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GRASP and IPCEF Promote ARF-to-Rac Signaling and Cell Migration by Coordinating the Association of ARNO/cytohesin 2 with Dock180

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ARFs are small GTPases that regulate vesicular trafficking, cell shape, and movement. ARFs are subject to extensive regulation by a large number of accessory proteins. The many different accessory proteins are likely specialized to regulate ARF signaling during particular processes. ARNO/cytohesin 2 is an ARF-activating protein that promotes cell migration and cell shape changes. We report here that protein–protein interactions mediated by the coiled-coil domain of ARNO are required for ARNO induced motility. ARNO lacking the coiled-coil domain does not promote migration and does not induce ARF-dependent Rac activation. We find that the coiled-coil domain promotes the assembly of a multiprotein complex containing both ARNO and the Rac-activating protein Dock180. Knockdown of either GRASP/Tamalin or IPCEF, two proteins known to bind to the coiled-coil of ARNO, prevents the association of ARNO and Dock180 and prevents ARNO-induced Rac activation. These data suggest that scaffold proteins can regulate ARF dependent processes by biasing ARF signaling toward particular outputs.

INTRODUCTION

ARFs (ADP ribosylation factors) are members of the Ras superfamily of small GTPases. The six mammalian ARFs are divided into three classes based upon sequence similarity. ARFs 1–3 make up class I, ARFs 4 and 5 comprise class II, and ARF6 is the sole class III member. The class I and II ARFs recruit vesicle coats and promote vesicle budding in the secretory system. ARF6 is located predominantly in the cell periphery. ARF6 regulates trafficking between the plasma membrane and endosomal systems (Donaldson and Honda, 2005).

In addition to regulating the endocytosis and recycling of plasma membrane proteins ARF6 also regulates the cortical actin cytoskeleton (Radhakrishna *et al.*, 1996; Frank *et al.*, 1998a,b; Song *et al.*, 1998; Radhakrishna *et al.*, 1999; Boshans *et al.*, 2000; Santy, 2002). ARF6-dependent actin rearrangements are critical during cell spreading, migration, and phagocytosis (Song *et al.*, 1998; Zhang *et al.*, 1998; Palacios *et al.*, 2001; Santy and Casanova, 2001; Beemiller *et al.*, 2006). Previous work has demonstrated cross-talk between ARF6 and the Rho-family GTPase Rac (Radhakrishna *et al.*, 1999; Zhang *et al.*, 1999; Santy and Casanova, 2001; Palacios and D'Souza-Schorey, 2003; Santy *et al.*, 2005; Koo *et al.*, 2007). These two small GTPases coordinate to regulate the cortical actin cytoskeleton and to alter cell shape.

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Abbreviations used: GEF, guanine nucleotide exchange factor, DSP dithiobis(succinimidylpropionate).

Like all GTPases, ARF6 cycles between an inactive GDP-bound state and an active GTP-bound state. Interconversion between these two states requires the actions of accessory proteins. Guanine nucleotide exchange factors (GEFs) promote the binding of GTP and the activation of the GTPase. GTPase-activating proteins (GAPs), on the other hand, induce the hydrolysis of the bound GTP, thereby inactivating the GTPase. Although there are only six ARFs, the human genome encodes 15 ARF GEFs and 20 ARF GAPs. This discrepancy suggests that ARFs are regulated by different GEFs and GAPs at particular subcellular locations or during particular processes (Donaldson and Honda, 2005; Casanova, 2007).

There are five families of ARF-GEFs: the GBF/BIG family, cytohesins, EFA6s, BRAGs, and Fbox8. Three of these families, the cytohesins, EFA6s, and BRAGs, have been reported to act in the cell periphery and to regulate endocytosis, recycling, and cell shape (Casanova, 2007). The cytohesins in particular have been implicated in the regulation of the actin cytoskeleton and cell shape. Cytohesins are recruited to the plasma membrane in response to growth factor signaling and induce rearrangements in the cortical actin cytoskeleton (Klarlund *et al.*, 1997, 1998; Frank *et al.*, 1998a,b; Venkateswarlu *et al.*, 1998a,b). We have previously shown that overexpression of ARNO/cytohesin 2 in Madin-Darby canine kidney (MDCK) cells promotes migration of these cells (Santy and Casanova, 2001). ARNO expression produces a scattering phenotype that resembles the action of the motility-promoting growth factor HGF.

Enhanced migration in response to ARNO expression requires ARF activation and the subsequent downstream activation of phospholipase D (PLD) and Rac (Santy and Casanova, 2001). ARNO-induced Rac activation can be blocked by coexpression of dominant negative mutants of Dock180 and Elmo (Santy *et al.*, 2005). Dock180 and Elmo act together as a bipartite Rac-GEF that has been extensively implicated as an activator of Rac during migration and

phagocytosis (Hasegawa *et al.*, 1996; Erickson *et al.*, 1997; Nolan *et al.*, 1998; Wu and Horvitz, 1998; Reddien and Horvitz, 2000; Gumienny *et al.*, 2001; Brugnera *et al.*, 2002). How ARNO and Dock180/Elmo coordinate to promote ARF-to-Rac cross-talk remains unclear.

Recent work on kinase signaling cascades has demonstrated that the output downstream of a particular kinase can be biased by protein–protein interactions. Scaffold proteins assemble the components of a particular signaling pathway into a complex and thereby promote signaling through that pathway (Morrison and Davis, 2003; Kolch, 2005; Dard and Peter, 2006; Pullikuth and Catling, 2007). We wondered whether similar processes bias ARF signaling downstream of ARNO toward Rac activation and motility.

We tested the hypothesis that protein–protein interactions direct ARF-dependent signaling downstream of ARNO toward Rac activation and motility. ARNO, like all the cytohesins, is made up of four distinct domains (see Figure 1). The sec7 domain is the catalytic ARF-GEF domain. The pleckstrin homology (PH) and polybasic domains mediate binding to membrane surfaces. The polybasic domain also functions as an intramolecular inhibitory domain (DiNitto *et al.*, 2007), and the PH domain also interacts with other proteins. The coiled-coil domain promotes dimerization and interacts with a number of other proteins (Casanova, 2007). The coiled-coil domain of ARNO has previously been shown to interact with several small scaffold proteins that contain multiple protein–protein interacting domains. These include GRASP/Tamalin (Nevrivy *et al.*, 2000; Kitano *et al.*, 2002, 2003), Pip3-E/IPCEF (Venkateswarlu, 2003), and CASP/Cybr/CYTIP (Mansour *et al.*, 2002; Tang *et al.*, 2002; Boehm *et al.*, 2003). These scaffold proteins can promote the recruitment of ARNO to the plasma membrane in response to growth factor or other signals (Venkateswarlu, 2003; Esteban *et al.*, 2006). These proteins can control the subcellular location of ARNO and could therefore modulate ARNO signaling. We therefore investigated the role of the coiled-coil domain in ARNO-induced motility and Rac activation.

