Proteomic Identification and Functional Characterization of a Novel ARF6 GTPase-activating Protein, ACAP4*

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ARF6 GTPase is a conserved regulator of membrane trafficking and actin-based cytoskeleton dynamics at the leading edge of migrating cells. A key determinant of ARF6 function is the lifetime of the GTP-bound active state, which is orchestrated by GTPase-activating protein (GAP) and GTP-GDP exchanging factor. However, very little is known about the molecular mechanisms underlying ARF6-mediated cell migration. To systematically analyze proteins that regulate ARF6 activity during cell migration, we performed a proteomic analysis of proteins selectively bound to active ARF6 using mass spectrometry and identified a novel ARF6-specific GAP, ACAP4. ACAP4 encodes 903 amino acids and contains two coiled coils, one pleckstrin homology domain, one GAP motif, and two ankyrin repeats. Our biochemical characterization demonstrated that ACAP4 has a phosphatidylinositol 4,5-bisphosphate-dependent GAP activity specific for ARF6. The co-localization of ACAP4 with ARF6 occurred in ruffling membranes formed upon ARF4 and epidermal growth factor stimulation. ACAP4 overexpression limited the recruitment of ARF6 to the membrane ruffles in the absence of epidermal growth factor stimulation. Expression of GTP hydrolysis-resistant ARF6T27N resulted in accumulations of ACAP4 and ARF6 in the cytoplasmic membrane, suggesting that GTP hydrolysis is required for the ARF6-dependent membrane remodeling. Significantly the depletion of ACAP4 by small interfering RNA or inhibition of ARF6 GTP hydrolysis by overexpressing GAP-deficient ACAP4 suppressed ARF6-dependent cell migration in wound healing, demonstrating the importance of ACAP4 in cell migration. Thus, our study sheds new light on the biological function of ARF6-mediated cell migration.

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During cell migration, coordination between membrane traffic, cell substrate adhesion, and actin reorganization is required for protrusive activity to occur at the leading edge of migrating cells (e.g., Ref. 1). Actin organization is regulated by small molecular weight GTPases such as the Rho and ARF1 protein families (e.g., Refs. 1 and 2). Recent findings show that a number of multidomain proteins characterized by an ARF GAP domain interact with both actin-regulating proteins as well as affect Rac-mediated protrusive activity and cell migration (3, 4). The ARF proteins are GTPases that function as regulators of membrane trafficking (5). ARF6, the sole member of the class III ARF family, resides on endosomes and the plasma membrane as well as regulates membrane trafficking between these compartments (6–9). ARF6 functions as a switch via GTP-GDP exchange and GTP hydrolysis. Correspondingly studies of its function have used expression of dominant negative GTP binding-deficient mutants (e.g., ARF6T27N) or of constitutively active GTPase-deficient mutants (e.g., ARF6G67L) to perturb membrane trafficking (e.g., Ref. 2). Activation of ARF6 promotes peripheral actin assembly (7), plasma membrane protrusions and invaginations (8, 9), and pinocytosis (10). However, the molecular mechanisms that direct membrane trafficking at sites of adhesion and actin cytoskeletal rearrangement at the cell edge during cell migration are still unknown. Given the participation of ARF proteins in regulating membrane trafficking, one appealing hypothesis is that ARF GTPase-activating proteins act as molecular devices that coordinate membrane traffic and cytoskeletal reorganization during cell motility in an ARF6-selective fashion. Although recent studies confirmed the involvement of ARF6 in tumor cell migration and invasion (11, 12), the mechanistic link between ARF6 function in cell migration remains to be established. Here we used proteomic analysis to identify proteins specifically interacting with an active form of ARF6 and discovered a novel ARF6-specific GTPase-activating protein, ACAP4. Our functional characterization indicated that ACAP4 integrates ARF6 function into cell migration.

1 The abbreviations used are: ARF, ADP-ribosylation factor; GAP, GTPase-activating protein; PH, pleckstrin homology; PIP₂, phosphatidylinositol 4,5-bisphosphate; EGF, epidermal growth factor; siRNA, small interfering RNA; ANK, ankyrin; HA, hemagglutinin; GFP, green fluorescence protein; HEK, human embryonic kidney; PA, phosphatidic acid; DMEM, Dulbecco’s modified Eagle’s medium.
The resulting supernatant was incubated with 30 μl of amylase-ARF6 beads at room temperature for 2 h. The beads were then washed with lysis buffer three times followed by boiling in 1× sample buffer. The samples were resolved on a 6–16% gradient SDS-polyacrylamide gel, and the protein band of interest was removed for mass spectrometric analyses as described previously (14).

Preparation of Samples for Mass Spectrometry—In-gel digestion was done essentially as described previously (14). Briefly protein band P1 was excised, chopped into small fragments with a razor blade, destained, and subjected to digestion by modified porcine trypsin (50–100 ng/digestion; Promega, Madison, WI). Peptides were recovered by three extractions of the digestion mixture with 50% acetonitrile plus 5% trifluoroacetic acid and desalted and concentrated using C18 ZipTips (Millipore Corp., Bedford, MA), eluting peptides in 50% (v/v) acetonitrile/water. All supernatants were pooled and concentrated to 5 μl in a SpeedVac and brought back up to 25 μl in 50% acetonitrile, 5% trifluoroacetic acid. The peptide mix was stored at −20 °C until analysis.

