A conserved C-terminal domain of EFA6-family ARF6-guanine nucleotide exchange factors induces lengthening of microvilli-like membrane protrusions

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Summary

We recently reported the identification of EFA6 (exchange factor for ARF6), a brain-specific Sec7-domain-containing guanine nucleotide exchange factor that works specifically on ARF6. Here, we have characterized the product of a broadly expressed gene encoding a novel 1056 amino-acid protein that we have named EFA6B. We show that EFA6B, which contains a Sec7 domain that is highly homologous to EFA6, works as an ARF6-specific guanine exchange factor in vitro. Like EFA6, which will be referred to as EFA6A from now on, EFA6B is involved in membrane recycling and colocalizes with ARF6 in actin-rich membrane ruffles and microvilli-like protrusions on the dorsal cell surface in transfected baby hamster kidney cells. Strikingly, homology between EFA6A and EFA6B is not limited to the Sec7 domain but extends to an adjacent pleckstrin homology (PH) domain and a ~150 amino-acid C-terminal region containing a predicted coiled coil motif. Association of EFA6A with membrane ruffles and microvilli-like structures depends on the PH domain, which probably interacts with phosphatidylinositol 4,5-biphosphate. Moreover, we show that overexpression of the PH domain/C-terminal region of EFA6A or EFA6B in the absence of the Sec7 domain promotes lengthening of dorsal microvillar protrusions. This morphological change requires the integrity of the coiled-coil motif. Lastly, database analysis reveals that the EFA6-family comprises at least four members in humans and is conserved in multicellular organisms throughout evolution. Our results suggest that EFA6 family guanine exchange factors are modular proteins that work through the coordinated action of the catalytic Sec7 domain to promote ARF6 activation, through the PH domain to regulate association with specific subdomains of the plasma membrane and through the C-terminal region to control actin cytoskeletal reorganization.

Key words: ADP-ribosylation factor 6, Sec7 domain, Actin cytoskeleton, Endocytosis, Guanine nucleotide exchange factor

Introduction

ADP ribosylation factors (ARFs) form a group of six low molecular weight Ras-related GTP-binding proteins required for intracellular transport (for reviews, see Chavrier and Goud, 1999; Donaldson and Jackson, 2000; Moss and Vaughan, 1998). Mainly through sequence similarities, ARF proteins have been classified into three classes: class I (ARF1,2,3), class II (ARF4,5) and class III (ARF6) (Tsuchiya et al., 1991). ARF1 is localized to the Golgi complex where it regulates vesicle formation. ARF6, the least conserved ARF protein, controls the morphology of the cell cortex by coordinating cortical actin cytoskeleton reorganization with plasma membrane remodelling (D'Souza-Schorey et al., 1997; Radhakrishna and Donaldson, 1997; Song et al., 1998). In agreement with its proposed function, ARF6 has been implicated in regulated exocytosis and Fc-receptor-mediated phagocytosis; two processes requiring membrane movement toward the cell surface and actin cytoskeleton reorganization (Bajno et al., 2000; Galas et al., 1997; Zhang et al., 1998).

As with other Ras-related G proteins, ARFs operate as binary switches: ARF with GDP is inactive, and replacement of bound GDP by GTP, which is catalysed by guanine nucleotide exchange factors (GEFs), produces active GTP-ARF. We recently reported the identification of EFA6 (Exchange factor for ARF6), a GEF that activates ARF6 in vitro and, like ARF6, is involved in membrane recycling and actin cytoskeleton remodelling (Franco et al., 1999). EFA6, like all the ARF GEFs identified so far, contains a 200 amino-acid domain, called the Sec7 domain, which is sufficient for exchange factor activity. Structural and biochemical data have shown that Sec7 domains harbour an α-helical structure forming a hydrophobic groove in which the switch regions of ARF insert (Béraud-Dufour et al., 1998; Cherfils et al., 1998; Goldberg, 1998; Mossessova et al., 1998). In addition to EFA6, members of the ARNO/cytohesin Sec7-domain-containing

GEF family also activate ARF6, although they appear to be more efficient for class I ARFs (Franco et al., 1998; Langille et al., 1999; Macia et al., 2001; Venkateswarlu and Cullen, 2000). Recently, a third ARF6 GEF has been identified, termed ARF-GEP$_{100}$, showing limited homology with the Sec7 domain of EFA6 and ARNO (Someya et al., 2001). Although reasons for such diversity appear unclear, it should be noticed that these GEFs differ in their expression profiles. EFA6 expression is restricted to the brain (Perletti et al., 1997), whereas ARNO/cytohesin GEFs and ARF-GEP$_{100}$ appear to be broadly expressed (Kolanus et al., 1996; Someya et al., 2001).

ARF6 GEFs can also be distinguished at the level of their intracellular distribution and regulation of membrane attachment. EFA6, ARNO and possibly ARF-GEP$_{100}$ contain a pleckstrin homology (PH) domain located C-terminally to the Sec7 domain (Chardin et al., 1996; Perletti et al., 1997; Someya et al., 2001). The PH domain is a protein module of ~120 amino acids found in many cytoskeletal and signaling proteins (Rebecchi and Scarlata, 1998). In the case of EFA6 and ARNO family members, this PH domain is required for localization to the plasma membrane (Franco et al., 1999; Venkateswarlu et al., 1998). Overexpressed ARNO3/GRP1 is mostly cytosolic and translocates transiently to the plasma membrane in response to cell activation with various agonists (Gray et al., 1999; Venkateswarlu et al., 1998). It appears that the GRP1-PH domain [especially in its diglycine form (Cullen and Chardin, 2000; Klarlund et al., 2000)] has a higher (650-fold) selectivity for phosphatidylinositol 3,4,5-triphosphate [PtdIns(3,4,5)P_3] over phosphatidylinositol 4,5-biphosphate [PtdIns(4,5)P_2]. This specificity, together with the fact that agonist-stimulated PtdIns(3,4,5)P_3 accumulation is rapid and transient, may provide a molecular basis for the regulation of ARNO/cytohesin family GEF association with the plasma membrane. In contrast to ARNO/cytohesin proteins, overexpressed EFA6 remains mostly bound to the plasma membrane (Franco et al., 1999), suggesting that the binding specificity of EFA6-PH domain is different. Whether EFA6-PH domain interacts with phosphoinositide(s), protein(s) or both is presently unknown. Finally, ARF-GEP$_{100}$ colocalizes partially with the early endosomal marker EEA-1, but in contrast to ARNO and EFA6, ARF-GEP$_{100}$ it could not be detected at the plasma membrane (Someya et al., 2001). Therefore, different agonists acting through distinct signalling pathways could promote ARF6 activation through the activity of EFA6, ARNO or ARF-GEP$_{100}$ and may lead to distinct cellular responses. Understanding how ARF6 GEFs control ARF6 activity would help to clarify the function of ARF6.

Expression of ARNO or EFA6 results in actin cytoskeleton reorganization, probably as a consequence of ARF6 activation (Franco et al., 1999; Frank et al., 1998b). However, deletion of the EFA6 Sec7 domain or substitution of the conserved glutamate residue (position 242) by a lysine, resulting in a catalytically inactive protein, induces the formation of numerous long and thin actin-rich extensions of the plasma membrane (Franco et al., 1999). Moreover, we found that the C-terminal region of EFA6 containing a putative coiled-coil motif is required for EFA6-mediated cytoskeletal reorganization (Franco et al., 1999). These finding suggest that EFA6, besides activating ARF6 through its Sec7 domain, may also exert a direct control over actin cytoskeletal organization depending on its C-terminal region.

On the basis of the observation that EFA6 expression is restricted to the brain, whereas ARF6 is expressed in a wide range of cell lines and tissues (Cavenagh et al., 1996; Tsuchiya et al., 1991), we tested the possibility that EFA6-like GEF(s) may regulate ARF6 activity outside of the brain. Here, we describe a novel broadly distributed ARF6 GEF, called EFA6B, containing a Sec7 domain highly related to EFA6, which will now be referred as EFA6A. The adjacent PH and C-terminal domains of EFA6A and EFA6B are also highly conserved. In cells, EFA6A and EFA6B colocalized with ARF6 at the plasma membrane where they accumulate in subdomains. These subdomains, which are enriched in actin filaments, correspond to microvilli-like structures. The conserved C-terminal region of EFA6A/B GEFs, when recruited to the membrane via the PH domain, triggered microvilli lengthening. Our results suggest that EFA6-like GEFs, which are conserved in evolution, are modular proteins consisting of an ARF6-specific Sec7 domain linked to a membrane-anchoring PH domain and a C-terminal region involved in actin cytoskeleton remodelling.