MATERIALS AND METHODS

Antibodies and Reagents

The 9e10 antibody against myc and mouse anti-HA (16B12) were purchased from Covance (Princeton, NJ). Mouse anti-Rac, mouse anti E-cadherin, and mouse anti-actin were obtained from BD (San Jose, CA). Goat anti-Dock180 (C-19, N-19), mouse anti-green fluorescent protein (GFP; B-2), and rabbit anti-GFP (fl) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-ARF6 and mouse anti-ARNO were a kind gift from Slyvain Bourgouin (Université Laval, Quebec, Canada). Polyclonal rabbit anti-ARNO was a gift from James Casanova (University of Virginia). M2 anti-flag resin was purchased from Sigma Aldrich (St. Louis, MO). DSP [dithiobis(succinimidylpropionate)] was obtained from Pierce/Thermo Scientific (Rockford, IL).

Expression Constructs

Recombinant adenoviruses for expressing full-length ARNO, ARNO E156K, or Dock180 under the control of the tetracycline responsive promoter have been previously described (Santy and Casanova, 2001; Santy *et al.*, 2005). For the Δcoiled-coil ARNO construct, PCR was used to amplify the region of human ARNO encoding amino acids 56–400 and to add a myc-tag at the 5′ end. For the ΔPH ARNO a stop codon was introduced at position 269 and a myc-tag added at the 5′ end by PCR. These products were cloned into pAdTet and recombinant adenoviruses produced as described (Hardy *et al.*, 1997). GFP-GRASP was obtained from Mark Leid (Oregon State University). An IPCEF cDNA was isolated by PCR from Marathon ready human brain cDNA (Clontech, Mountain View, CA). The cDNA was sequenced and is identical to the human Pip3-E sequence (NM_015553). The HA-tag was added to the N-terminus by PCR. Both scaffold cDNAs were cloned into pAdlox and recombinant adenoviruses produced as described previously (Hardy *et al.*, 1997). GFP-Elmo and GFP-Elmo-T625 were obtained from Kodi Ravichandran (University of Virginia). The GFP-Elmo constructs were subcloned into pAdTet and adenoviruses produced as described above. Truncated Dock180 constructs were created by PCR and cloned into pcDNA3.

Cell Culture

The T23 line of MDCKII cells that expresses the tetracycline responsive transactivator and Tet-off MCF-7 cells were obtained from James Casanova. MDCKs were maintained in DMEM with 10% FBS. MCF-7s were maintained in DMEM/F12 with 10% FBS and nonessential amino acids. Cells were maintained at 37°C and 5% CO_2. Cell culture media was purchased from Mediatech (Manassas, VA), and FBS was purchased from Hyclone (Rockford, IL).

siRNA Knockdown

The siRNAs targeting human and dog GRASP (target sequence GCTTTGAGATCCAGACTTA), human and dog Pip3-E/IPCEF (target sequence CACATCAGAAAGTGGATTT), human and dog CASP (target sequence CTGGTGATGTCCTTGCAAA), firefly luciferase, and a scrambled nontargeting control (siControl 1) were obtained from Dharmacon (Lafayette, CO). Fluorescent Block-It Alexafluor Red control siRNA was obtained from Invitrogen (Carlsbad, CA). siRNAs were transfected into MCF-7 cells using LipoRNAi max, and into MDCK cells using Lipofectamine2000 (Invitrogen). Transfections were carried out using the manufacturer's suggested protocol for reverse transfection. For GTPase pulldown assays and immunofluorescence, 3×10^5 MDCK cells were transfected with 100 pmol of siRNA in 35-mm dishes. After 48 h of knockdown the cells were trypsinized and replated onto two 60-mm dishes. Pulldown assays were performed 18 h after replating. For immunoprecipitations (IPs) 4×10^6 MCF-7 cells were transfected with 300 pmol siRNA in 15-cm dishes. After 48 h of knockdown the cells were infected with adenoviruses encoding ARNO and Dock180. IPs were performed 18 h after infection.

Immunofluorescence

MDCK cells were grown on glass coverslips, infected, and fixed and stained as previously described (Santy and Casanova, 2001). Cells were observed and photographed using a Nikon Eclipse E800 digital camera (Melville, NY) equipped with a Diagnostic Instruments Spot II (Sterling Heights, MI) or a Zeiss Axioplan microscope (Thornwood, NY) equipped with a Diagnostic Instruments Spot RT3. The brightness and contrast of the entire image was adjusted, and scale bars were added using ImageJ (http://rsb.info.nih.gov/ij/; Abramoff *et al.*, 2004). Multipanel figures were assembled using Adobe Illustrator CS2 (San Jose, CA).

GTPase Pulldown Assays

Active ARF6 was isolated by binding to glutathione *S*-transferase (GST)-GGA3. Active Rac was isolated by binding to GST-PBD. Pulldown assays were performed as previously described (Santy and Casanova, 2001). Western blots of the pulldowns and saved samples of the starting lysate were analyzed by densitometry using ImageJ (Abramoff *et al.*, 2004). Levels of active GTPase were first normalized to the amount of GTPase present in the starting lysate. Normalized levels of active GTPase in ARNO-expressing cells were divided by the normalized level of active GTPase in the control cells to give the fold activation of the GTPase induced by ARNO. Differences in GTPase activation were analyzed for significance using a paired *t* test on the indicated number of independent pulldown experiments.

Cross-linking and IP

Tet-off MCF-7s or MDCK cells were infected with adenoviruses encoding myc-ARNO and flag-Dock180 for 18 h (MCF-7s) or 3 h (MDCKs). Alternatively, expression constructs were transfected into MDCK cells using Lipofectamine LTX according to the manufacture's instructions, and cells were allowed to express for 18 h. Interacting proteins were cross-linked by treating the cells with the cell-permeable cross-linker DSP. Briefly, the cells were rinsed with PBS and then incubated with PBS, 150 μM DSP for 30 min. Cells were subsequently lysed in 1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 2 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.1 mM PMSF. Unsolubilized material was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. A small aliquot of the cleared lysate was saved, and the remainder of the lysate was incubated with M2 anti-flag resin for 2 h at 4°C. IPs were washed three times with lysis buffer and one time with TBS. Precipitated proteins were eluted into SDS-PAGE sample buffer. IP and lysate samples were boiled for 3 min to reverse the DSP cross-links and then analyzed by Western blot.

