFA6-PH domain (data not shown). This suggests that the PI(3,4,5)P$_3$ level is very low in BHK cells. In addition, the phosphatidylinositol 3-kinase inhibitor wortmannin did not modify the plasma membrane localization of the EFA6-PH domain (data not shown). Moreover, even in stimulated cells, PI(4,5)P$_2$ is more abundant than PI(3,4,5)P$_3$ (35). Thus, we conclude that PI(4,5)P$_2$ recruits EFA6 to the plasma membrane where it activates Arf6.

To demonstrate that the direct interaction between EFA6 and PI(4,5)P$_2$ was mediated by the PH domain, we used purified GST fusion of EFA6-PH and pure lipid vesicles. In a set of surface plasmon resonance experiments, we observed that the EFA6-PH domain bound strongly to lipid layers containing 10% PI(4,5)P$_2$, whereas it bound weakly to PI-containing vesicles (Fig. 3B). To quantify the affinity of the EFA6-PH/PI(4,5)P$_2$ interaction, we performed the experiment at different PH domain concentrations (Fig. 3C). We calculated an apparent $K_d$ of about 0.5 $\mu$M very similar to the one of the PLC$\delta$-PH (0.2 $\mu$M) determined by the same approach (26). Thus, our data showed that the PH domain of EFA6 binds to PI(4,5)P$_2$ with a high affinity.

The PH Domain of EFA6 Interacts with the Cytoskeleton—Next, because EFA6 is known to be involved in actin organiza-
two PH domains fused to GFP (Table 1). It should be noted that our FRAP experiment does not allow discrimination between the lateral diffusion of a strongly membrane-associated molecule versus the membrane association/dissociation of a weakly membrane-bound molecule. However, McLaughlin et al. (36) have recently studied the cell surface mobility of PI(4,5)P₂ using another experimental approach and found a lateral diffusion coefficient of about 0.8 μm²/s. We found exactly the same value for the PLC₇-PH domain (Table 1), suggesting that our value reflects the diffusion of the PLC₇-PH domain in association with PI(4,5)P₂. We observed that the PH domain of EFA6 was about two times less mobile than PLC₇-PH, confirming that EFA6-PH could interact with another compound than the PI(4,5)P₂ at the cell surface. We next observed that the addition of cytochalasin D, an F-actin depolymerizing drug, increased the lateral diffusion of EFA6 (Table 1). Altogether, these results suggested that in addition to PI(4,5)P₂, EFA6-PH could interact with a cytoskeletal element most likely the F-actin itself.

The PH Domain of EFA6 Localizes and Co-immunoprecipitates with F-actin—By immunofluorescence analysis, we observed that the EFA6-PH domain strongly co-localizes with F-actin in microvilli-like structures at the cell surface (Fig. 5A), suggesting that EFA6-PH and actin filaments are present in the same regions of the plasma membrane. By conducting co-immunoprecipitation experiments in BHK cells expressing GFP alone or fused to various EFA6 constructs, we observed that F-actin co-immunoprecipitated with EFA6 and notably with its PH domain but not with the PLC₇-PH (Fig. 5B). These results indicate that the EFA6-PH domain could be engaged in an actin-containing complex at the plasma membrane.

The PH Domain of EFA6 Interacts Directly with F-actin—Next we tested whether the interaction between actin and the EFA6-PH domain was direct. We used purified actin polymerized in KME buffer and took advantage of the property of actin filaments to sediment. In our co-sedimentation assay, we observed that the F-actin was able to pellet GST fusion proteins of full-length EFA6 and of the isolated PH+C region of EFA6 but not the GST alone (Fig. 6A). The same results were also obtained with His-tagged version of EFA6 (data not shown). These results demonstrated that EFA6, via its PH+C region, binds directly to F-actin. Next, we tried to identify which of the PH or the C domain interacted with F-actin. As shown in Fig. 6B, we observed that the PH but not the C terminus domain was able to co-sediment with F-actin. However, we noticed that the same con-

![FIGURE 4. EFA6 and its PH domain are mainly Triton X-10-insoluble. A and B, BHK cells were transfected with expression plasmids encoding PLC₆-PH, EFA6-PH, or EFA6 full-length fused to GFP. A, the cells were homogenized, and the post-nuclear supernatants were centrifuged at 100,000 × g to separate a membrane (m) and a cytosolic (c) fraction. B, BHK cells were lysed at 4 °C in Triton X-100-containing buffer and centrifuged to separate Triton-X-100-soluble (s) and -insoluble (i) fractions. The different fractions were immunoblotted using anti-GFP mouse monoclonal antibody.