Materials and Methods

Cell culture, antibodies and reagents

Baby hamster kidney cells (BHK) were maintained in GME-M (GIBCO BRL) with 10% volume/volume Tryptose broth and 5% volume/volume FCS supplemented with 2 mM L-glutamine. Jurkat cells (a kind gift of C. Hivroz) were cultured in RPMI 1640 medium plus Glutamax (GIBCO BRL), supplemented with 10% volume/volume FCS and 10 mM Hepes, pH 7.2. Polyclonal antibodies against EFA6B were raised against a C-terminal peptide (LGREAGGTREPKLS, position 1000-1013 of EFA6B amino acid sequence) and were immunopurified on the peptide immobilized on a Hi-Trap NHS-activated HP column (Amersham Pharmacia Biotech). Monoclonal antibodies against HA (clone 3F10) and VSV-G epitope-tags (clone P5D4) were purchased from Roche Molecular Biochemicals. Latrunculin, Texas-Red-conjugated wheat germ agglutinin and Texas-Red-conjugated phalloidin were from Molecular Probe. Cy3-conjugated antibodies were from Jackson ImmunoResearch. HRP-conjugated secondary antibodies were from Sigma Chemical Co.

cDNA cloning

EFA6B was cloned from a human placenta cDNA library (CLONTECH) using a 555 bp *Pvu*II-*Pst*I fragment corresponding to the Sec7 domain of human EFA6A as a probe. Hybridisation was performed in 5×SSPE/10× Denhardt/2% SDS for 18 hours at 65°C. Filters were washed with 2×SSC/O.5% SDS at 25°C for 30 minutes and 0.5×SSC/O.5% SDS at 55°C for 60 minutes, followed by autoradiography at –80°C. Eight positive clones were isolated and sequenced from both ends. A 5′ end ~1.45 kb *Eco*RI fragment was derived from the longest clone (~3.1 kb). A ~2 kb cDNA was isolated and found to overlap over a region of ~1.3 kb at the 5′ end of the 3.1 kb clone. The combined ~3.8 kb sequence contained an open reading frame encoding a predicted protein of 1056 amino acids. The cDNA clone reported here is present in the EMBL database under accession number AJ459781. It is almost identical to a sequence of Genbank/EMBL/DDBJ called TIC (accession no. U63127).

DNA constructs and transfection

EGFP-tagged EFA6A, EFA6A-PH (residues 351-494), -PHCTer (residues 349-645), -CTer (residues 478-645), -PHCT1 (349-617), -PHCT2 (residues 349-587), -PHCT3 (residues 349-547), -PHCT4 (349-520), EFA6B, EFA6B-PHCTer (residues 752-1056), and EFA6B-CTer (residues 879-1056) were generated by PCR with

oligonucleotide primers incorporating restriction sites. After digestion, the PCR products were inserted into the multi-cloning sites of pEGFP-C3 (CLONTECH) to give in-frame fusion with the N-terminal EGFP tag. All constructs were verified by double-stranded DNA sequencing.

Cells were transfected using Fugene-6 (Roche) according to the manufacturer's instructions. Western blotting analysis of transfected cell extracts using anti-GFP antibodies showed that all proteins migrated at their expected size during SDS-PAGE (data not shown).

Northern blot analysis

A human multiple tissue Northern blot (CLONTECH) was hybridised with DNA probes corresponding to a 1230 bp fragment of human EFA6A (corresponding to residues 1-410) or a 364 bp fragment of human EFA6C (corresponding to the Sec7 domain) or a 215 bp fragment from human EFA6B (residues 637-708) or a human β-actin cDNA probe as a control. The DNA probes were labeled with [α-^{32}P]dCTP (3000 mCi/mol) using Random Primer labelling kit (Roche Molecular Biochemicals), and hybridisation was performed at 68°C in 5xSSPE/2% SDS/10× Denhardt. The membrane was washed with 0.5°SSC/0.1% SDS at 65°C for 60 minutes, followed by autoradiography at –80°C. The expression profile quantified by RT-PCR ELISA concerning clone KIAA0942 (EFA6D) is available at http://www.kazusa.or.jp/huge/gfpage/KIAA0942/.

Analysis of EFA6B expression by immunoblotting

Jurkat or BHK (25×10^6) cells, or BHK cells transfected with pSRα-VSVG-EFA6B (2×10^6 cells) were lysed in RIPA buffer (1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS, 50 mM Tris pH7.5, 100 mM NaCl, 1 mM EDTA, protease inhibitors). After clearing DNA by centrifugation, lysates were first incubated with Pansorbin (Calbiochem) and then 5 μg/ml rabbit affinity-purified anti-EFA6B, mouse monoclonal anti-VSV-G epitope antibodies (clone P5D4) or irrelevant (anti-Sec10) antibodies were added for 2 hours at 4°C. Protein G Sepharose (Amersham Pharmacia Biotech) was added for 1 hour at 4°C, and Sepharose beads were finally washed once in RIPA buffer and twice in PBS. Proteins were separated by SDS-PAGE on 8% polycacrylamide gel and transferred on PVDF membrane. For immunoblotting analysis, anti-EFA6B or anti-VSV-G antibodies (1 μg/ml) were used, followed by ECL procedure (Amersham Pharmacia Biotech).

GTPγS binding assay

Myristoylated ARF1 and ARF6 were produced and purified as described previously (Chavrier and Franco, 2001). A portion of EFA6B coding sequence starting at an internal methionine residue (position 245) and including the Sec7 domain was subcloned into pET3a. pET3aEFA6A (Franco et al., 1999) and -EFA6B(245-1056) were transformed into *Escherichia coli* BL21 (DE3) strain, and protein expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactoside to a 0.2 l culture for 2 hours at 37°C. Then, bacteria were lysed using a French press, and the lysate produced was centrifuged at 20,000 ***g***. EFA6B(245-1056) was recovered in the insoluble fraction. Pellets containing EFA6A or EFA6B(245-1056) were solubilized in urea buffer (Tris/HCl 50 mM pH 8.0; 1 mM $MgCl_2$; 1 mM DTT; 10 M urea) and ultracentrifuged (350,000 ***g*** for 20 minutes in TL100.3 Beckman rotor). The urea concentration of the supernatants was decreased by successive dialysis in order to obtain soluble EFA6A and EFA6B(245-1056) proteins in the absence of urea. After dialysis, samples were ultracentrifuged (350,000 ***g*** for 20 minutes in TL100.3 Beckman rotor), and supernatants were used as source of GEFs in the nucleotide exchange experiments. The concentration of GEFs was determined by scanning Coomassie-stained SDS gel.

[^{35}S]GTPγS binding measurements were performed as previously described (Franco et al., 1999). Briefly, myrARF1 or myrARF6 (1 μM) were incubated at 30°C in 50 mM Hepes/NaOH pH7.5, 1 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 10 μM [^{35}S]GTPγS (1000 cpm/pmol, NEN) supplemented with 1.5 mg/ml azolectin vesicles (Sigma). EFA6A or EFA6B(245-1056) were added to a final estimated concentration of 200 nM. At indicated times, aliquots of 25 μl were measured for radioactivity.

Immunofluorescence and time-lapse video microscopy

For immunofluorescence analysis, transfected cells were fixed in 3% paraformaldehyde 36 hours after transfection. Cells were processed for immunofluorescence microscopy as described previously (Guillemot et al., 1997). Images were collected on a Leica DMRB microscope equipped with a cooled CCD camera (MicroMAX 5 MHz, Princeton Instruments, inc) with a 100×/1.4 PL Apo lens and Metaview software (Universal Imaging). For confocal microscopy, optical sections were taken with a Leica TCS SP2 confocal microscope.

Scanning electron microscopy

BHK cells were transfected with EGFP-EFA6A (or EFA6A-PHCTer) together with CD25/$FKBP_2$, a chimeric surface protein comprising the extracellular and transmembrane regions of human CD25 (Castellano et al., 1999). Before fixation, cells were incubated with biotinylated anti-CD25 monoclonal antibody (5 μg/ml in complete medium, clone B1.49.9, Beckman Coulter) for 30 minutes at 4°C. Cells were washed in complete medium and incubated for 30 minutes at 4°C with 2.5 μm diameter streptavidin-coated latex beads (Bangs Laboratories, Inc.) after centrifugation at 400 ***g*** for 4 minutes to sediment the beads. After extensive washing in complete medium, and a final wash in PBS, cells were fixed and processed for scanning electron microscope (SEM) analysis as described previously (Guillemot et al., 1997).

Results

Cloning, sequence analysis and expression of EFA6B

In an attempt to identify new EFA6-related ARF6 GEFs, we screened a human placenta cDNA library with a probe corresponding to the EFA6 Sec7 domain. Stringency conditions were selected to minimize hybridisation to Sec7-domain-encoding sequences corresponding to the related ARNO/cytohesin GEF family. A ~3.2 kb cDNA was isolated, and its nucleotide sequence revealed that it comprised the C-terminal half of a new Sec7 domain that is highly related to EFA6 (see below). Further screening with a probe spanning this partial Sec7 domain led to the isolation of a ~2.5 kb cDNA corresponding to the 5′ end of the mRNA. The merged cDNAs (accession number: AJ459781), which are almost identical to a sequence of the database called TIC (for telomeric of interleukin-1 cluster, accession no. U63127), contain an ORF

Table 1. Sequence identity (and similarity) between Sec7 domains of EFA6

	EFA6B	EFA6C	EFA6D	Y55D9A.1	CG6941	ARNO
EFA6A	63 (77)	70 (82)	66 (84)	45 (60)	61 (79)	35 (57)
EFA6B		61 (74)	60 (76)	44 (61)	65 (78)	36 (60)
EFA6C			70 (82)	47 (62)	61 (77)	37 (58)
EFA6D				41 (59)	61 (77)	34 (59)
Y55D9A.1					44 (67)	31 (54)
CG6941						32 (58)

encoding a 1056 amino-acid protein with a predicted M_r of 90,446 (Fig. 1A). Sequence analysis confirmed the presence of a Sec7 domain (residues 554-747, highlighted in yellow in Fig. 1A) with high similarity to the EFA6 Sec7 domain (63% identity, 77% similarity; Table 1). In addition, the complete sequence of a cDNA released in the database under accession no. AL136559 encodes another EFA6-related GEF (predicted protein: 771 amino acids (Wiemann et al., 2001), 70% identity with EFA6 Sec7 domain; Table 1). EFA6 will now be referred as EFA6A, TIC to as EFA6B, and we propose to call the protein