GST Pulldown

The region of ARNO encoding the N-terminal 60 amino acids was amplified by PCR and inserted in frame into pGEX-2T to produce a construct encoding GST-coiled-coil. GST and GST-coiled-coil were purified as described for GST-GGA3. MDCK cells were infected with adenoviruses encoding Dock180 for 18 h. Cells were lysed as described above for IP. The postnuclear supernatant was incubated with glutathione Sepharose and 30 μg GST or 30 μg GST-coiled-coil for 5 h at 4°C. Pulldowns were washed and blotted with goat anti-Dock180.

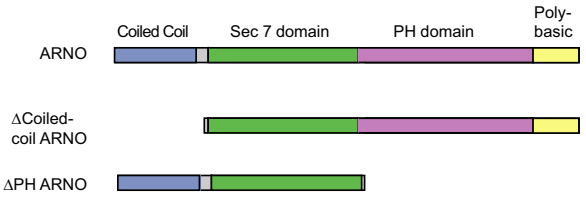

Figure 1. Structure of ARNO constructs used in this study. The domain structures of full-length, Δcoiled-coil, and ΔPH ARNO are depicted. The coiled-coil domain is involved in protein–protein interactions. The Sec7 domain is the catalytic ARF GEF domain. The PH and polybasic domains interact with phosphoinositides and acidic phospholipids, respectively.

Migration Assays

Migration was tested using a transwell migration assay as previously described (Santy and Casanova, 2001).

Cell Fractionation

MDCK cells were infected with adenoviruses encoding the indicated proteins for 3 h. The cells were then scraped off the plate in 250 mM sucrose, 3 mM imidazole, pH 7.4, 1 mM EDTA, 1 mM DTT, 2 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.1 mM PMSF. Cells were broken by eight passages through a 22-gauge needle. Unbroken cells and nuclei were removed by centrifugation for 2 × 10 min at 12,000 × *g* at 4°C. The postnuclear supernatant was separated into total membranes and cytosol by ultracentrifugation for 1 h at 100,000 × *g* in a TLA 100.3 rotor at 4°C. Membrane and cytosol fractions were resuspended in equal volumes of SDS-PAGE sample buffer and analyzed by Western blot.

RT-PCR

Cells were transfected with siRNAs as described above. Forty-eight hours later RNA was isolated using the RNeasy kit (Qiagen, Germantown, MD). RT-PCR was performed using the One-Step RT-PCR kit (Qiagen) with 0.5 μg RNA as template. Primers to amplify human GRASP (NM_181711, bp 329–576), human CASP (NM_004288, bp 210–685) human IPCEF (NM_015553, bp 177–834), dog GRASP (XM_845242, bp 105–353), and dog IPCEF (XM_541159, bp 2155–2614) were ordered from Integrated DNA Technologies (Coralville, IA). Readymade primers for amplifying GapDH were also obtained from Integrated DNA Technologies.

RESULTS

The Coiled-Coil Domain of ARNO Promotes ARF-to-Rac Cross-Talk

We hypothesize that protein–protein interactions bias ARF signaling downstream of ARNO toward modulating the actin cytoskeleton and promoting migration. ARNO contains one major protein-interacting domain, the coiled-coil domain (Figure 1). Therefore we tested whether a truncation mutant of ARNO lacking this domain can induce epithelial motility. As we have previously shown, overexpression of wild-type (WT) ARNO produces the formation of large-fan shaped lamellipodia and a scattering phenotype in MDCK cells (Figure 2A, rows 1 and 2; Santy and Casanova, 2001). Also as we have previously shown, this phenotype requires ARF activation, as a point mutant of ARNO, E156K, that cannot activate ARFs does not induce scattering (Figure 2A, row 3). We have found that overexpressing a truncation of ARNO lacking the coiled-coil domain (Δcoiled-coil) also fails to produce scattering. MDCK cells expressing this truncation resemble the control cells or cells expressing the inactive ARNO point mutant (Figure 2A, row 4). MDCK cells expressing Δcoiled coil-ARNO do not produce large lamellipodia and remain anchored to their neighbors. The lack of a scattering phenotype suggests that unlike WT ARNO, the Δcoiled coil-ARNO truncation does not promote epithelial migration. We tested the migration of these cells using a transwell migration assay. As can be seen in Figure 2B,