MALDI-TOF Mass Spectrometric Identification of a Novel ARF6 GTPase-activating Protein, ACAP4—Aliquots of unseparated tryptic digests were co-crystallized with cyano-4-hydroxycinnamic acid and analyzed using a MALDI delayed extraction reflector TOF instrument (Voyager DE-STR mass spectrometer; Perseptive Biosystems, Framingham, MA) equipped with a nitrogen laser. Measurements were performed in a positive ionization mode. All MALDI spectra were externally calibrated using a standard peptide mixture (Sigma). Some PSD spectra were acquired on a TofSpec SE MALDI-TOF mass spectrometer (Micromass, Manchester, UK).

Data base interrogations based on experimentally determined peptide masses were carried out using MS-Fit, and PSD data interrogation was performed using MS-Tag; both software programs were developed in the University of California San Francisco Mass Spectrometry Facility and are available on the World Wide Web at prospector.ucsf.edu. Both the National Center for Biotechnology Information protein data base and Swiss-Prot data base were searched. Search parameters included the putative protein molecular weight and a peptide mass tolerance of 100–200 ppm.

Sequence Analysis—Plekstrin homology (PH), ARF GAP, and ankryn (ANK) repeat domains were identified with the program Pfam run at the Sanger Center and with ProfileScan run on the Institut Suisse de Recherche Experimentale sur le Cancer server (15). As described previously, the predicted ANK repeats agree, to a large extent, with the published structure for the ARF GAP and ANK repeat domains of PyK2-associated protein (16). Coiled coils were identified with the program COILS (17), which was accessed through the ExPASy Molecular Biology Server of the Swiss Institute for Bioinformatics and run with the MTIDK matrix (matrix derived from myosins, tropomyosins, intermediate filaments, desmosomal proteins, and kinases). A 28-residue window was used to detect potential coiled coil domains, and a 21-residue window was used to identify the ends. A probability of 0.6 with less than a 25% change with weighting was used as a cutoff. ACAP1 and ACAP2 sequences were aligned using the GAP module within a Genetics Computer Group run on the National Center for Biotechnology Information (NCBI) server. Multiple alignments were performed by ClustalW on the NCBI server.

MATTERIAls AND METHODS

Affinity Chromatography—GST fusion proteins for ARF6 were produced and purified as described previously (13). GST-ARF6(G67L) was used as an affinity matrix to isolate proteins interacting with active ARF6 from HeLa cells, whereas GST-ARF6(G27N) was used as control. Briefly, interphase HeLa cells were extracted with lysis buffer containing 50 mM Tris-HCl (pH 8.9), 100 mM NaCl, 0.1% Triton X-100 plus proteinase inhibitor mixture (pepstatin A, leupeptin, aprotinin, and chymostatin at final concentrations of 5 μg/ml each). The cell lysates were clarified using an Eppendorf centrifuge at 13,000 rpm for 10 min. The resulting supernatant was incubated with 30 μl of amylase-ARF6 beads at room temperature for 2 h. The beads were then washed with lysis buffer three times followed by boiling in 1× sample buffer. The samples were resolved on a 6–16% gradient SDS-polyacrylamide gel, and the protein band of interest was removed for mass spectrometric analyses as described previously (14).

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Molecular Biology and Protein Expression—Human clones of ARF1 and ARF5 were obtained from a HeLa cell cDNA library, and ARF6 was a gift from Drs. Julie Donaldson and Harish Radhakrishna (National Institutes of Health). ACAP4 was purchased from Open Biosystems (Huntsville, AL). Point mutations were introduced using the QuikChange method (Stratagene). The following mammalian expression constructs were generated using HA (Sigma), 3×FLAG (Sigma), and pEGFP-N1 (BD Biosciences): constitutively active (Q71L) and dominant negative (N126I) ARF1 mutants, constitutively active (Q71L) and dominant negative (N126I) ARF5 mutants, constitutively active (Q67L) and dominant negative (T27N) ARF6 mutants, p3×FLAG-tagged full-length ACAP4, GAP-deficient mutants (R469G), and two deletion mutants (ΔPH and ΔGAP). Site-directed mutation was done using a standard molecular biology protocol. All constructs were confirmed by DNA sequencing.
terminated by diluting the samples into ice-cold 10 mM Tris, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT. The purified FLAG-ACAP4 proteins were added to the GAP assay at 0.3 μM to avoid the saturation of enzyme. Protein-bound nucleotide was trapped on nitrocellulose filters as described previously (e.g., Ref. 19), and the nucleotide was then extracted from the filters into 1 ml of 2 M formic acid. This treatment was shown to release 95–100% of the filter-bound radioactivity without changing the nature of radiolabeled nucleotide (e.g., Ref. 19). Nucleotides were fractionated by thin layer chromatography on a polyethyleneimine cellulose plate developed in 1 M LiCl, 1 M formic acid. Quantification of GDP and GTP was carried out by scintillation counting or on a PhosphorImager (Amersham Biosciences).

Phospholipids were solubilized in Triton X-100 and added as mixed micelles. Briefly phospholipids (PIP₂ and PA) in 95:5 chloroform/methanol were dried under a stream of nitrogen. To prepare liposomes, the lipids were then resuspended in 25 mM Tris, pH 8.0, followed by sonication in a G112SP1 ultrasonic cleaner (Laboratory Supplies Co., Hicksville, NY) for 5 min. To prepare micelles, the lipids were solubilized in 1% Triton X-100. The final concentration of Triton X-100 in GAP assay was 0.1% (v/v). The experiments revealed that half-maximal ACAP4 GAP activity was achieved with 10–20 μM PIP₂ in the presence of 50 μM PA. The GAP activity reported was normalized to the amount of ACAP4 protein quantified by Western blot analysis.