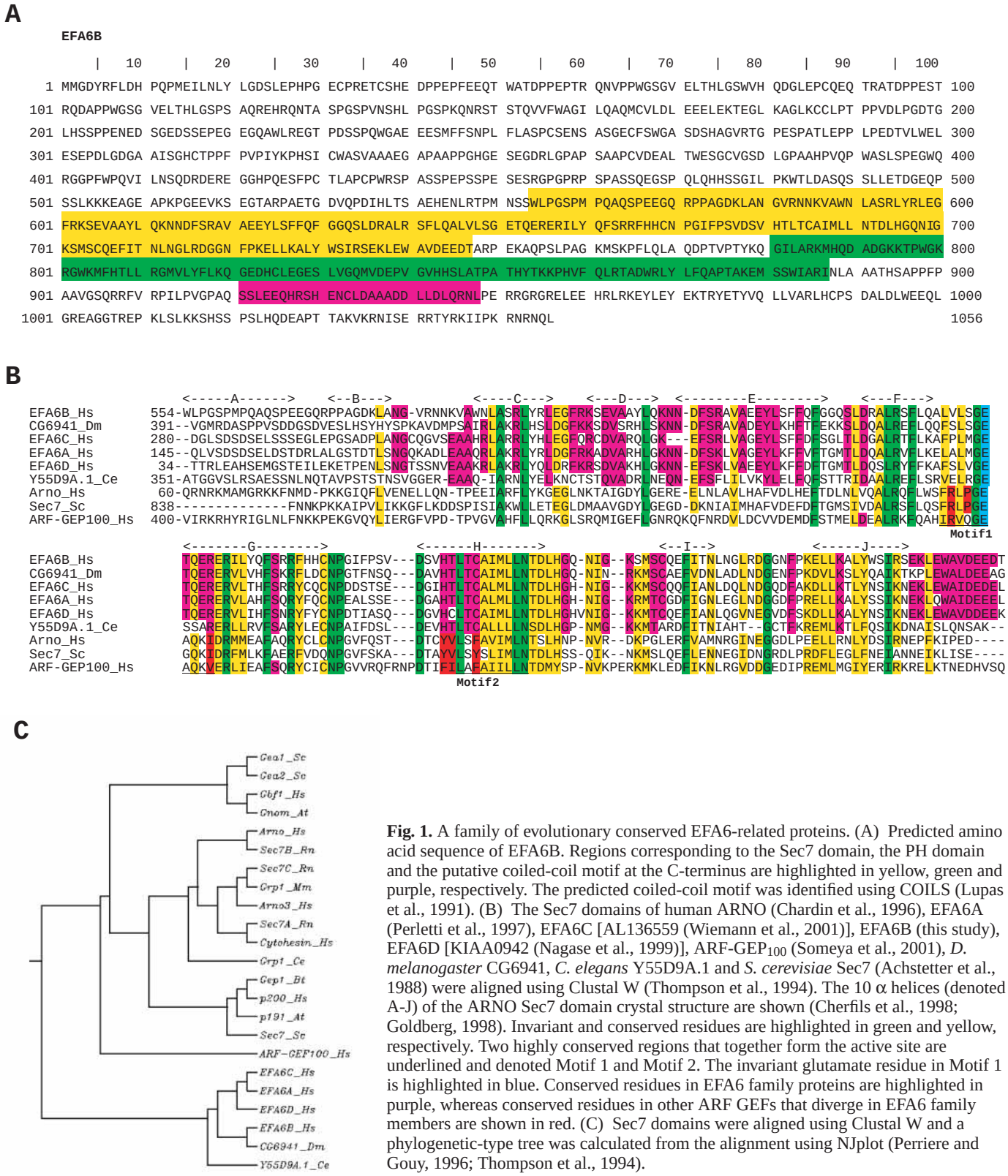


Fig. 1. A family of evolutionary conserved EFA6-related proteins. (A) Predicted amino acid sequence of EFA6B. Regions corresponding to the Sec7 domain, the PH domain and the putative coiled-coil motif at the C-terminus are highlighted in yellow, green and purple, respectively. The predicted coiled-coil motif was identified using COILS (Lupas et al., 1991). (B) The Sec7 domains of human ARNO (Chardin et al., 1996), EFA6A (Perletti et al., 1997), EFA6C [AL136559 (Wiemann et al., 2001)], EFA6B (this study), EFA6D [KIAA0942 (Nagase et al., 1999)], ARF-GEP$_{100}$ (Someya et al., 2001), *D. melanogaster* CG6941, *C. elegans* Y55D9A.1 and *S. cerevisiae* Sec7 (Achstetter et al., 1988) were aligned using Clustal W (Thompson et al., 1994). The 10 α helices (denoted A-J) of the ARNO Sec7 domain crystal structure are shown (Cherfils et al., 1998; Goldberg, 1998). Invariant and conserved residues are highlighted in green and yellow, respectively. Two highly conserved regions that together form the active site are underlined and denoted Motif 1 and Motif 2. The invariant glutamate residue in Motif 1 is highlighted in blue. Conserved residues in EFA6 family proteins are highlighted in purple, whereas conserved residues in other ARF GEFs that diverge in EFA6 family members are shown in red. (C) Sec7 domains were aligned using Clustal W and a phylogenetic-type tree was calculated from the alignment using NJplot (Perriere and Gouy, 1996; Thompson et al., 1994).

predicted from clone AL136559, EFA6C. Finally, a human cDNA encoding a fourth member of the EFA6 family (66% identity; Table 1), EFA6D, is also present in the database [clone KIAA0942, which originates from the human brain (Nagase et al., 1999); the partial coding sequence is 551 amino acids].

Human EFA6-like Sec7 domains were aligned with various other Sec7 domains using Clustal W (Thompson et al., 1994), and on the basis of the alignment a phylogenetic-type tree was calculated using NJplot (Perriere and Gouy, 1996) (for space reasons a subset of the complete clustal alignment is shown in Fig. 1B). Human EFA6 Sec7 domains could be grouped into a family that is clearly distinct from the ARNO/cytohesin/GRP-1 family (Fig. 1C). Interestingly, the Sec7 domains of *Caenorhabditis elegans* Y55D9A.1 (accession no. AL032649, 816 amino acids) and *Drosophila melanogaster* CG6941 (900 amino acids) proteins were also grouped together with human EFA6-like domains, indicating that this subgroup has been conserved throughout evolution (see below). The conservation between EFA6 family Sec7 domains is clearly apparent in the clustal alignment shown in Fig. 1B. Besides residues that are conserved in all Sec7 domains (invariant residues are shown in green and conserved residues in yellow, respectively), some positions are conserved only within the EFA6 family (highlighted in purple in Fig. 1B). Moreover, the homology between EFA6-related proteins was not limited to the Sec7 domain. Significant homology was observed in a pleckstrin homology (PH) domain adjacent to the Sec7 domain (see schematic representation of EFA6-family protein structure in Fig. 9A and Fig. 9B for an alignment of EFA6 family PH domains) and in the C-terminal region containing a predicted coiled-coil motif (aligned in Fig. 9C). By contrast, no significant similarity was found in the N-terminal domains of EFA6 family proteins. Altogether, these observations indicate that the overall organisation of a module consisting of the Sec7 and PH domains, as well as the C-terminal region, is conserved in EFA6 family proteins throughout evolution.

Expression of the EFA6 family genes in human tissues was analysed by northern blotting. As previously reported, mRNA for EFA6A was mostly detected in the brain (Perletti et al., 1997). In addition, a smaller message was detected in the small intestine and colon (Fig. 2A). EFA6C expression was also restricted to the brain (Fig. 2C). By contrast, EFA6B had a broad expression, which mirrored that of EFA6A. The highest EFA6B levels were found in placenta, pancreas, spleen, thymus and peripheral blood. The message was also detected in lung, liver, kidney, prostates but was barely detectable in heart, brain, ovary, small intestine and colon, whereas a smaller transcript was detected in testis (Fig. 2B). The multiple size EFA6A and EFA6B mRNAs detected by northern blot analysis may represent differently