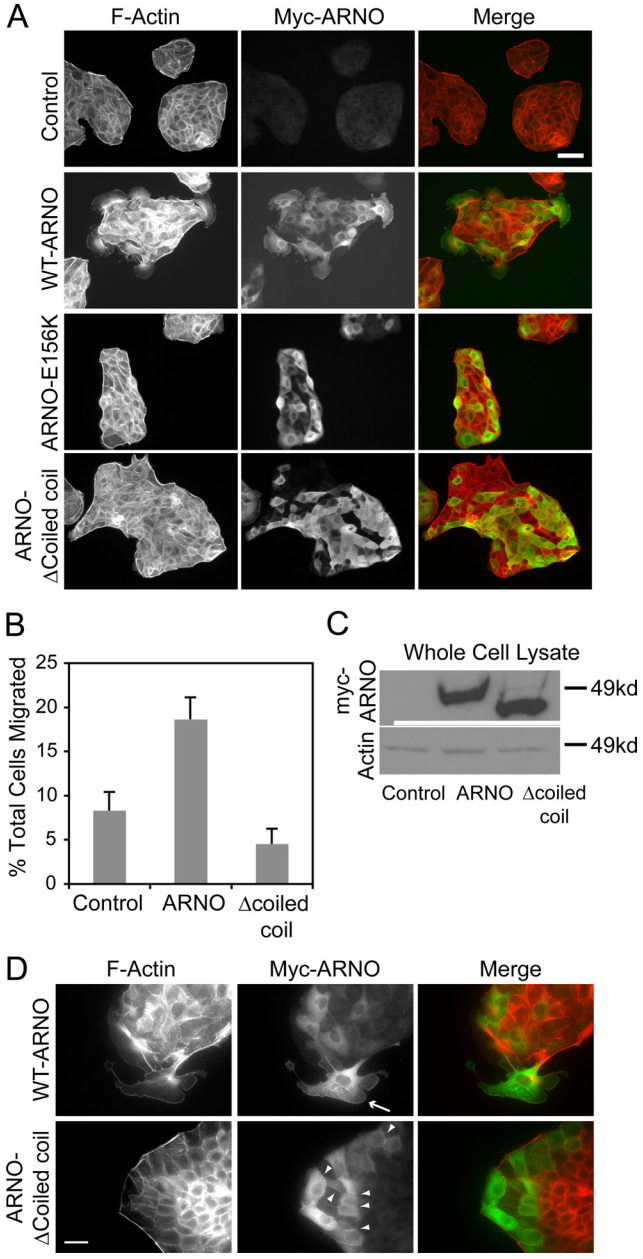

Figure 2. The coiled-coil domain of ARNO is necessary for the induction of epithelial motility. (A) MDCK cells were infected with adenoviruses encoding the indicated ARNO constructs for 3 h. Cells were then fixed and stained with mouse anti-myc followed by Alexa-488–conjugated anti-mouse antibody and rhodamine-phalloidin. Control cells were infected with adenovirus encoding WT ARNO in the presence of doxycycline to suppress transgene expression. Bar, 50 μm. (B) Motility of cells expressing the indicated constructs was tested using the transwell assay as described in *Materials and Methods*. The percent of cells migrating through the filter in 18 h are indicated. Data shown are mean ± SD of triplicate samples. (C) Expression levels of the myc-tagged ARNO constructs and actin in the cells subjected to the transwell assay shown in B were visualized by Western blot of saved cell samples with mouse anti-myc and mouse anti-actin antibodies. (D) MDCK cells were processed for immunofluorescence as in A. Bar, 25 μM.

although full-length ARNO significantly increases the number of cells that migrate through the filter, cells expressing Δcoiled-coil ARNO show no increase in migration compared

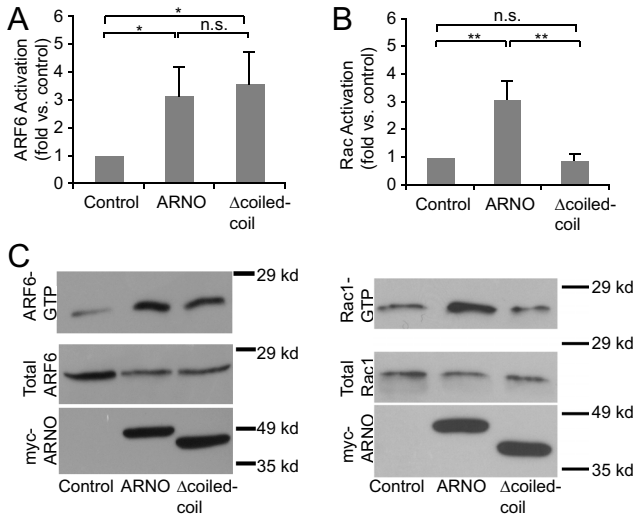

Figure 3. ARNO lacking the coiled-coil domain is deficient at inducing Rac activation. MDCK cells were infected with adenovirus encoding indicated ARNO constructs in the absence of doxycycline or with WT ARNO in the presence of doxycycline (control) for 3 h. Cells were then lysed and active ARF6 or Rac isolated by pulldown as described in *Materials and Methods.* (A and B) Activation of ARF 6 (A) or Rac1 (B) in cells expressing full-length or Δcoiled-coil ARNO. Data shown are mean ± SE of eight independent experiments. Data were analyzed for statistically significant differences using a paired *t* test. *$p < 0.05$; **$p < 0.01$; n.s.; not significant. (C) Representative gels from the pulldown experiments presented in A and B.

with the control cells. Both full-length and Δcoiled-coil ARNO show some perinuclear accumulation (Figure 2C). Full-length ARNO is also localized to the plasma membrane at the leading edge of the lamellipodia (Figure 2C, arrow), whereas Δcoiled-coil ARNO can be seen at the plasma membrane between adjacent cells (Figure 2C, arrowheads).

Overexpression of ARNO in MDCK cells not only activates ARF6, but also produces an increase in the level of active Rac (Santy and Casanova, 2001). This enhanced Rac activity is required for the scattering phenotype and increased motility seen in the ARNO-expressing cells (Santy and Casanova, 2001). The Dock180/Elmo complex is required for Rac activation downstream of ARNO and ARF6 in MDCK cells (Santy *et al.*, 2005). We therefore tested the hypothesis that ARF-to-Rac signaling is impaired in the cells expressing Δcoiled coil-ARNO compared with cells expressing full-length ARNO. The cells expressing Δcoiled coil-ARNO activate endogeneous ARF6 to a level comparable to that seen in cells expressing full-length ARNO (Figure 3A). This is expected because Δcoiled coil-ARNO still has the ARF-activating Sec7 domain and the membrane-binding PH and polybasic domains. Significantly we find that cells expressing Δcoiled coil-ARNO have levels of active Rac that are similar to those seen in control cells and that are significantly lower than those seen in cells expressing full-length ARNO (Figure 3B). We conclude from this data that protein–protein interactions mediated by ARNO's coiled-coil domain are critical for promoting efficient ARF-to-Rac signaling.

The Coiled-Coil Domain Assembles ARNO and Dock180 into a Complex

We have previously demonstrated that the Dock180/Elmo complex is required for ARF-to-Rac signaling downstream of ARNO in MDCK cells (Santy *et al.*, 2005). One possible mechanism for promoting efficient ARF-to-Rac signaling would be for both exchange factors to associate in a larger multiprotein complex. This would ensure that ARF activation occurs in the same area of the cell where Dock180 is available to respond and activate Rac. We investigated this possibility by determining whether Dock180 and ARNO could be coimmunoprecipitated. Cells expressing myc-ARNO and flag-tagged Dock180 were lysed, and the postnuclear supernatant was subjected to IP. Neither ARNO nor Dock180 is precipitated by an antibody against GFP (Supplemental Figure S1A). However, we found that when Flag-Dock180 is precipitated with M2 anti-Flag, a small amount of ARNO is coimmunoprecipitated (Supplemental Figure S1A). The amount of ARNO coimmunoprecipitated with Dock180 could be enhanced by treating the cells with DSP, a cell-permeable cross-linker, before lysis (Supplemental Figure S1A,B). ARNO could be coimmunoprecipitated with Dock180 from both MCF-7 and MDCK cells (Supplemental Figure S1A,B), and ARNO could be coimmunoprecipitated with both overexpressed and endogenous Dock180 (Supplemental Figure S1, A and C). These data suggest that ARNO and Dock180 can associate in a larger multiprotein complex and that this association may be transient, weak, or mediated by intervening protein(s). We tested whether the coiled-coiled domain of ARNO is required for this association. We found that although full-length ARNO can be coimmunoprecipitated with Dock180, Δcoiled-coil ARNO cannot (Figure 4A). Therefore we conclude that protein–protein interactions of ARNO's coiled-coil domain promote ARF-to-Rac signaling by bringing together ARNO and Dock180.