![TABLE 1 Lateral mobilities of EFA6 and PLC₆ PH domains measured by FRAP](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAAEAABAAAQAAB9Kr29AAAAGXRFWHRTb2Z0d2FyZQBBZG9iZSBJbWFnZVJlYWR5ccllPAAAAyBpVFh0WE1MOmNvbS5hZG9iZS5jb20AAHwSVRFVHUmYzRiAAAAhSUrUhUERVRhVFR0lFWE1MR0lFQ0tEQ0tFSwAAAAASUVORK5CYII=)

| Lateral diffusion coefficients μm²/s | n  |
|-------------------------------------|----|
| EFA6-PH                            | 0.401 ± 0.180 | 12 |
| EFA6-PH + cytochalasin D            | 0.644 ± 0.261 | 8  |
| PLC₆-PH                            | 0.835 ± 0.234 | 24 |

![FIGURE 5. Actin co-localizes and co-immunoprecipitates with EFA6-PH domain. A, BHK cells were transfected with expression plasmids encoding EFA6-PH fused to GFP. 24 h after transfection cells were fixed and probed with Texas Red-labeled phalloidin to visualize F-actin and imaged by confocal microscopy. B, lysates of BHK cells expressing GFP alone (as the control in the left and right panels) or fused to PLC₆-PH domain (as the control in right panel), EFA6, EFA6-PH, or EFA6-PH+PH-C were immunoprecipitated (IP) with an anti-GFP antibody. Immunoprecipitates were resolved on SDS-PAGE, blotted on nitrocellulose membranes, and probed first with an anti-actin antibody and reprobed with an anti-GFP. 4% of the input (cell lysates) were also immunoblotted with anti-GFP to ensure that the different constructs were expressed and anti-actin to ensure that the amount of actin in each conditions was equivalent.](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAAEAABAAAQAAB9Kr29AAAAGXRFWHRTb2Z0d2FyZQBBZG9iZSBJbWFnZVJlYWR5ccllPAAAAyBpVFh0WE1MOmNvbS5hZG9iZS5jb20AAHwSVRFVHUmYzRiAAAAhSUrUhUERVRhVFR0lFWE1MR0lFQ0tEQ0tFSwAAAAASUVORK5CYII=)
The concentration of F-actin was able to pellet a higher amount of the PH/H11001C than the PH domain alone. It suggested that the presence of the C terminus domain participates in the binding to F-actin. To determine an apparent affinity between the PH/H11001C domain and the F-actin, we performed the co-sedimentation assay at different actin concentrations (Fig. 6C). From this experiment, after subtraction of the background due to the aggregated GST-PH/H11001C protein, we extracted a $K_d$ of about 0.8 $\mu$M. Finally, we tested the two PI(4,5)P$_2$ binding-defective mutants of the PH domain for actin binding. Both mutants were still able to interact with F-actin (Fig. 6C, right panel), indicating that the two basic residues (Arg-386 and Lys-387) are solely required for the interaction to the PI(4,5)P$_2$ but not the F-actin.