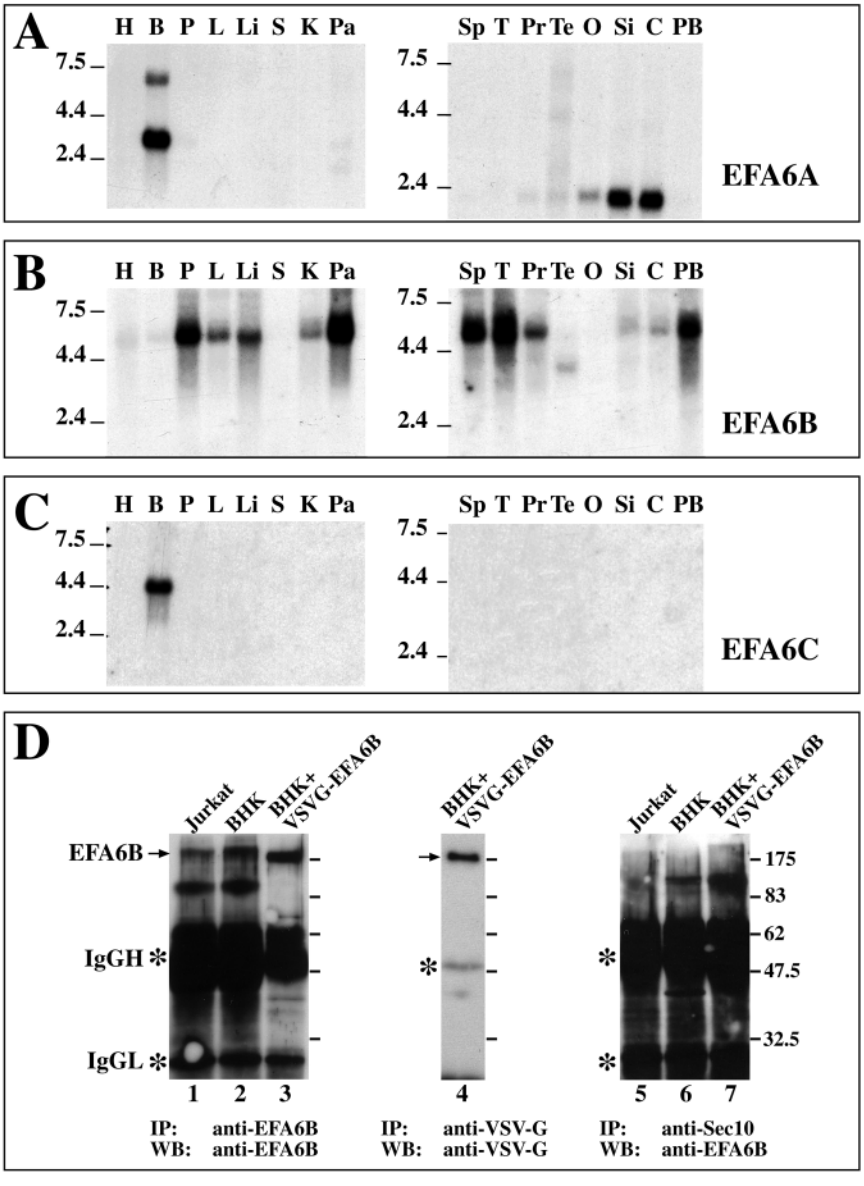


Fig. 2. mRNA and protein expression analysis of EFA6-like genes. Poly(A)$^+$ RNA from the indicated human tissues was hybridised with probes derived from EFA6A (A), EFA6B (B) and EFA6C (C) cDNAs. The blots were also probed with β-actin cDNA to ensure that loading was equal between the different tissues (data not shown). H, heart; B, brain; P, placenta; L, lung; Li, liver; S, skeletal muscle; K, kidney; Pa, pancreas; Sp, spleen; T, thymus; Pr, prostate; Te, testis; O, ovary; Si, small intestine; C, colon; PB, peripheral blood. D shows immunoprecipitations from lysates of Jurkat human T lymphocytes (lanes 1 and 5), BHK cells (lanes 2 and 6) or BHK cells overexpressing N-terminally VSV-G-tagged EFA6B (lanes 3, 4, 7) using anti-EFA6B (lanes 1-3), anti VSV-G tag (lane 4) or anti-Sec10 irrelevant antibodies (lanes 5-7). EFA6B and VSVG-EFA6B in the immunoprecipitates were detected with anti-EFA6B antibodies (lanes 1-3 and 5-7). Alternatively, VSVG-EFA6B was detected with an anti-VSVG antibody (lane 4). EFA6B (predicted molecular weight 116.34 kDa) migrates as a ~180 kDa species in SDS-PAGE (arrows). Asterisks denote heavy (H) and light (L) immunoglobulin chains.

spliced forms. EFA6D expression profile available from the HUGE Database (clone KIAA0942, see Materials and Methods section) revealed that this member of the EFA6 family is mostly expressed in the brain and, to lower extent in liver, the kidney, testis and ovary.

Expression of EFA6B at the protein level was assessed by immunoprecipitation using specific antibodies (Fig. 2D). Antibodies directed against a C-terminal peptide derived from the human EFA6B polypeptide sequence (see Materials and Methods) detected a protein migrating with an apparent molecular weight of ~180 kDa in lysates of Jurkat human T lymphocytes and BHK cells (Fig. 2D, lane 1 and 2, respectively). Noticeably, the nucleotide sequence of EFA6B cDNA predicts a protein of molecular weight 116.34 kDa. Therefore, EFA6B that was N-terminally tagged with VSVG was overexpressed in BHK cells as a control. The overexpressed protein was immunoprecipated with anti-EFA6B (lane 3) or anti-VSV-G antibodies (lane 4) and detected by immunoblotting with the corresponding antibodies revealing a protein migrating with an apparent molecular weight similar to the endogenous polypeptide. In contrast, the 180 kDa species was not detected when irrelevant antibodies were used for immunoprecipitation (lanes 5 to 7). Altogether, these results indicate that EFA6B is expressed in Jurkat and BHK cells and migrate with a ~180 kDa apparent molecular weight in SDS-PAGE.

EFA6 and EFA6B are ARF6-specific GEFs associated with dynamic plasmalemmal subdomains

Given the strong similarity between the Sec7 domain of EFA6A and EFA6B, we assessed the ability of EFA6B to catalyse nucleotide exchange by measuring guanine 5′-[γ-thio]-triphosphate binding on recombinant myristoylated ARF6 (mARF6) or mARF1. The data presented in Fig. 3A show that like EFA6A (open square), recombinant EFA6B (with the N-terminal region deleted, i.e. EFA6B/245-1056) stimulated nucleotide exchange on mARF6 in vitro (open diamond). In the same conditions, EFA6A and EFA6B/245-1056 had very low guanine nucleotide exchange activity on mARF1 (Fig. 3B).

The intracellular distribution of EGFP-tagged EFA6A and EFA6B was then compared with that of their substrate ARF6 using immunofluorescence microscopy analysis. As previously shown (Franco et al., 1999), overexpressed ARF6 and EFA6A were found at the plasmalemma. In addition to a homogenous distribution at the cell surface, both proteins appeared to accumulate in subdomains of the dorsal cell surface and cell periphery at specific sites corresponding to microvilli-like protrusions and membrane ruffles, respectively (Fig. 4A,B). A very similar distribution was also observed in the case of EGFP-EFA6B (Fig. 4C,D). The punctuate localisation of EFA6A/EFA6B at the plasma membrane coincided with that of cell-surface glycoproteins stained with wheat germ agglutinin, confirming the surface distribution of the ARF6 GEFs (data not shown). When live BHK cells expressing EGFP-tagged EFA6A were imaged by high-resolution fluorescence video-microscopy, we could observe bright fluorescent ribbon-like structures on the dorsal cell-surface that corresponded to membrane ruffles developing and moving laterally in the plane of the membrane. These structures were highly dynamic, as new ruffles continuously formed

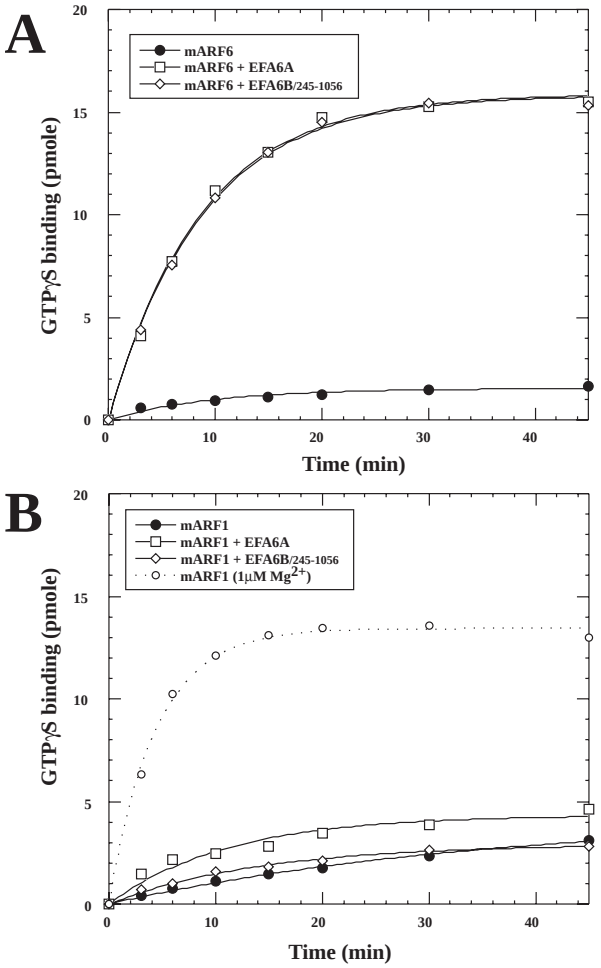


Fig. 3. Measurement of [^{35}S]GTPγS binding to myristoylated ARF6 (mARF6) (A) or mARF1 (B) in the absence (●) or presence of purified recombinant EFA6A (□) or EFA6B/245-1056 (◇). Spontaneous GTPγS binding on mARF1 in low free Mg^{2+} concentration (1 μM) (○).

and collapsed (Fig. 5). Smaller fluorescent structures corresponding to microvilli-like protrusions were also visible on the cell's dorsal surface. Treatment of the cells with latrunculin D totally abolished movement of all these structures, suggesting that actin filament dynamics are essential for movement of EFA6A-enriched microvilli and membrane ruffles (data not shown). Altogether, these findings indicate that both EFA6A and EFA6B GEFs colocalized with their ARF6 substrate at the plasmalemma and suggest that these proteins are enriched in specific subdomains corresponding to membrane ruffle-like structures and dorsal microvilli-like protrusions.