Protein–protein interactions of ARNO's coiled-coil domain have been reported to promote the recruitment of ARNO to the plasma membrane or to particular membrane subdomains (Venkateswarlu, 2003; Shmuel *et al.*, 2006). Therefore it is possible that membrane binding is required for the interaction of ARNO and Dock180 and that the only role of the coiled-coil domain is to promote membrane association. To address this possibility, we used another truncation mutant of ARNO, ARNO ΔPH. The ΔPH mutant lacks the two membrane association domains: the PH and polybasic domains (Figure 1). We found that the ΔPH ARNO was still able to bind to Dock180 (Figure 4A). We also confirmed the subcellular location of these proteins by fractionation. MDCK cells expressing full-length, Δcoiled-coil, or ΔPH ARNO were lysed by passage through a 22-gauge needle. The postnuclear supernatant was fractionated into cytosolic and total membrane fractions by ultracentrifugation. The cell fractions were Western-blotted to determine the locations of E-cadherin (membrane), B-actin (cytosol), and the various ARNO constructs (Figure 4B). Both full-length and Δcoiled-coil ARNO have a significant membrane-bound population. The ΔPH ARNO, on the other hand, is entirely cytosolic (Figure 4B). Therefore membrane association is not required for the interaction of ARNO and Dock180 and protein–protein interactions mediated by ARNO's coiled-coil domain are necessary. We were also able to isolate Dock180 by incubation of a Dock180-expressing cell lysate with a fusion of GST to the ARNO coiled-coil domain (Figure 4C). These data suggest that the coiled-coil domain of ARNO is sufficient to mediate the interaction of ARNO with Dock180.

We next determined the region of Dock180 that is required for the interaction with ARNO. We cotransfected MDCK cells with ARNO and flag-tagged truncation mutants of Dock180 (Figure 5A). We tested whether ARNO is coimmunoprecipitated with the Dock180 truncations. We first

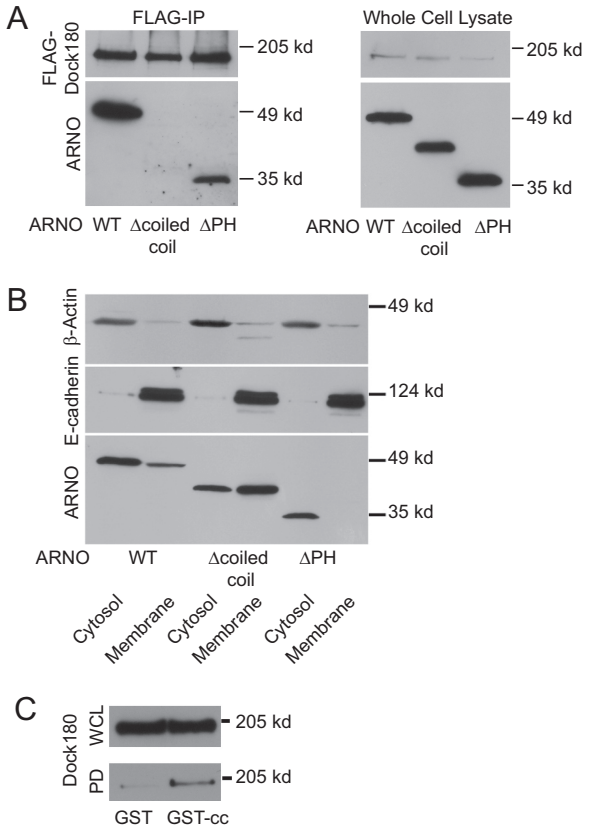

Figure 4. The coiled-coil domain of ARNO is required for interaction with Dock180. (A) MDCK cells expressing flag-Dock180 and the indicated myc-tagged ARNO constructs were cross-linked, lysed, and incubated with M2 anti-flag resin as described in *Materials and Methods.* The immunoprecipitates were blotted with goat anti-Dock180 and rabbit anti-ARNO. Saved samples of the starting lysate were blotted with goat anti-Dock180 and mouse anti-myc antibodies. (B) Subcellular location of full-length ARNO and ARNO truncation mutants. MDCK cells were infected adenovirus encoding the indicated myc-tagged ARNO constructs for 3 h. Cells were then lysed and separated into membrane and cytosol fractions by ultracentrifugation as described in *Materials and Methods.* Equal proportions of the membrane and cytosol fractions were Western-blotted with mouse anti E-cadherin (membrane marker), mouse anti B-actin (cytosol marker), and mouse anti-myc to detect the location of the ARNO proteins. (C) Dock180 can be isolated from a cell lysate by the ARNO coiled-coil domain. MDCK cells expressing Dock180 were lysed and the postnuclear supernatant incubated with glutathione-Sepharose and either 30 μg of GST or 30 μg of GST fused to the ARNO coiled-coil domain. Starting lysates (WCL) and the isolated pulldown samples (PD) were blotted with goat anti-Dock180.

tested Dock DOHRS, a C-terminal truncation of Dock180, that lacks the Crk-binding domain (Grimsley *et al.,* 2004). This mutant retained the ability to coimmunoprecipitate ARNO (Figure 5B, top). Next we tried a series of truncation mutants including Δ357-Dock, lacking the N-terminal 357 amino acids, Δ900-Dock, lacking the N-terminal 900 amino acids, and Dock-N-1086 containing the first 1086 amino acids. Both the Δ357 and Δ900 mutants failed to coimmunoprecipitate ARNO, suggesting that the association of ARNO with Dock180 requires the first 357 amino acids. In support of this conclusion the Dock-N-1086 construct retains the ability to coimmunoprecipitate ARNO even though it is expressed at very low levels (Figure 5B, bottom).

The N-terminal 357 amino acids of Dock180 is the region of Dock180 that is required for interaction with Elmo (Brugnera *et al.,* 2002). Additionally this region contains an SH3 domain at its N-terminal end. Therefore we investigated the possibility that ARNO interacts with Dock180 via Elmo. If Elmo bridges the interaction between ARNO and Dock180, then a mutant of Elmo that cannot bind to Dock180 should disrupt this interaction. Elmo T625 is a truncation mutant that lacks the Dock180-binding domain (Brugnera *et al.,* 2002). Expression of either WT Elmo or Elmo T625 did not prevent coIP of ARNO with Dock180 (Figure 5C). Therefore we conclude that the N-terminus of Dock180 is required for the interaction of ARNO and Dock180 and that this association is independent of Elmo.