**Cell Culture and Transfection**—HeLa cells were cultured in Advanced DMEM (Invitrogen) containing 10% fetal bovine serum. ARF6 and ACAP4 derivatives were transfected using Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science) into synchronized HeLa cells (20). Double-stranded 19-nucleotide RNA duplex targeted to ACAP4 (5’-CTCCACAGGACGATATA-3’) was purchased from Dharmaco Research Inc. (Boulder, CO) and used for silencing of ACAP4. As a control, either a duplex targeting cyclophilin or scrambled sequence was used. All small interfering RNA (siRNA) duplexes were transfected as described previously (14). Briefly HeLa cells were grown to ~40% confluency in DMEM with 10% fetal bovine serum at 37 °C in 5% CO₂ followed by transfection of siRNA duplex. Pilot experiments indicated that treatment of 100 nm siRNA duplex for 36 h resulted in a maximal suppression of ACAP4 protein expression. Thus, we used this condition for wound healing migration assay.

Adenoviral infections of HeLa cells were performed 5 h postplating. Cells were grown to 50% confluency and infected with the control virus alone or the recombinant adenoviral constructs incorporating wild type GFP-ACAP4 and GFP-ACAP4ΔR469G mutant. Infection was executed by using 3 × 10⁶ particles/ml of viruses. Cultures were incubated at 37 °C for 12 h and then changed to fresh medium without viruses. We chose our experimental conditions based on the level of expression of GFP-ACAP4 and GFP-ACAP4ΔR469G proteins as determined by intensity of fluorescence measured with a Spex fluorometer and the general appearance of the cells. Direct observation of GFP and subsequent immunostaining indicated that 92 ± 4% of HeLa cells were expressing the ACAP4 constructs.

**Immunoprecipitation**—HeLa cells were grown to ~45% confluency in DMEM with 10% fetal bovine serum at 37 °C in 5% CO₂. Cells were then transfected with HA-tagged ARF1, ARF5, ARF6, and their mutant constructs, respectively, by FuGENE 6 (Roche Applied Science). Cells were collected 22 h after transfection, and cell lysates were prepared in lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 2 mM EGTA, 0.1% Triton X-100) containing protease inhibitor mixture (Sigma) and clarified by centrifugation at 16,000 × g for 5 min at 4 °C. HA-tagged fusion proteins were incubated with HA antibody-coupled agarose beads. The beads were washed five times with lysis buffer and then boiled in SDS-PAGE sample buffer for 2 min. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane for Western blotting of HA tag, ARF6, and ACAP4.

FLAG-ACAP4 proteins were purified from HEK 293 cells transiently transfected to express wild type and mutant ACAP4. Twenty-four hours after transfection, cell lysates were prepared and incubated with agarose beads coupled with FLAG (M2) antibodies (Sigma). The agarose beads were then washed with GAP assay buffer (40 mM HEPES, pH 7.4, 0.1% Triton X-100, 100 mM NaCl, 1 mM EDTA, 2.5 mM MgCl₂, 1 mM GTP, 1 mM DTT) and eluted with FLAG peptide (Sigma) in GAP buffer.

**Immunofluorescence Microscopy**—HeLa cells were fixed in 4% formaldehyde plus 0.025% glutaraldehyde (Sigma). Coverslips were then permeabilized with 0.2% Triton X-100 and blocked with 0.05% Tween 20 in PBS (TPBS) with 1% BSA (Sigma). Cells were incubated with primary antibodies in a humidified chamber for 1 h and then washed three times in TPBS. Texas Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) were used as the secondary antibodies for visualization of appropriate antigens. DNA was stained with 4’,6-diamidino-2-phenylindole (Sigma). Slides were examined with a Zeiss Axiovert 200 fluorescence microscope, and images were collected and analyzed with Image-5 (Carl Zeiss; Ref. 20). ARF6-dependent changes in the actin cytoskeleton were measured in HeLa cells as described previously (e.g., Ref. 4).

**Migration Assay**—For the wound healing migration assay, confluent HeLa cells on coverslips were starved overnight in DMEM with 0.01% BSA. After marring coverslips with a linear scratch by a sterile pipette tip, cells were stimulated with 20% fetal bovine serum at 37 °C for the times indicated. Images were taken with the 10x objective of an inverted microscope (Axiovert200) coupled to an AxioCam-HS digital camera (Carl Zeiss).

**Western Blotting**—Samples were subjected to SDS-PAGE on a 6–16% gradient gel and transferred to nitrocellulose membrane. Proteins were probed with the appropriate primary antibodies followed by 125I-protein A. Immunoreactive signals were visualized by autoradiography on Kodak BioMax MS film for 6–8 h at −80 °C with an intensifying screen as described previously (20).

**Antibodies**—Anti-ACAP1 antibody was generated by immunizing two rabbits with purified His-tagged ACAP4 protein. The rabbit IgG fraction was purified using the affinity beads coupled with GST-tagged ACAP4 protein as described previously (21). In some cases, mouse IgG from ACAP4-immunized mice was used for immunofluorescence and Western blotting. The specificity of the ACAP4 antibodies was tested by Western blotting using HeLa cell lysates. The other antibodies used in this study were as follows: mouse monoclonal anti-ARF6 antibody (Novus Biologicals, Inc; 1:1,000), anti-FLAG M2 gel and mouse monoclonal anti-FLAG antibody M2 (Sigma; 1:2,000), mouse monoclonal anti-εzin antibody 6H111(1:5,000; Ref. 14), rabbit anti-ACAP1 antibodies (1:1,000; Ref. 22), Alexa Fluor 488-conjugated phalloidin (Molecular Probes; 1:500), goat anti-rabbit IgG antibody conjugated with horseradish peroxidase, Texas Red-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-rabbit IgG, and rhodamine-conjugated goat anti-rabbit IgG (Jackson Immunoresearch).