We then compared the distribution of EFA6A and EFA6B with that of F-actin as cortical cytoskeleton is known as a major organizer of epithelial-cell microvilli and plasmalemmal protrusions of non-epithelial cells (Bretscher, 1999). Staining with phalloidin to label filamentous actin and confocal sectioning through the dorsal cell surface demonstrated that EFA6A and EFA6B colocalized with F-actin in membrane ruffles and dorsal microvilli-like protrusions in transfected BHK cells (Fig. 6A-C, Fig. 6M-O, respectively). We next

sought to identify the regions of EFA6A required for the localization in microvilli-like structures. We had previously shown that deletion of the PH domain prevented membrane localization of EFA6A (Franco et al., 1999). Therefore, we expressed the isolated EGFP-tagged EFA6A-PH domain (residues 351-494; Fig. 9A,B), and its localization was analysed by confocal microscopy of fixed BHK cells stained for F-actin. Similar to the EFA6A distribution, the EFA6A PH domain demonstrated accumulation in scattered actin-rich microvilli-like protrusions at the plasma membrane (compare Fig. 6A-C with 6D-F). Therefore, the PH domain makes a major contribution to the localization of EFA6A to plasma membrane subdomains. Interestingly, the distribution of the isolated PH domain from phospholipase Cδ (PLCδ) was similar to EFA6A, whereas the GRP1/ARN03 PH domain was mostly cytosolic in these conditions (data not shown). Given the fact that the PH domain of PLCδ has a high affinity for PtdIns(4,5)P_2 and has been characterized as a marker for membrane-associated PtdIns(4,5)P_2 (Varnai and Balla, 1998), whereas the GRP1 PH domain has a much higher affinity for PtdIns(3,4,5)P_3 than for PtdIns(4,5)P_2 (Kavran et al., 1998), our results suggest that binding of PtdIns(4,5)P_2 to the conserved PH domain of EFA6A is probably a major determinant of membrane attachment and microvilli-like distribution of ARF6 GEFs.

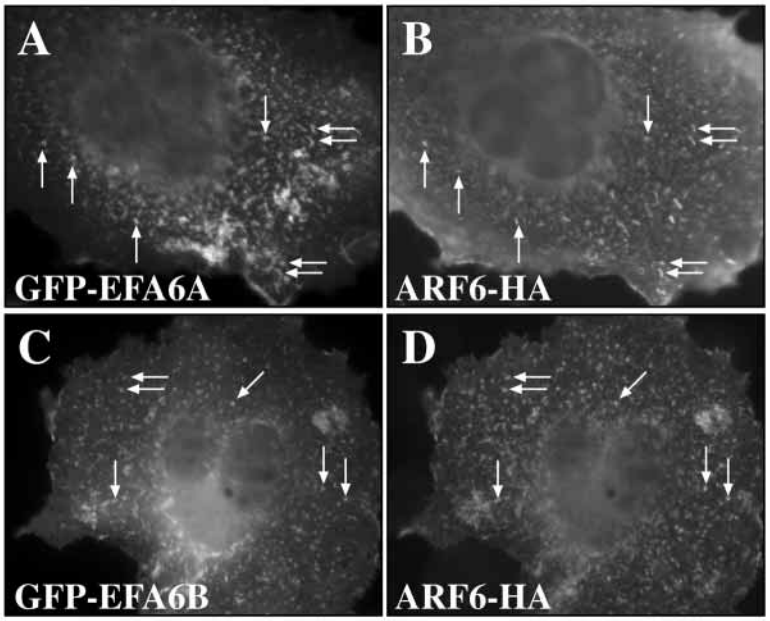


Fig. 4. EFA6A and EFA6B colocalise with ARF6 in microvilli-like structures at the plasma membrane. (A-D) Localization of C-terminally HA-tagged ARF6 and EGFP-EFA6A (A,B), or -EFA6B (C,D) in BHK cells. Micrographs show that surface microvilli-like structures are enriched in ARF6 and EFA6A or EFA6B (arrows). The focus was set at the level of the dorsal plasma membrane. Bar, 10 μm.

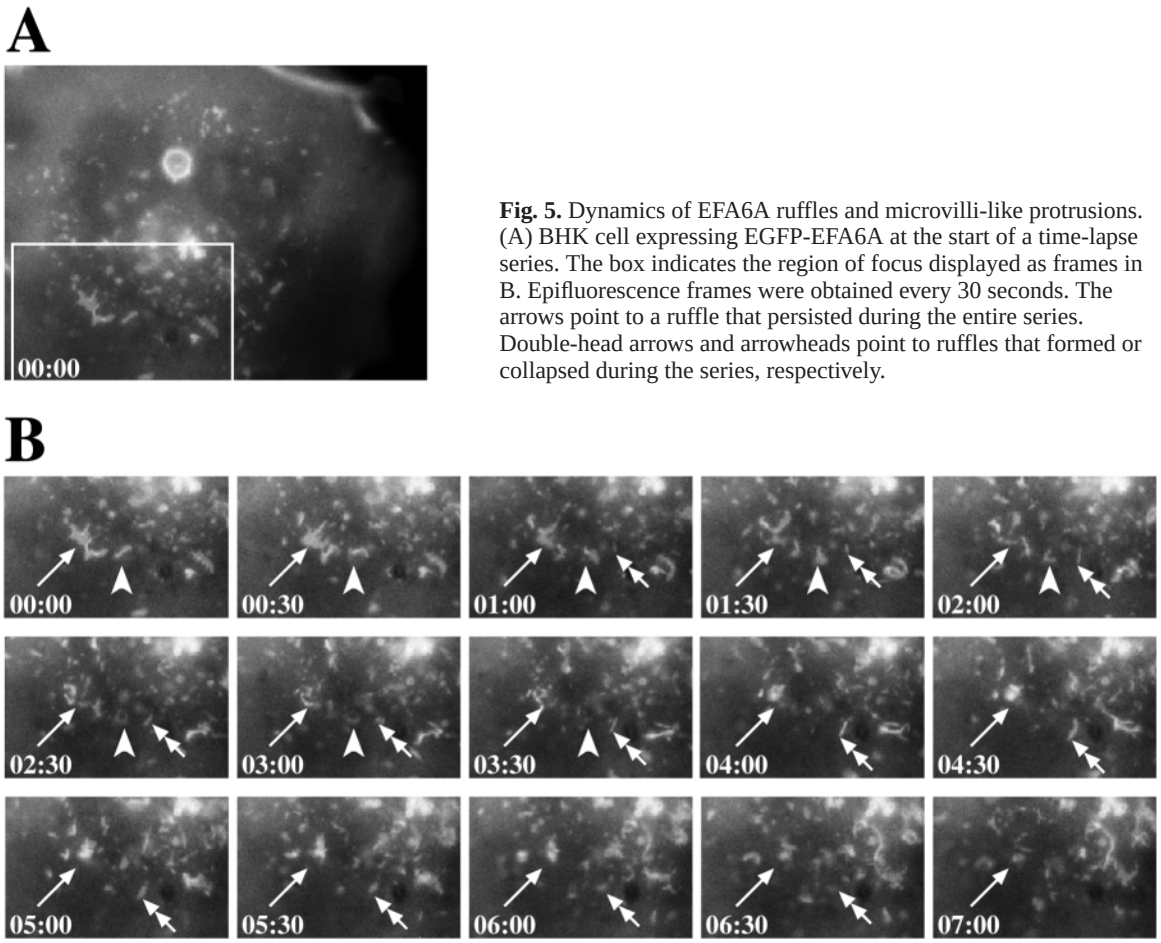
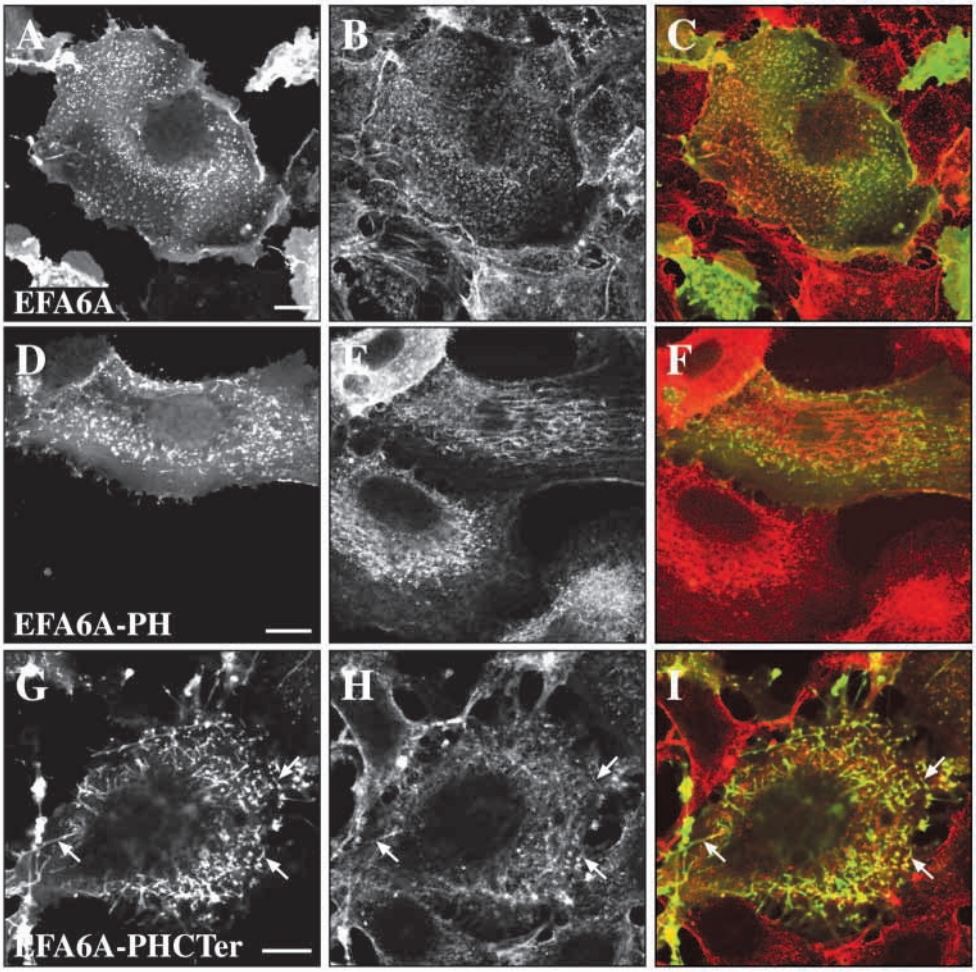


Fig. 5. Dynamics of EFA6A ruffles and microvilli-like protrusions. (A) BHK cell expressing EGFP-EFA6A at the start of a time-lapse series. The box indicates the region of focus displayed as frames in B. Epifluorescence frames were obtained every 30 seconds. The arrows point to a ruffle that persisted during the entire series. Double-head arrows and arrowheads point to ruffles that formed or collapsed during the series, respectively.