Scaffold Proteins Mediate the Association of ARNO with Dock180 and Promote ARF-to-Rac Cross-Talk

Given the small amount of ARNO that was coimmunoprecipitated with Dock180, we suspected that the interaction is not direct. Several small scaffold proteins have been identified that bind to ARNO's coiled-coil domain including GRASP, IPCEF, and CASP (Nevrivy *et al.,* 2000; Mansour *et al.,* 2002; Venkateswarlu, 2003). We therefore investigated the possibility that one of these scaffold proteins acts as a bridge linking ARNO and Dock180. The mRNA for these proteins can be almost completely eliminated by the transfection of siRNAs into MCF-7 cells (Supplemental Figure S2). We transfected MCF-7 cells with siRNAs targeting one of the scaffold proteins to knock down expression of these proteins or with siRNA directed against firefly luciferase as a nontargeting siRNA control. After 48 h of knockdown these cells were infected with recombinant adenoviruses encoding myc-ARNO and flag-Dock180 and incubated for an additional 18 h. The cells were then treated with cross-linker and lysed, and the Dock180 was immunoprecipitated by incubation with M2 anti-flag resin. Western blotting of the immunoprecipitate with polyclonal antiserum directed against ARNO revealed that knockdown of either IPCEF or GRASP prevented association of ARNO with Dock180 (Figure 6A). Cells transfected with siRNA targeting CASP had lower expression levels of ARNO and Dock180; nevertheless ARNO could still be coimmunoprecipitated with Dock180 (Figure 6A). CASP is specifically expressed in the immune system (Heufler *et al.,* 2008). Using RT-PCR we could amplify a small region of this gene from MCF-7 RNA; however, we were unable to find any evidence for expression of this RNA in MDCK cells using multiple primer sets (data not shown). Therefore we conclude that CASP is not involved in promoting the interaction of ARNO and Dock180 or in promoting ARF6 to Rac cross-talk in epithelial cells.

If GRASP and IPCEF are involved in the assembly of a multi-GEF complex containing ARNO and Dock180, then they should also be present in a Dock180 IP. MDCK cells were infected with adenoviruses encoding flag-Dock180 and either GFP-GRASP or HA-IPCEF for 18 h. The cells were then treated with DSP cross-linker, and IP was performed with either mouse anti-myc as a negative control or M2 anti-Flag to precipitate Dock180. Western blotting revealed that none of the proteins was isolated with anti-myc antibody, whereas both GRASP and IPCEF could be coIPed with Dock180 (Figure 6B).

We predicted that because knockdown of IPCEF or GRASP impairs the assembly of ARNO and Dock180 into a larger complex, knockdown of these proteins would also disrupt ARF-to-Rac signaling. We used the Rac pulldown assay to test this prediction in MDCK cells, because these cells show robust ARNO-induced Rac activation. Both