**EFA6 Interacts Simultaneously with F-actin and the Phospholipids**—Then we asked whether the presence of F-actin could modulate the catalytic activity of EFA6 on Arf6 in the presence of phospholipid liposomes. We observed that the addition of filamentous actin did not affect either the spontaneous or the EFA6-catalyzed activation of Arf6 in the presence of phospholipid vesicles. Binding of $[^{35}\text{S}]$GTP$\gamma$S to 2 $\mu$M recombinant Arf6GDP was determined as described under procedures in the presence of 0.4 mM azolectin vesicles with (●, ○) or without (○, □) 20 nM EFA6 and with (○, ●) or without (□, ■) 5 $\mu$M filamentous actin. B, co-sedimentation of fluorescent mixed micelles and EFA6 with F-actin. 0.8 g/liter azolectin-cholate (1/4) micelles were incubated with or without F-actin (4 $\mu$M) and GST-EFA6 (2 $\mu$M). After sedimentation, the fluorescence in the pellet was measured and quantified as described under “Experimental Procedures.” NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)). C, electron micrograph of actin filaments incubated in the presence of azolectin vesicles and in the presence (right) or the absence (left) of EFA6. Scale bar, 2 $\mu$m.
partners. This result suggests that EFA6 interacts simultaneously with F-actin and phospholipids.

To confirm the formation of a ternary complex between EFA6, F-actin, and phospholipids, we first performed a co-sedimentation experiment. We incubated fluorescent mixed micelles in the presence or not of purified recombinant EFA6 and F-actin. After centrifugation, in the absence of any protein the micelles were found essentially in the soluble fraction (Fig. 7B). In the presence of purified F-actin, we observed that the fluorescent micelles were found in the pellet only when EFA6 was added, suggesting that EFA6 interacted simultaneously with F-actin and phospholipids. We noticed that in the absence of F-actin, a small amount of EFA6 (probably an aggregated form) sedimented (not shown), bringing together a small quantity of fluorescent micelles in the pellet.

Second, we incubated phospholipid vesicles and F-actin in the presence or absence of purified GST-EFA6. The samples were then negatively stained and analyzed by electron microscopy (Fig. 7C). We observed that the addition of EFA6 drives the aggregation of actin filaments with phospholipid vesicles. Indeed, in the absence of EFA6, actin filaments and liposomes were uniformly distributed on the grid (Fig. 7C, left panel), whereas in its presence the filaments and liposomes were reassembled, indicating that EFA6 could link the liposomes to the actin filaments.

**DISCUSSION**

To understand the molecular basis of the Arf6/EFA6 pathway at the plasma membrane, it is important to determine how EFA6 localization is controlled. The present study demonstrates that the PH domain is necessary and sufficient to target EFA6 to the plasma membrane via its binding to phosphoinositides, most likely PI(4,5)P2 and cortical filamentous actin.

The PH domain binds PI(4,5)P2 with a high affinity (~0.5 μM), comparable with the one of the PLCδ1-PH domain, which is commonly used as a model for PH to PI(4,5)P2 interaction (30). By analogy with PLCδ8 and Arno3/Grp1, we identified two basic residues, Arg-386 and Lys-387, crucial for targeting EFA6 to the plasma membrane. These amino acids are conserved in the β1-β2 loop and are probably involved in the binding of the 4- and 5-phosphate groups of PI(4,5)P2 (37, 38). Finally, PI(4,5)P2 hydrolysis by the activation of endogenous PLC induces the release of EFA6 from the plasma membrane to the cytoplasm, indicating that PI(4,5)P2 controls the plasma membrane localization of EFA6. These results are coherent as PI(4,5)P2 appears to be enriched at the plasma membrane and is responsible for the recruitment of most of the proteins involved in endocytosis and of several actin cytoskeleton regulators (32), two processes in which EFA6 is involved (17, 31, 39).

In addition to PI(4,5)P2 binding, we also demonstrated that the EFA6-PH domain is able to interact directly with filamentous actin. We demonstrated this interaction in vitro by pull-down and co-sedimentation experiments using purified proteins. We determined an apparent affinity of the PH + C domain for F-actin of about 0.8 μM. In the cells we showed that in the absence of PI(4,5)P2, F-actin binding is not sufficient to recruit EFA6 to the F-actin-enriched regions of the plasma membrane nor to stress fibers. Moreover, we observed that the addition of actin-disrupting drugs affected the pattern of distribution of EFA6 at the plasma membrane without decreasing its membrane association as judged by cytosol/membrane fractionation (not shown). Thus, we think that the EFA6-PH/F-actin interaction rather contributes to the concentration of EFA6 in the small actin-rich microvilli of the plasma membrane.