**RESULTS**

**Identification of a Novel GTPase-activating Protein, ACAP4**—Cycling between GTP- and GDP-bound forms of ARF6 regulates the recruitment of various effectors to cellular membrane by which ARF6 modulates cellular dynamics. The binding of GTP to ARF6 results in a conformational change, which leads to an activation of ARF6 (e.g., Refs. 1 and 2). To further understand the role of ARF6 in cellular dynamics and isolate the proteins bound to the GTP-bound "active" ARF6, we generated an affinity matrix-immobilized recombinant...
FIG. 1. Proteomic identification of a novel ARF6-activating protein, ACAP4, from an active ARF6 affinity matrix. A, HeLa cell Triton X-100 extracts were applied to ARF6T27N (GDP-bound-mimicking mutant) or ARF6Q67L (GTP-bound-mimicking mutant) affinity columns. After binding, columns were extensively washed, and bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE. The indicated protein (P1) was extracted from the gel and digested with trypsin, and the amino acid sequence of peptides was determined by MALDI-TOF mass spectrometry.

B, MALDI MS spectrum of the tryptic peptides identified in the 95-kDa protein band P1 from the active ARF6-interacting proteins. Protein band of 95 kDa, noted as P1 in Fig. 1A, was subjected to in-gel trypsin digestion, and resultant peptides were analyzed by MALDI MS. Mass values (m/z) and putative amino acid position assignments are indicated above peaks; assignments were made using the MS-Fit function of the Protein Prospector package.

C, the peptides from mass spectra of protein band P1 in-gel digest match a previously uncharacterized open reading frame, AAH60786, that encodes a 903-amino acid protein of unknown function. We designated AAH60786 as ACAP4 for its structure containing ARF GAP (orange), coiled coil (yellow), ANK repeat (pink), and PH domain (green). Amino acid sequences of ACAP4 with various structural motifs are highlighted as indicated above.

D, schematic diagram of ACAP4 structural features. The structure of ACAP4 was identified using the on-line data bases of conserved protein domains named Pfam, ProfileScan, and COILS. Based on the amino acid sequence comparison, ACAP4 contains coiled coil, PH, ARF GAP, and ANK repeat domains. E, comparison of ACAP4 with other ARF GAP family proteins. F, the GAP sequences were aligned using ClustalW. Identities between ACAP4 and DDF1-GAP are shaded in gray. SH3, Src homology 3.

ACAP4 Controls Cell Migration
TABLE I

Proteins bound to ARF6 GTPase identified by mass spectrometry

Proteins bound to ARF6<sup>Q67L</sup> affinity matrix were fractionated by SDS-PAGE as illustrated in Fig. 1A. Individual bands were removed for in-gel digestion, and the resulting peptides were identified by MALDI-TOF and LC-MS/MS mass spectrometric analyses. The protein names, their predicted molecular mass in Da (MM), and their NCBI accession numbers are indicated. The protein probability score, sequence coverage, sequenced peptides, number of peptides matched, and the total number of peptides detected are also listed.