The conserved C-terminal coiled-coil motif of EFA6-family GEFs triggers microvilli lengthening



The C-terminal region distal to the PH domain is conserved in all EFA6 family GEFs (Fig. 9A,C; see Discussion), suggesting an important function for this domain. Furthermore, we previously found that this region is required for induction of membrane protrusion upon EFA6A overexpression (Franco et al., 1999). Therefore, constructs corresponding to the EFA6A C-terminal region fused to the PH domain (EFA6A-PHCTer, residues 349-645), or alone (EFA6A-CTer, residues 478-645) were expressed in BHK cells as EGFP fusion proteins, and their distribution was compared with F-actin. EFA6A-PHCTer accumulated in long finger-like extensions that were induced at the dorsal surface of transfected cells (Fig. 6G, arrows). Most of these extensions were clearly stained for F-actin (Fig. 6H,I, arrows). By contrast, the distribution of EFA6A-CTer was diffuse and excluded from intracellular vacuole profiles, suggesting that this construct was mostly cytosolic (Fig. 6J-L). In addition, EFA6A-CTer did not induce any morphological change in the transfected cells. These results indicate that the C-terminal region of EFA6A, when bound to the membrane via the PH domain, is able to trigger the formation of finger-like extensions of the plasma membrane. Similarly, a construct corresponding to the isolated PH domain and C-terminal region (PHCTer) of EFA6B (residues 752-1056) induced the formation of finger-like extensions and was found within these structures (Fig. 6P-R, arrows). Cells expressing the EFA6B-CTer construct with the PH domain deleted (residues 879-1056) exhibited diffuse cytosolic staining without morphological change (data not shown). We also noticed that EFA6B-PHCTer demonstrated a strong nuclear accumulation (Fig. 6P), whereas neither full-length EFA6B nor EFA6BCter showed such a distribution (Fig. 6M) (data not shown). A possible explanation is that a cryptic nuclear targeting motif may become exposed in EFA6B-PHCTer. Altogether, these findings suggest a role for the conserved C-terminal regions of EFA6A and EFA6B in mediating morphological changes at the plasma membrane when bound to the membrane via the adjacent PH domain in the absence of Sec7 domain activity.

The morphology of these structures was further characterized by examining the surface of EFA6A and EFA6A-PHCTer-expressing cells by scanning electron microscopy. Transfected cells were identified using antibody-coupled beads recognizing the extracellular region of a transmembrane protein that was co-expressed with the EFA6A constructs (see the Materials and Methods section). In general, EFA6A-expressing cells had more microvilli-like protrusions on their dorsal surface than control cells expressing the transmembrane protein alone, but these structures were of similar size (compare Fig. 7A,B with 7C,D). Strikingly, expression of EFA6A-PHCTer triggered the appearance of a ‘hedgehog-like’ morphology with the cell surface being covered by long (1-3 μm length) finger-like membrane extensions. Longer extensions, which may have been flattened during the fixation procedure, were laid down on the cell surface (Fig. 7E,F, arrows), whereas smaller structures were stood up (double-head arrows). The diameter of the longer structures was similar to microvilli-like protrusions of control cells or cells overexpressing native EFA6A, suggesting that EFA6A-PHCTer increased the length of pre-existing microvilli-like structures (arrows). Altogether, these findings indicate that overexpressed EFA6A accumulates in subdomains of the plasma membrane where it promotes formation of actin-rich plasmalemmal microvilli-like protrusions. In addition, they suggest that membrane attachment of the C-terminal region of EFA6A in these subdomains via the PH domain induces a dramatic remodelling of the plasma membrane by promoting lengthening of these microvilli.

In order to establish the domain(s) of the C-terminal region

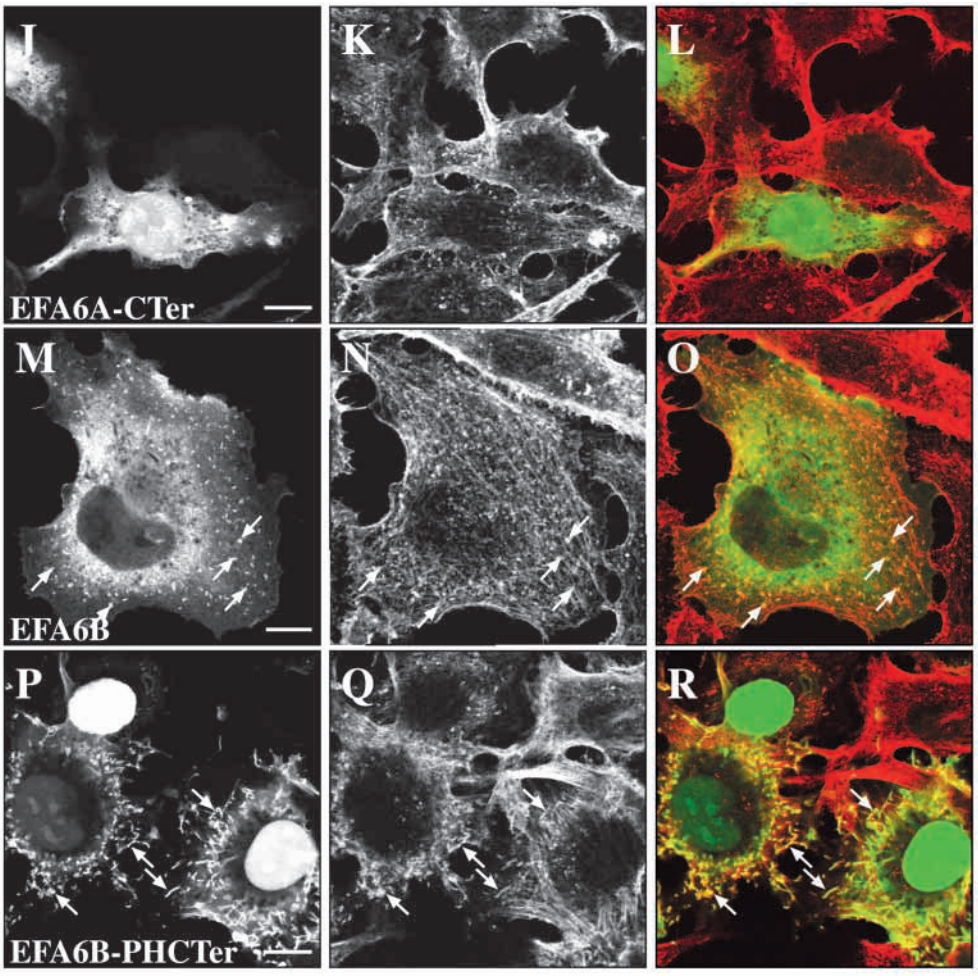


Fig. 6. Effect of EFA6A and EFA6B variants on cortical actin cytoskeleton morphology. BHK cells were transfected with EGFP-tagged EFA6A (A-C); EFA6A variants corresponding to the isolated PH domain (EFA6A-PH, D-F); the C-terminal region including the PH domain (EFA6A-PHCTer, G-I); the C-terminal region with the PH domain deleted (EFA6A-CTer, J-L), EFA6B (M-O); or the C-terminal region and adjacent PH domain of EFA6B (P-R). After fixation, cells were stained with Texas-Red-conjugated phalloidin to visualize actin filaments (B,E,H,K,N,Q). Localization of EGFP constructs is shown in panels A,D,G,J,M, P. Panels C,F,I,L,O,R show the superimposed images (EGFP constructs are show in green, and F-actin is shown in red). Confocal sections were taken in the dorsal plane of the cells. Bar, 10 μm.

required for microvilli lengthening, we assessed the effect of constructs derived from EFA6A-PHCTer-presenting serial truncations from the C-terminus. These constructs expressed as GFP-fusion protein (PHCT1-3, starting at position 349 and ending at position 617, 587, and 547, respectively) were able to induce dorsal finger-like extensions (Fig. 8A-C), except for the shortest one (PHCT4, residues 349-520), which had the putative coil-coiled motif deleted (Fig. 8D), indicating that the conserved C-terminal coil-coiled region is essential for microvilli lengthening.