Altogether these results imply that the EFA6-PH domain could be a sensor of specific lipids and proteins at the plasma membrane. Actin and PI(4,5)P2 partners work as the “coincidence detectors” described by Lemmon (40). Until now, such duality for a PH domain has only been described for another Arf-GEF of the Arno family (15, 16). In this case the PH domain was shown to bind phosphoinositides and, surprisingly, two members of the Arf family in their GTP bound state, Arf6 and Arl4. Thus, using two distinct and specific molecules (phosphoinositides and small G-protein), the Arf exchange factor Arno is targeted to selective cell compartment.

In addition to localization, we were also interested in understanding how the exchange factor activity is controlled. We observed that the presence of PI(4,5)P2 and PI(3,4,5)P3 in lipid membranes strongly increased the catalytic activity of EFA6 on Arf6. This phosphoinositide-stimulatory effect could simply be explained by the membrane recruitment of the exchange factor where it encounters its substrate, the myristoylated Arf6-GDP. However, one cannot exclude that the interaction between the PH domain and phosphoinositides affects the three-dimensional structure of EFA6 and increases the GEF activity of the Sec7 domain. In cells, a way for PI(4,5)P2 to control EFA6 activity might rely on a positive feedback loop. It is well established that the activation of Arf6 leads to a PI(4,5)P2 production via the activation of a phosphatidylinositol 4-phosphate 5-kinase (41). This loop could control the formation of a local pool of activated Arf6 involved in both clathrin coat assembly and actin remodeling. Nevertheless, the fact that PI(4,5)P2 is relatively abundant at the plasma membrane (42) and that the affinity between EFA6 and PI(4,5)P2 is high (~0.5 μM as determined in this study) could suggest that EFA6 is constitutively located and active at the plasma membrane. A more credible explanation would be that in vivo the PI(4,5)P2 recruitment is not sufficient to reveal the full GEF activity of EFA6, and an additional regulatory mechanism could affect the activity of the Sec7 domain. Such control has been recently shown for members of the Arno family (43). Indeed, Dinitto et al. (43) demonstrated that the C-terminal amphipathic helix of Arno3 physically interacted with the Sec7 domain, blocking access to Arf protein. This autoinhibition could be reversed by the binding of phosphatidylinositol 4-phosphate and Arf6-GTP to the PH and C-terminal helix. A similar activating mechanism could regulate the GEF activity of EFA6 once at the plasma membrane. In contrast to PI(4,5)P2, the interaction with F-actin did not increase the nucleotide exchange activity of EFA6. This could suggest that actin acts only as a hook to localize EFA6 in specialized regions of the plasma membrane. On the other hand, EFA6 might regulate the polymerization state or at least the structural organization of the actin filaments. The fact that EFA6 physically links plasma membrane phospholipids with actin filaments might constitute the first step of the molecular mechanism that accounts for the role of EFA6 in coordinating the plasma mem-
brane transport with actin cytoskeleton remodeling. Could a direct interaction with F-actin be responsible for the actin reorganization observed in the cells when EFA6 is overexpressed? It has been shown that among the few PH domains known to bind F-actin, the PH domain of the oxysterol-binding protein is able to bundle F-actin in vitro (13). We, therefore, asked whether EFA6 could similarly organize actin filaments. Unfortunately, by electron microscopy analysis, we observed no effect of the addition of EFA6 (see Fig. 7). This result indicates that EFA6 is not able to remodel actin filaments by itself. Thus, in the cells, the EFA6-induced reorganization of actin cytoskeleton might involve EFA6-binding proteins. It has been reported recently that EFA6 binds to the actin-cross-linker protein α-actinin (44). It is now tempting to think that this EFA6/α-actinin interaction could constitute a major way for EFA6 to control the organization of the actin cytoskeleton.

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