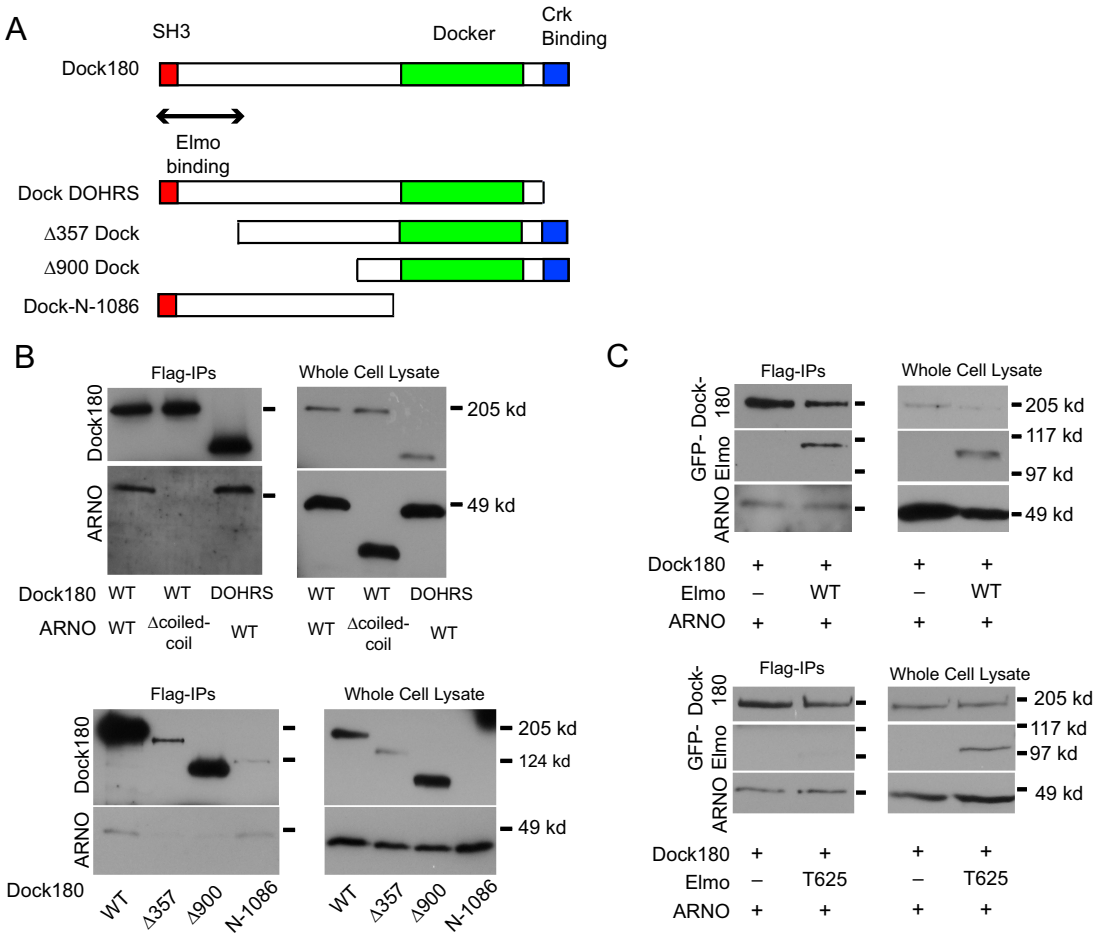

Figure 5. The N-terminus of Dock180 is required for interaction with ARNO. (A) Dock180 truncation constructs used in these experiments. All constructs are Flag-tagged at the N-terminus. (B) MDCK cells were cotransfected with the indicated Dock180 and pTRE-ARNO constructs in the presence of 2 ng/ml doxycycline to repress ARNO expression. After 18 h the doxycycline was removed, and ARNO allowed to express for 4 h. Cells were then treated with cross-linker, lysed, and incubated with M2 anti-flag as described in *Materials and Methods.* The immunoprecipitates were blotted with rabbit anti-ARNO and goat anti-Dock180 antibodies. A mixture of Dock180 antibodies directed against both N- and C-terminal epitopes was used to allow visualization of all truncations. (C) MDCK cells were infected with adenoviruses encoding Dock180, ARNO, and the indicated Elmo constructs for 4 h (WT Elmo) or for 18 h in the presence of 1 ng/ml doxycycline to moderate expression (T625). Cells were cross-linked, lysed, and immunoprecipitated as above. Immunoprecipitates and aliquots of the starting lysate were blotted with goat anti-Dock180, rabbit anti-ARNO, and rabbit anti-GFP.

GRASP and IPCEF mRNA levels can be reduced in MDCK cells by transfection of siRNAs (Supplemental Figure S2). MDCK cells were transfected with siRNAs targeting IPCEF, GRASP, or a nontargeting siRNA. Two days later the cells were split onto duplicate plates and allowed to recover overnight. The cells were then infected with adenovirus that inducibly expresses ARNO for 3 h. ARNO expression was induced in one of the duplicate plates. The cells were then lysed, and active Rac was isolated by binding to GST-PBD. Levels of active Rac were normalized to the level of Rac in the starting whole-cell lysate. Multiple independent knockdown experiments were analyzed using a paired *t* test to determine whether reduced GRASP or IPCEF expression impairs ARNO-induced Rac activation. Knockdown of either IPCEF or GRASP significantly reduced Rac activation in the ARNO-expressing cells (Figure 7).

Finally we confirmed that knockdown of IPCEF and GRASP impairs the ability of ARNO to produce fan-shaped lamellipodia and a scattering phenotype. MDCK cells were transfected with siRNAs targeting IPCEF, GRASP, CASP, or a nontargeting siRNA and treated as described for the Rac pulldown. After 3 h of ARNO expression the cells were fixed processed for indirect immunofluorescence. Control and CASP siRNA-treated cells show ARNO induced scattering, whereas ARNO-expressing cells transfected with siRNAs targeting IPCEF or GRASP are impaired in the production of lamellipodia and scattering (Figure 8). These data support the conclusion that protein–protein interactions mediated by the coiled-coil domain of ARNO promote ARF-to-Rac signaling. Additionally both IPCEF and GRASP are necessary to assemble a multiprotein complex containing ARNO and Dock180 and to produce efficient ARF-to-Rac cross-talk downstream of ARNO.

DISCUSSION

We have shown in this study that protein–protein interactions involving the coiled-coil domain of ARNO are critical for promoting ARF-to-Rac cross-talk downstream of ARNO. ARNO and Dock180 are present together in a larger multiprotein complex. Assembly of this complex requires the coiled-coil domain and two scaffold proteins, GRASP and

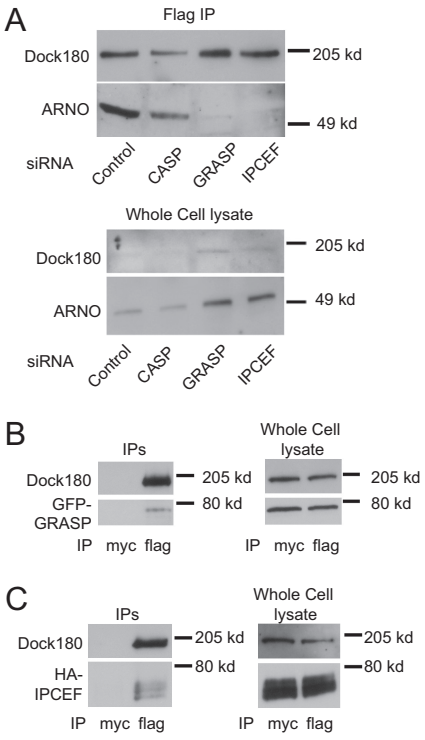

Figure 6. Knockdown of GRASP or IPCEF impairs interaction of ARNO and Dock180. (A) MCF-7 cells were transfected with siRNAs targeting firefly luciferase (control) or the indicated proteins as described in *Materials and Methods.* After 48 h the cells were then infected with adenoviruses encoding ARNO and Dock180 for 18 h. Cells were then cross-linked, lysed, and incubated with M2 anti-flag resin as described in *Materials and Methods*. The immunoprecipitates were blotted with goat anti-Dock180 and rabbit anti-ARNO. Samples of the starting lysates were blotted with goat anti-Dock180 and mouse anti-myc. (B and C) GRASP (B) and IPCEF (C) are coimmunoprecipitated with Dock180. MDCK cells were infected with adenoviruses encoding flag-Dock180 and either GFP-GRASP or HA-IPCEF for 18 h. Cells were treated with cross-linker, lysed, and incubated with mouse anti-myc or M2 anti-flag as indicated. Immunoprecipitates and samples of starting lysates were Western-blotted with goat anti-Dock180 and mouse anti-GFP or mouse anti-HA.