Discussion

The EFA6 GEF family comprises proteins conserved in multicellular organisms throughout evolution. In humans, this family consists of four members. Besides the prototypic EFA6 member (Franco et al., 1999), which we call EFA6A, and EFA6B, which we have characterized in this study, databases contain sequences of EFA6C [accession no. AL136559771 (Wiemann et al., 2001)] and EFA6D [clone KIAA0942 (Nagase et al., 1999)]. At the transcriptional level, EFA6A expression is restricted to the brain and intestine (Perletti et al., 1997) (this study), whereas EFA6B is broadly expressed but not detectable in the brain. Finally, EFA6C and -D are mostly expressed in the brain, although on the basis of the data available at the Huge Database, EFA6D mRNA is also detected in the liver, kidney, testis and ovary. The hallmark of human EFA6 family proteins is a conserved C-terminal module comprising a Sec7 domain and an adjacent PH domain, followed by a ~150 amino-acid C-terminal region containing a putative coiled-coil motif (Fig. 9A). The C-terminal module is also conserved throughout evolution: in *C. elegans* for which the complete genome is available, there is only one EFA6 family member called Y55D9A.1, and this also the case, so far, in *Drosophila* (CG6941). In contrast, N-terminal regions of EFA6 family proteins are divergent. Here, we show that each of the three structural domains of the C-terminal module of human EFA6A/B contributes to a distinct function.

EFA6 family members contain a ~200 amino-acid Sec7 domain positioned right in the centre of the protein (Fig. 9A). The Sec7 domain is found in all ARF GEFs described so far. Human EFA6 Sec7 domains are 60-70% identical to each other and only ~30-35% identical to ARNO/cytohesin Sec7 domains (Fig. 1B,C; Table 1). As we previously reported in the case of EFA6A (Franco et al., 1999; Macia et al., 2001), EFA6B exhibits GEF activity in vitro only with ARF6 (class III ARF) and not with ARF1 (class I ARF), raising the question of how EFA6 GEFs discriminate between two ARF proteins that share 67% sequence identity. Determination of the three-dimensional structure of a complex between yeast Gea2 Sec7 domain and human ARF1 revealed that residues from switch-1 and -2 regions of ARF1 insert in a hydrophobic groove within the

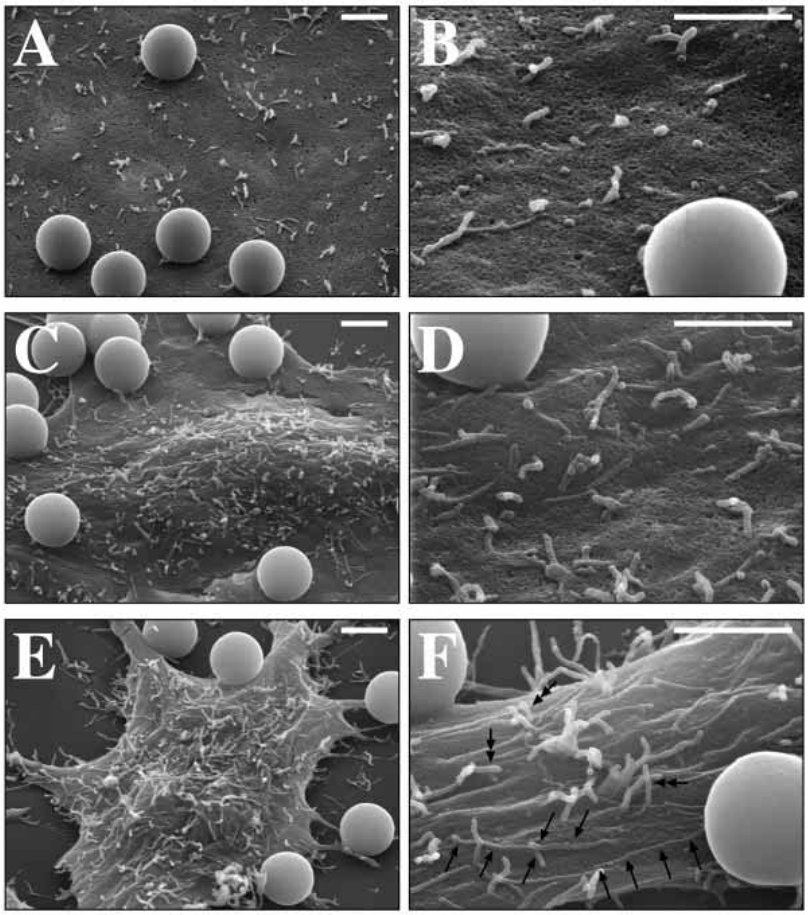


Fig. 7. Scanning EM analysis of microvilli-like protrusions in BHK cells expressing EFA6 or EFA6-PHCTer. BHK cells were transfected to express a CD25-derived transmembrane protein (see Materials and Methods) alone (A,B) or together with EFA6A (C,D) or EFA6A-PHCTer (E,F). Transfected cells were identified using anti-CD25-coated latex beads. EFA6A-expressing cells exhibited microvilli with a comparable length but the microvilli were present at a higher density than in control cells. EFA6A-PHCTer-expressing cells show a 'hedgehog-like' morphology with numerous, long finger-like structures covering the cell surface. The arrows point at lengthened microvilli structures laying down on the surface of EFA6A-PHCTer-expressing cells. Double-head arrows indicate smaller erected microvilli-like protrusions. Bars, 2 μm.

Sec7 domain (Béraud-Dufour et al., 1998; Goldberg, 1998; Mossessova et al., 1998). This groove, formed by a loop between α helices F and G (motif 1; Fig. 1B) and a central portion of helix H (motif 2) (Cherfils et al., 1998; Mossessova et al., 1998) is conserved in all known Sec7 domains (Fig. 1B; invariant residues are highlighted in green, and conserved residues in yellow). The invariant glutamate residue in motif 1 (in blue in Fig. 1B) is critical for guanine nucleotide exchange, and it acts by inserting directly in the ARF nucleotide-binding pocket and thereby promoting nucleotide dissociation (Béraud-Dufour et al., 1998; Goldberg, 1998; Mossessova et al., 1998). By contrast, some residues that are identical in EFA6-family Sec7 domains (highlighted in purple in Fig. 1B) differ in other Sec7-domain-containing GEFs. These residues may contribute to ARF substrate specificity. In addition, although the amino-acid sequence of the switch-1/2 regions of ARF1 and ARF6 are very similar (Tsuchiya et al., 1991), the recent determination of the three-dimensional structure of GDP-bound ARF6 has revealed differences from the structure of GDP-ARF1 that may explain its specificity (Ménétrey et al., 2000).

Adjacent to the Sec7 domain, there is a PH domain whose sequence is highly conserved amongst EFA6 family members. (The EFA6A PH domain is ~70% identical to EFA6C/EFA6D, ~50% identical to EFA6B/CG6941 and ~40% identical to Y55D9A.1.) This domain is most

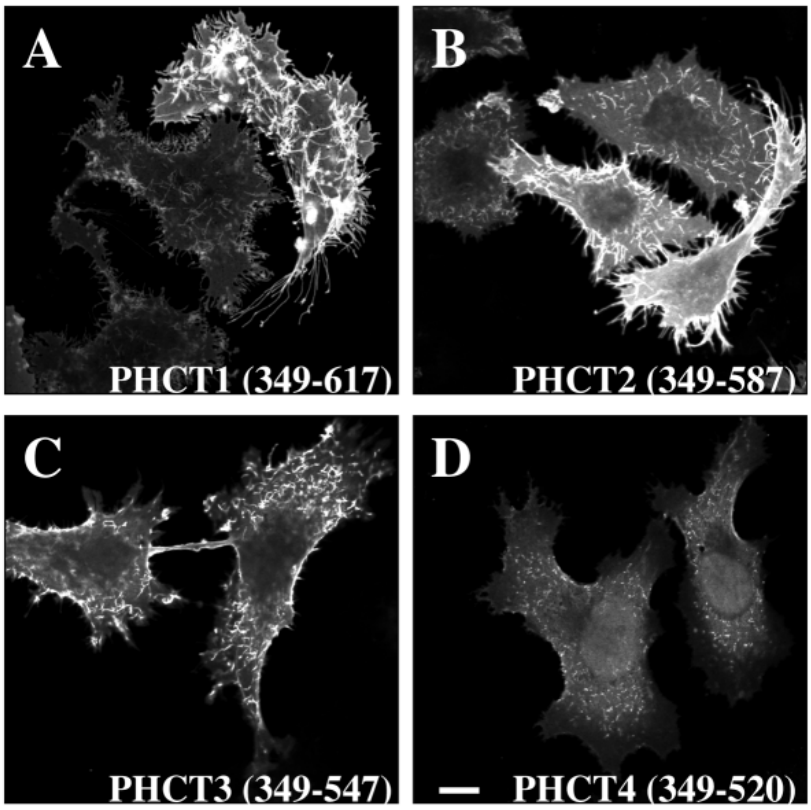


Fig. 8. Localization of EGFP-EFA6A-PHCT1 (A), PHCT2 (B), PHCT3 (C) and PHCT4 (D) in BHK cells. (These constructs, which include the PH domain, start at position 349 and their C-terminus is indicated, see also Fig. 9C). Expression of PHCT1-3 constructs induced the formation of finger-like protrusions on the dorsal cell surface. By contrast, deletion of the conserved C-terminal coiled-coil motif in PHCT4 prevented the induction of these structures, and this construct accumulated in short microvilli-like protrusions. Confocal sections were taken in the dorsal plane of the cells. Bar, 10 μm.