| Band no. | NCBI accession no. | MM       | Protein identified | Protein probability score | Sequence coverage and sequenced peptides                                                                 | No. of matched | Peptides detected |
|----------|-------------------|----------|--------------------|---------------------------|----------------------------------------------------------------------------------------------------------|----------------|-------------------|
| P2       | gi|12408656         | 81,838   | Calpain I          | 5.3e−011                   | HENAIK
          |       |          |                   |                            | YLGQDYEQLVR
          |       |          |                   |                            | LVFVHSAEGNEFWSALLEK
          |       |          |                   |                            | VNGSYEALSGGSTSEGFEFTGGVTEWYELRK
          |       |          |                   |                            | LEICNLTPDALK
          |       |          |                   |                            | NYPATFWVPQFK
          |       |          |                   |                            | ESGCSFVLALMQK
          |       |          |                   |                            | ARSEQFINLR
          |       |          |                   |                            | ARSEQFINLREVSTR
          |       |          |                   |                            | SEQFINLR
          |       |          |                   |                            | SEQFINLREVSTR
          |       |          |                   |                            | FRLPPGEYVVVFSTEPNKEDDFVL
          |       |          |                   |                            | EFSEK
          |       |          |                   |                            | SMVNLMDRDNGK |
| P3       | gi|38570142         | 77,246   | PALS1              | 6.0e−006                   | 19.7%
          |       |          |                   |                            | EMADVCPGDGLTR
          |       |          |                   |                            | ARDIPLGATVR
          |       |          |                   |                            | NEMDSVIIR
          |       |          |                   |                            | SGLLHEGDEVLIEGIEIR
          |       |          |                   |                            | AHFDYDPDDPYPCR
          |       |          |                   |                            | EGDPDNQPLAGLVPGK
          |       |          |                   |                            | FASAHPHTR
          |       |          |                   |                            | DHYFVSR
          |       |          |                   |                            | QAFEDIAAGK
          |       |          |                   |                            | FIEHGEFEK
          |       |          |                   |                            | NLYGTSIDSVR |
| P4       | gi|46249758         | 67,332   | Ezrin              | 3.7e−008                   | 39%
          |       |          |                   |                            | PKPINVRVTTMDAELFAIQPDN
          |       |          |                   |                            | TTTMDAELFAIQPDNT
          |       |          |                   |                            | TTQLFLGHLHYV
          |       |          |                   |                            | VDK
          |       |          |                   |                            | TIGLRREWYLGLHYNK
          |       |          |                   |                            | EWYFGHLHYV
          |       |          |                   |                            | NK
          |       |          |                   |                            | AKFYPDVAELIQDITOK
          |       |          |                   |                            | EYFPEVADVAELIQDITOK
          |       |          |                   |                            | EGILSDEYCPPETAVLLGSYA
          |       |          |                   |                            | VQAK
          |       |          |                   |                            | DQWEDIQWVHA
          |       |          |                   |                            | ER
          |       |          |                   |                            | IAOQLEMYGINFYEIK
          |       |          |                   |                            | GTDLWLGVALGLN
          |       |          |                   |                            | NIEK
          |       |          |                   |                            | IGFPSWEIR
          |       |          |                   |                            | IGFPWEIRISIFNDK
          |       |          |                   |                            | KAPDFVFYAPR
          |       |          |                   |                            | APDFVFYAPR
          |       |          |                   |                            | ILQLCMGNHEL
          |       |          |                   |                            | YMR
          |       |          |                   |                            | ILQLCMGNHEL
          |       |          |                   |                            | YMR
          |       |          |                   |                            | QAVDQIKSQEQLA
          |       |          |                   |                            | ELAEYTAK
          |       |          |                   |                            | AKEAQQDLK
          |       |          |                   |                            | VR
          |       |          |                   |                            | QRIDEFEAL |
| P5       | gi|22749235         | 74,604   | FLJ38281           | 2.5e−002                   | 4.1%
          |       |          |                   |                            | QCGKAFESCSSSVR
          |       |          |                   |                            | QCGKAFESCSSSVR |

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FIG. 2. **ACAP4 is a novel GTPase-activating protein specific for ARF6.** A, ACAP4 co-precipitates with ARF6 but not ARF1 or ARF5. HeLa cells were transfected with HA-tagged ARF1, ARF5, or ARF6. Cells were then extracted with Triton X-100, and HA-tagged ARF proteins were precipitated with anti-HA monoclonal antibody. Co-precipitating proteins were then immunoblotted for the presence of ACAP4 (upper panel) or HA-tagged ARF proteins (lower panel). B, ACAP4 interacts in vivo with ARF6 in a GTP-dependent fashion. HeLa cells were either mock-transfected (MOCK) or transfected with HA-tagged ARF6, ARF6Q67L, or ARF6T27N. Cells were then extracted with Triton X-100, and HA-tagged ARF6 proteins were precipitated with anti-HA monoclonal antibody. Co-precipitating proteins were then immunoblotted for the presence of ACAP4 (upper panel) or ARF6 proteins (lower panel). C, ACAP4 displays an ARF6-dependent GAP activity. GAP assays were performed using affinity-purified FLAG-ACAP4 protein expressed in HEK 293 cells as described under “Materials and Methods.” Bacterially recombinant GST-ARF6 (2 μg) was used in each reaction that included 10 μM PIP2, 50 μM PA, and 1 μg of ACAP4. Affinity-purified FLAG-ACAP4 proteins are shown in the upper panel; GAP activity was judged by the catalysis of GTP to GDP in thin layer chromatography (lower panel). A conserved Arg in the GAP domain is essential for ACAP4 activity. D, the ARF6-activated GAP activity was quantified and normalized to the amount of ACAP4 proteins analyzed by Western blotting. The error bars represent S.E.; n = 3 preparations. E, PI(2,5)P2 and PA
ACAP4 Controls Cell Migration

ARF6<sup>G67L</sup> protein from bacteria and carried out a proteomic search for proteins in HeLa cell extracts that bind to the active form of ARF6. To distinguish the putative ARF6-binding proteins from nonspecific binding, we compared eluates from ARF6<sup>G67L</sup> and ARF6<sup>T25N</sup> affinity columns by SDS-PAGE. As shown in Fig. 1A, several major protein bands including a 95-kDa protein (indicated as P1) were found to be reproducibly enriched on the ARF6<sup>G67L</sup> matrix. For protein identification, the P1 along with four other bands was removed from the acrylamide gel and digested in-gel by trypsin, and the resulting peptide fragments were extracted and analyzed by MALDI-TOF MS (Fig. 1B). The most abundant peptides were chosen for PSD analysis to obtain sequence data and identify the peptides using the MS-Tag program. For example, the PSD spectrum of the peptide, corresponding to an m/z of 1,926.018, uniquely identified its sequence as AIHSSGLGHTQVENEEQYR, amino acids 61–77 of a previously uncharacterized open reading frame of AAH60786. As highlighted in Fig. 1C, 10 additional peptides (e.g. GAGLAREEILEGDOAILQR, VTGGIPGEVDAQMQVR, AQAQLRPFFIEK, LAASVHALHQAEDELOQ, YGCLTISHTHPPVK, LTLLTCQVRNPREEK, KCFDLVTHNR, and DYIMKYVEHR) were identified as tryptic fragments of AAH60786 on the basis of MALDI MS and PSD analysis. These peptides account for ~17% of the sequence of AAH60786 and span the NH<sub>2</sub>–terminal half of the protein. We summarize the mass spectrometric identification of other proteins bound to active ARF6 protein in Table I.

AAH60786 encodes a 903-amino acid protein of unknown function. Computational analysis show that AAH60786 contains one putative ARF GTPase-activating protein domain, one PH domain, two ankyrin repeats, and two coiled coil regions in its amino-terminal half (Fig. 1, C and D). Because AAH60786 distinguishes it from other ARF-activating proteins such as Arf GAPs containing SH3, ANK repeat, and pH domain; Arf GAPs and Rho GAPs with ankyrin repeat and pH domains; and PAG3 but shows similarity to ACAPs, we reasoned that a region of AAH60786 other than the PH domain of ACAP4 did not alter the GAP activity. Therefore, ACAP4 bears biochemical properties that are consistent with an arginine finger-based catalysis. Like ACAPs, the GAP activity of ACAP4 depends on the active ARF6 protein (Fig. 2E). ACAP4 showed similar phospholipid dependence when ARF6 was used as a substrate with a half-maximal activity achieved with 10 μM PIP<sub>2</sub> in the presence of 50 μM PA (Fig. 2E). Because deletion of the PH domain of ACAP4 did not alter the GAP activity (e.g. Fig. 2D), we reasoned that a region of ACAP4 other than the PH domain is responsible for regulating the PIP<sub>2</sub>-dependent GAP activity shown in Fig. 2E. To examine whether ACAP4 is specific for ARF6, we carried out a GAP activity assay using ARF1, ARF5, and ARF6 as substrates in the presence of PIP<sub>2</sub> and PA. As shown in Fig. 2F, ACAP4 utilized ARF6 as a substrate about 15-fold better than did ARF1 and ARF5. Thus, we concluded that ACAP4 is an ARF6-selective GAP in vitro.

ACAP4 Regulates ARF6-dependent Membrane Cytoskeletal Remodeling—ARF6 plays a role in cell periphery mem-

dependence of ACAP4. ARF6 was used as a substrate for ACAP4. GAP activity was assayed as a function of PIP<sub>2</sub> concentrations as indicated. The reactions contained 50 μM PA where indicated. The rates are given in hydrolyses/min. F, substrate selectivity on ACAP4. GAP assays were performed using FLAG-tagged ACAP4 expressed in HEK 293 cells and purified as described under “Materials and Methods.” Bacterially recombinant ARF1, ARF5, and ARF6 (2 μg each) were used in a reaction that included 10 μM PIP<sub>2</sub>, 50 μM PA, and ~1 μg of ACAP4. ACAP4 was added in quantities to give a reaction rate of 0.25 hydrolyses/min. GAP activity was quantified and normalized to the amount of ACAP4 proteins analyzed by Western blotting. The error bars represent S.E.; n = 3 preparations. Immunoprep., immunoprecipitation; ip, immunoprecipitate; WT, wild type.
brane cytoskeletal dynamics (e.g. Refs. 4 and 23–25). Examination of subcellular localization of ACAP4 relative to ARF6 revealed a co-distribution of the two proteins primarily in the cytoplasm of HeLa cells (Fig. 3A). To ascertain the subcellular localization of ACAP4 relative to filamentous actin, HeLa cells were transfected with FLAG-tagged ACAP4 and GAP-deficient mutants, stained for ACAP4 using an anti-FLAG antibody (red), and probed for F-actin using Alexa Fluor 488-coupled phallolidin (green). The majority of cells exhibited a diffuse cytoplasmic distribution of ACAP4 (Fig. 3B, a–c) that is similar to that of endogenous ACAP4 (Fig. 3A, b). Expression of GAP-deficient ACAP4 mutants (ACAP4ΔGAP or ACAP4R469G) did not significantly affect cell morphology, actin organization, or localization compared with wild type (Fig. 3B, a′–c′ and a″–c″).

HeLa cells have been used as a model system for studying ARF6 as treatment with tetrafluoroaluminate (AlF₄⁻) results in acute formation of actin-rich membrane protrusions (e.g. Refs. 4 and 23–25). If ACAP4 is able to act as a GAP for ARF6 in vivo, then expression of this protein should result in the modulation of ARF6 molecular dynamics. Consistent with this prediction, expression of full-length ACAP4 inhibited the formation of protrusions in response to AlF₄ treatment (Fig. 3C, a–c). This effect was not observed in cells expressing the GAP-inactive mutant ACAP4R469G (Fig. 3C, a′–c′, arrows) or the GAP deletion mutant ACAP4ΔGAP (Fig. 3C, a″–c″, arrows). The localization of ACAP4 mutant lacking the GAP domain to the protrusion suggests that interaction with ARF6 is not required for localizing ACAP4 to the protrusions. In fact, the mutant form of ACAP4 lacking the PH domain failed to localize at the protrusions (Fig. 3C, a″–c″). We quantified the proportion of cells making protrusions in each case and compared them with those formed in cells expressing ARF6 alone. As shown in Fig. 3D, a summary from three different experiments indicates that ACAP4 was very effective at inhibiting the protrusions. This inhibitory effect was dependent on GAP activity because mutants that lacked GAP activity had no effect.