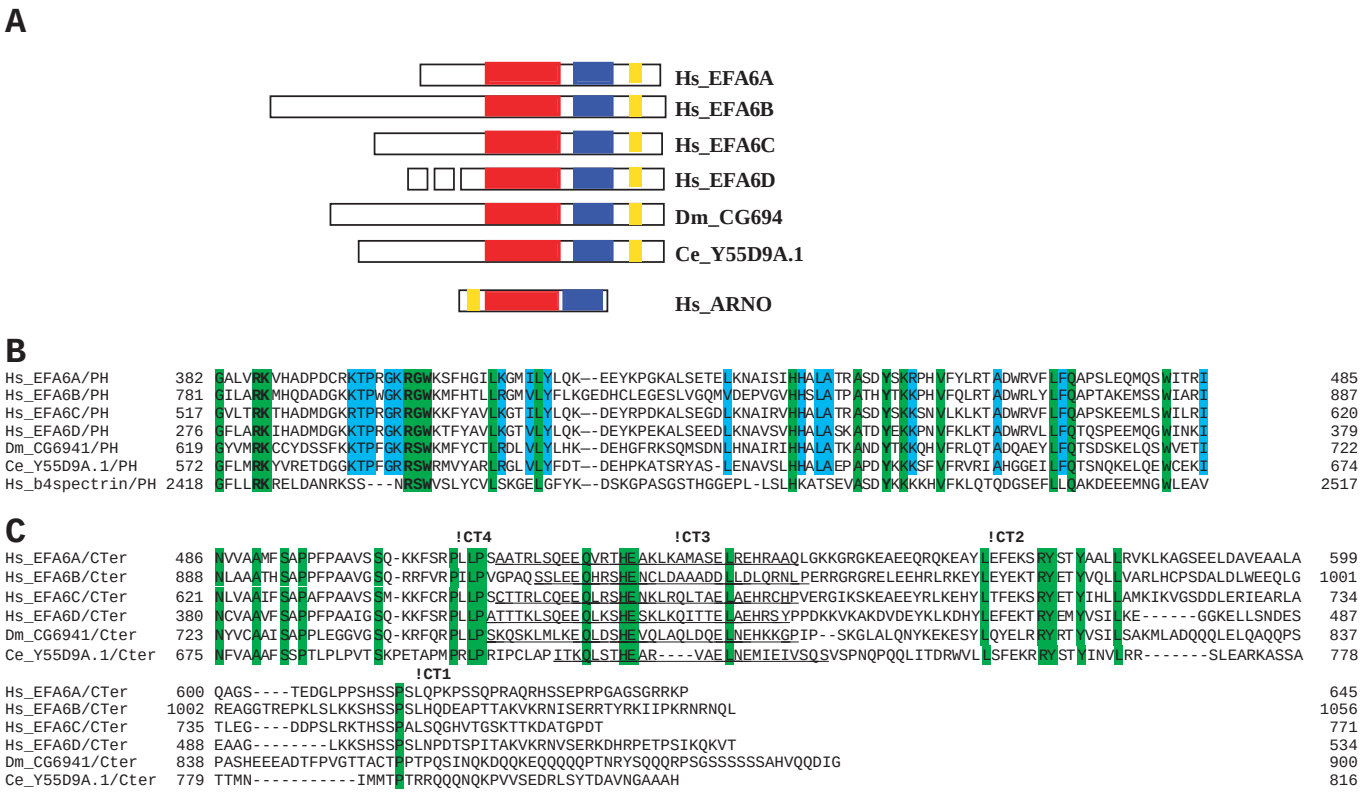


Fig. 9. Overall organization of EFA6 family GEFs. Alignment of the Sec7 domain, PH domain and C-terminal region sequences. (A) A schematic structure of the EFA6 family and ARN0 GEFs. Sec7 domains, PH domains and putative coiled-coil motifs are depicted by red, blue and yellow boxes, respectively. (B) Amino-acid sequences of the PH domains of human EFA6 family members, *D. melanogaster* CG6941 gene product, *C. elegans* Y55D9A.1 and human beta IV Σ1 spectrin (Berghs et al., 2000) were aligned using Clustal W. Conserved residues in the seven PH domains are highlighted in green. Residues of the spectrin PH domain involved in interactions with inositol (1,4,5)P_3 phosphates (Hyvonen et al., 1995), and which are conserved in EFA6 family PH domains, are shown in bold. Invariant or similar residues amongst EFA6 family PH domains are highlighted in blue. (C) Amino-acid sequences of the conserved C-terminal regions of EFA6-family GEFs were aligned using Clustal W. Invariant residues are highlighted in green. Putative coil-coiled motifs identified using COILS are underlined. The terminus of C-terminal deletion variants of EFA6A (designed CT1 to 4) is indicated by an exclamation mark.

similar to the human βIV spectrin PH domain (Berghs et al., 2000) (34% identity; Fig. 9B). The well characterized PH domain of β-spectrin binds to membrane PtdIns(4,5)P_2 with high affinity, and residues known to form critical contacts with the PtdIns(4,5)P_2 head group in β-spectrin and PLC δ PH domains (Hyvonen et al., 1995; Rameh et al., 1997) are strikingly conserved in all EFA6 family PH domains (Fig. 9B, bold characters). In fact, several observations suggest that the PH domain of EFA6 GEFs supports membrane association by interacting with PtdIns(4,5)P_2. First, we previously reported that deletion of a region of EFA6A comprising its PH domain prevented membrane localization (Franco et al., 1999). Second, we have now observed that overexpressed EFA6A- and PLC δ-PH domains have similar a distribution in BHK cells (data not shown). Third, we found that EFA6A and EFA6B (as well as their isolated PH domain) accumulate in dynamic actin-rich membrane ruffles and microvilli-like structures. Similarly, the GFP-tagged PLC δ-PH domain, which was used to follow the dynamics of PtdIns(4,5)P_2 in living cells (Varnai and Balla, 1998), revealed non-uniform fluorescence in distinct structures identified as ruffles and microvilli-like protrusions from the dorsal cell surface (Tall et al., 2000). Altogether, our findings indicate that EFA6A and EFA6B localize to subdomains of the plasma membrane, probably through interaction of their PH domain with membrane-bound PtdIns(4,5)P_2. This localization may reflect a non-uniform distribution of PtdIns(4,5)P_2, which appears to be enriched in highly dynamic regions of the plasma membrane (Tall et al., 2000). Finally, it is also quite remarkable that the PH domain of ARNO/cytohesin GEFs (specially in its diglycine form) (Cullen and Chardin, 2000; Klarlund et al., 2000) is highly selective for PtdIns(3,4,5)P_3. Accordingly, membrane recruitment of ARNO/cytohesin GEFs was found to require phosphoinositide 3-kinase activation (Gray et al., 1999; Venkateswarlu et al., 1998). Given the fact that ARNO has been shown to activate ARF6 to some extent (Frank et al., 1998a), these and our findings, which point to distinct regulatory mechanisms for membrane association and activation for EFA6 versus ARNO/cytohesin family members, suggest that various signalling pathways may operate through distinct GEF families resulting in ARF6 activation.

In addition to the PH domain, all EFA6 family GEFs share a conserved ~150 amino-acids C-terminal region, which includes a ~30 amino-acid motif with a predicted coiled-coil structure (Fig. 9A,C). This conservation, combined with our initial observation that deletion of this region abolished EFA6-induced actin cytoskeleton reorganization (Franco et al., 1999), points to an essential function for the C-terminus. In agreement with this assumption, we found that overexpression of the C-

terminal region of EFA6A/B fused to the adjacent PH domain triggers lengthening of microvilli-like structures at the plasma membrane, and this requires the integrity of the putative coiled-coil motif. By contrast, the C-terminal region of EFA6A (or EFA6B, data not shown), expressed without the PH domain, accumulates in the cytosol and is ineffective in inducing the 'hedgehog-like' morphology, indicating that membrane targeting of the C-terminal region is required for microvilli lengthening. The possibility that microvilli lengthening results from dominant inhibition of EFA6A (or EFA6B)-GEF activity, and hence inhibition of ARF6 activation by the overexpressed C-terminus is unlikely, as expression of a dominant inhibitory mutant form of ARF6 (ARF6T27N), which also results in ARF6 inactivation, does not induce the 'hedgehog-like' phenotype (data not shown). In addition, microvilli lengthening is abolished when the PH domain/C-terminal module of EFA6A (PHCter) is expressed together with a constitutively activated mutant of ARF6 (ARF6Q67L; data not shown). Altogether, our data are consistent with a dual function for EFA6 GEFs as shown by ARF6 activation by the Sec7 domain and the direct effect of the C-terminus on the actin cytoskeleton in microvilli-like structures. Uncoupling these two functions either by deletion of the C-terminal region or by deletion/mutagenesis of the Sec7 domain, prevents membrane ruffling formation or triggers microvilli lengthening, respectively [(Franco et al., 1999), this study]. Although the mechanism underlying the regulation of actin cytoskeleton reorganization by the conserved C-terminal region is presently unknown, the fact that it requires the integrity of the coiled-coil motif is remarkable. Coiled coils are α helical structures involved in homo- or heterooligomeric association of helices (Lupas, 1996). So far, we have not been able to detect EFA6 oligomers (C.C. and P.C., unpublished), and we are actively searching for proteins interacting with this region of EFA6 that could control the morphology of microvilli-like structures.

This study suggests that EFA6 family proteins could influence the composition and organization of membrane subdomains at the cell cortex by coordinating phosphoinositide signalling and ARF6-mediated events on membrane remodelling. Our results should help clarify the function of EFA6 family GEFs in cellular processes requiring membrane movement to the cell surface and actin cytoskeleton remodeling, such as regulated exocytosis or phagocytosis, which are all known to depend on ARF6 activity (Bajno et al., 2000; Galas et al., 1997; Zhang et al., 1998).

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