To test whether the functional state of ARF6 modulates the association of ACAP4 with the plasma membrane, we also assessed the distribution of ACAP4 with respect to ARF6 in co-transfected cells. Overexpression of ARF6 resulted in an accumulation of exogenous ARF6 in the plasma membrane (Fig. 3E, a, arrow). In the co-transfected cells, ACAP4 was primarily cytosolic, whereas wild type ARF6 was mainly localized to cell periphery membranes (Fig. 3E, d and e). ARF6 and ACAP4 were also co-localized to small protrusion buds on the plasma membrane (Fig. 3E, f, arrows). Overexpression of constitutively active ARF6 mutant ARF6Q67L resulted in formation of large vacuolar membrane structures (Fig. 3E, g–i, arrow; Ref. 10). In these cells, ARF6 and ACAP4 displayed greater co-distribution (Fig. 3E, i, arrows). This is consistent with our findings that ACAP4 bound better to active ARF6Q67L compared with wild type ARF6, which was demonstrated by the immunoprecipitation (Fig. 2B).

If ACAP4 functions at promoting ARF6 GTPase cycling, inactivation of its GAP activity would enhance the action of ARF6 at the plasma membrane. Consistent with this prediction, a single point mutant of ACAP4, ACAP4R469G, which lacked GAP activity, further stimulated ARF6-dependent protrusions in the absence of AlF₄ treatment (Fig. 3E, j–l, arrows). Examination of HeLa cells expressing an ACAP4 mutant lacking the GAP domain indicated that the mutant ACAP4 co-localizes with ARF6 to ruffles (Fig. 3E, m–o, arrows). Thus, we conclude that ACAP4 is an ARF6-specific GAP and functions in the ARF6-mediated membrane cytoskeletal remodeling.

**ACAP4 Regulates EGF-stimulated Cytoskeleton-Membrane Reorganization**—It has been shown that ARF6 activation by growth factor EGF triggers active cytoskeletal and membrane rearrangement and ruffling (e.g. Ref. 26). To evaluate the involvement of ACAP4 in EGF-stimulated membrane ruffling, we also assessed the distribution of ACAP4 with respect to ARF6 in co-transfected cells. In serum-starved cells, both ACAP4 and ARF6 were primarily cytosolic with some concentration in endosome-like structure (Fig. 4A, a–c). EGF stimu-
lation triggered redistribution of both ARF6 and ACAP4 from the cytoplasm to the ruffles on the plasma membrane (Fig. 4A, a’–c’, arrows). Consistent with ACAP4 function in controlling the molecular dynamics of ARF6, membrane ruffles were extremely pronounced in the GAP-deficient mutant (ACAP4R469G)-expressing cells (Fig. 4A, a’–c’, arrows). To examine whether actin-based cytoskeleton is also modulated by EGF stimulation via ACAP4-dependent mechanisms, we carried out double labeling of F-actin and ACAP4. As shown in Fig. 4B, labeling of F-actin confirmed that the membrane ruffles are rich in actin cytoskeleton based on the phalloidin labeling (a’ and a”, arrows). A summary from three different experiments indicates that ACAP4 was very effective at inhibiting EGF-stimulated membrane ruffles. Taken together, these data show that ACAP4 is an important regulator of growth factor-regulated membrane cytoskeletal remodeling.

**ACAP4 Is Important for Cell Migration**—Recent studies show the requirement of ARF6 in tumor cell migration (11, 12). To examine the function of endogenous ACAP4 underlying cell migration, HeLa cells were depleted of ACAP4 by transfection with siRNA duplexes. Western blotting revealed that ACAP4 was efficiently depleted by specific siRNAs but not by scrambled sequences, whereas the levels of ezrin and ARF6 were unaffected (Fig. 5A). Importantly the protein level of another ARF6 GAP, ACAP1, which is implicated in cell migration, was also not altered by ACAP4 siRNAs (Fig. 5B). In ACAP4-depleted cells, membrane ruffles were formed and identical to those observed in ACAP4R469G-expressing cells (Fig. 4A). However, depletion of ACAP4 protein inhibited the cell migration as determined by wound healing assay (Fig. 5C). We scored cells that had migrated to wound area in three independent experiments; these results are presented in Fig. 5D. This phenotype was validated by overexpressing GAP-deficient ACAP4R469G, suggesting that ARF6 GAP activity is important for migratory activity (Fig. 5E). In fact, the level of inhibition of migration observed in ACAP4-depleted cells was

**Fig. 4.** ACAP4 mediates EGF-stimulated membrane cytoskeletal rearrangement. **A**, HeLa cells were co-transfected with HA-tagged ARF6 and FLAG-tagged ACAP4 along with their derivatives. Twenty-four hours after the transfection, the HeLa cells were serum-deprived for 4–6 h (a–c) followed by stimulation with 200 ng/ml EGF for 5 min (a’–c’; a”–c”). Stimulated cells were fixed and permeabilized for visualization of ARF6 (red) and ACAP4 (green) proteins. Bars, 10 μm. **B**, HeLa cells were transfected with FLAG-tagged ACAP4 along with their derivatives. Twenty-four hours after the transfection, HeLa cells were serum-deprived for 4–6 h (a–c) followed by stimulation with 200 ng/ml EGF for 5 min (a’–c’; a”–c”). Stimulated cells were fixed and permeabilized for visualization of ACAP4 (green) and F-actin (red) proteins. Bars, 10 μm. **C**, quantitation of the effect of ACAP4 on EGF-stimulated membrane protrusions. HeLa cells were transfected with ARF6 and ACAP4 for 24 h and treated with EGF (200 ng/ml) for 5 min prior to fixation. The data are presented as the fraction of cells forming protrusions when co-expressed with the indicated ACAP4 protein normalized to the fraction of cells expressing ARF6 alone. The error bars represent S.E.; n = 3 preparations.
FIG. 5. ACAP4 is essential for cell migration. A, HeLa cells were transfected with the ACAP4 siRNA oligonucleotides for 36 h and subjected to SDS-PAGE and immunoblotting. Left panel, immunoblot for ACAP4; central panels, immunoblot for ezrin; right panel, immunoblot for ARF6. Scrambled oligonucleotides were used as controls. B, HeLa cells were transfected with the ACAP4 siRNA oligonucleotides for 36 h and subjected to SDS-PAGE and immunoblotting. Scrambled oligonucleotides were used as controls. Immunoblot of ACAP1 shows specificity of the siRNA treatment. C, depletion of ACAP4 inhibits wound-healing cell migration. HeLa cells treated with siRNA against ACAP4 or a scrambled control were examined in the wound healing assay. Results are representative of three independent experiments. D, ACAP4 is required for cell migration. Quantitative analyses of three independent siRNA experiments. The number of migrating cells depleted of ACAP4 to the wound area was compared with that of scrambled siRNA-treated cells and then expressed as a percentage. The mean with S.E. was then derived from three independent experiments. E, overexpression of GAP-deficient mutant ACAP4R469G inhibits wound-healing cell migration. HeLa cells infected with adenoviral constructs to overexpress wild type ACAP4 or GAP-deficient mutant ACAP4R469G were examined in the wound healing assay. Results are representative of three independent experiments. F, ACAP4 GAP activity is required for cell migration. Quantitative analyses of three independent exogenous ACAP4 expression experiments are shown. The number of migrating cells expressing GAP-deficient mutant ACAP4R469G to the wound area was compared with that of wild type ACAP4-expressing cells and then expressed as a percentage. The mean with S.E. was then derived from three independent experiments. WT, wild type.
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consistent with that seen in ACAP4^{R469G} overexpressing cells (Fig. 5F). Therefore, these data suggest that endogenous ACAP4 is an important regulator responsible for the ARF6-dependent cell migration.

DISCUSSION

We identified ACAP4 as a specific GAP for ARF6 with an essential role in controlling membrane cytoskeletal remodeling and cell migration via an ARF6-dependent pathway. A number of previous observations have identified several GAPs for ARF6 and shown their function in the cell periphery (e.g. Refs. 4 and 5) and in promoting endocytic recycling (22). We designed a novel proteomic search for proteins that specifically bind to active ARF6 and identified a novel ARF6 GTPase-activating protein, ACAP4, along with several other potential ARF6- and/or ACAP4-associated proteins (Table I). The implication of our findings is that the ARF6 GTP hydrolysis itself is important for the membrane cytoskeletal remodeling underlying cell migratory activity. ACAP4 may contribute to maintaining a limiting amount of ARF6 in GTP-loaded form by accelerating its GTP hydrolysis activity. This raises the question of how ACAP4 activity is orchestrated. Previous studies show that phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of ARF6 in membrane ruffle formation and triggers the recruitment of a diverse but interactive set of signaling molecules into sites of active cytoskeletal and membrane rearrangement (e.g. Ref. 26). The tightly controlled retention of ACAP4 to the membrane ruffle upon EGF stimulation raises the possibility that spatial elevation of PIP2 levels at the membrane ruffles may allow ACAP4 to anchor to the membrane for spatial activation of ACAP4 in vivo. Consistent with this hypothesis, ACAP4 contains four potential PIP2 binding sites in the PH domain, and the mutant form of ACAP4 lacking the PH domain failed to localize to the membrane protrusions. In this regard, ACAP4 may provide a link between growth factor-receptor signaling and the regulation of membrane cytoskeletal dynamics at the peripheral membrane. Whether or not this PIP2 binding is the mechanism accounting for ACAP4 regulation will be an important avenue for further investigation.

We propose that ACAP4 is required for ARF6-mediated migratory activity and plays an important role in linking growth factor signaling to membrane cytoskeletal dynamics. On one hand, ACAP4 is required for maintaining ARF6 GTPase cycling during migratory processes through orchestrating plasma membrane cytoskeletal dynamics. On the other hand, through its multiple structural motifs, ACAP4 may be involved in the assembly of a signaling protein complex at membrane ruffles. ACAP4 is likely a regulated link between membrane proteins and the actin-based cytoskeleton and may also participate in signal transduction pathways. In fact, our proteomic identification of membrane cytoskeletal linker ezrin in the ARF6-ACAP4 protein complex supports such a notion. Recent studies suggest that ezrin is necessary for osteosarcoma metastasis in animals (27, 28). It would be of great interest to elucidate how ezrin interacts with ACAP4 and ARF6 and whether such interactions orchestrate cell migratory activity. Future experiments will be directed to elucidate how ACAP4 interacts with other proteins identified in Table I. A combination of biochemical characterization with real time analysis of molecular dynamics will enable us to consolidate these protein-protein interactions into a model for ARF6-ACAP4 protein complex signaling in cell migration.

Finally it is worth noting that ACAP4 (under the name UPLC1) has been identified as a gene that is overexpressed 3–11-fold over the normal level in human liver cancers (29). Notably a higher level of ACAP4 protein expression is correlated with the high level of ARF6 protein and with the invasive phenotype of some breast and melanoma cancer cell lines (11, 12). These findings raise the important possibility that ACAP4 plays a role in tumor progression and leukocyte motility, perhaps by affecting the cellular plasticity and thus contributing to cell migration and acquisition of invasive phenotypes.

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