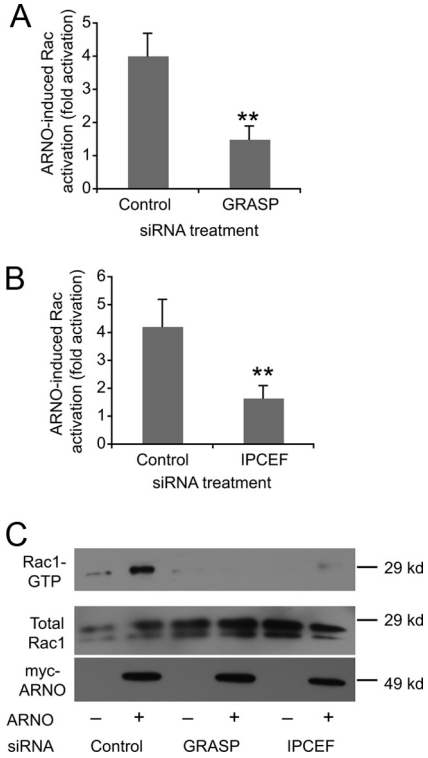

Figure 7. Knockdown of GRASP or IPCEF impairs ARNO-induced Rac activation. MDCK cells were transfected with the indicated siRNAs for 48 h. The cells were then split onto duplicate plates. After 18 h of recovery they were infected with adenovirus encoding ARNO in the presence or absence of doxycycline to repress transgene expression. After 3 h of expression cells were lysed, and active Rac was isolated by pulldown as described in *Materials and Methods*. Levels of active GTPase were normalized to the level of total GTPase in the starting lysate. (A and B) Activation of Rac by ARNO is impaired by knockdown of GRASP (A) or IPCEF (B). Data shown are the mean ± SE of the fold activation of Rac in ARNO-expressing cells compared with control cells. The effect of knockdown on ARNO-induced Rac activation was analyzed using a paired *t* test on multiple independent knockdown experiments. $^{**}p < 0.01$, n = 14 (GRASP knockdowns) or n = 12 (IPCEF knockdowns). (C) Representative gel from the pulldown experiments quantified in A and B.

IPCEF, that each bind to this domain. Knockdown of either scaffold inhibits activation of Rac downstream of ARNO and ARF.

The Role of ARF Activation in ARNO-induced Rac Activation

The experiments presented here define a scaffolding function for the coiled-coil domain of ARNO in assembling a complex of proteins containing the Rac-GEF Dock180. These data could be taken to indicate that ARNO-induced Rac activation is independent of ARNO's ARF-GEF activity. However, a point mutant of ARNO that lacks ARF-GEF function, E156K, fails to produce scattering when expressed in MDCK cells (Figure 2A, row 3; Santy and Casanova, 2001). Furthermore, we have previously demonstrated that this mutant does not enhance motility or lead to increased Rac activation in MDCK cells (Santy and Casanova, 2001). Therefore both the ARF-activating and scaffolding functions of ARNO are required for ARNO-induced Rac activation. Neither one alone is sufficient to produce robust activation of Rac.

The exact role of ARF activation in ARNO-induced Rac activation remains unclear but there are several possible actions of active ARFs that might be required. First, ARF6 activation has been shown to drive the recycling of lipid raft domains from an endosomal compartment to the plasma membrane (Balasubramanian *et al.,* 2007). The recycling of these raft domains is required for Rac1 recruitment to the plasma membrane and for Rac1 activation (Balasubramanian *et al.,* 2007). Additionally ARFs can directly modulate membrane lipid composition through activation of PLD and phosphoinositol-4-P-5 kinase to produce phosphatidic acid and phosphoinositol-(4,5)P_2, respectively (Exton, 1997; Honda *et al.,* 1999). We have previously shown that PLD is not required for ARNO-induced Rac activation (Santy and Casanova, 2001); however, the role of phosphoinositol-4-P-5 kinase in this process has not been investigated. The combined ARF-activating and -scaffolding functions of ARNO could ensure that membrane domains that recruit Rac and the Rac-GEF Dock180 are located in the same area of the plasma membrane.

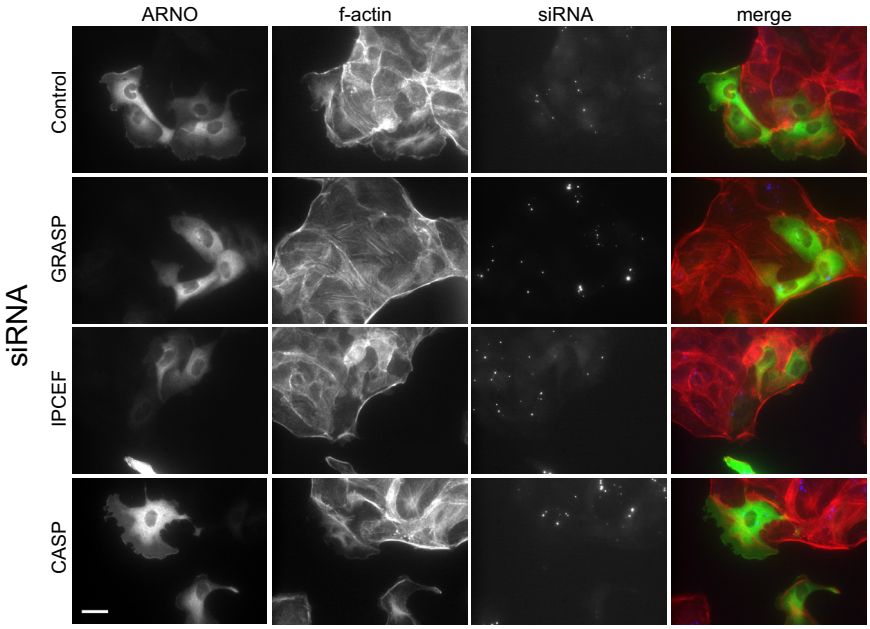

Figure 8. Knockdown of GRASP or IPCEF prevents ARNO from scattering MDCK cells. MDCK cells were transfected with 80 pmol of the indicated siRNA plus 20 pmol of Block-It Alexafluor red, fluorescent control siRNA, as a transfection marker. Forty-eight hours after transfection cells were replated onto coverslips and infected with adenovirus encoding ARNO in the presence of doxycycline to prevent transgene expression. After 18 h of recovery the doxycycline was removed, and the cells were allowed to express ARNO for 3 h. Cells were then fixed and stained with mouse anti-ARNO followed by Alexa-488–conjugated anti-mouse secondary antibody and Alexa-647–conjugated phalloidin. In the merge ARNO is psuedocolored green, f-actin is psuedocolored red, and the fluorescent siRNA is psuedocolored blue. Bar, 25 μM.

The exact ARF that is required for ARNO-induced Rac activation is also ambiguous. The cytohesin family can activate all ARFs in vitro, but in cells ARNO is localized at the cell periphery along with ARF6 (Chardin *et al.*, 1996; Frank *et al.*, 1998a; Cohen *et al.*, 2007). We have previously shown that MDCK cells expressing ARNO have a robust activation of endogenous ARF6 and no detectable activation of endogenous ARF1 (Santy and Casanova, 2001). However, given that the vast majority of ARF1 is located at the Golgi, the pulldown assay used in these experiments might be unable to distinguish the activation of a small pool of peripheral ARF1. Indeed null mutants of Steppke, the *Drosophila* cytohesin, have significant defects in growth and insulin signaling, whereas a *Drosophila* mutant of ARF6 shows more modest defects limited to defective cytokinesis during sperm formation (Fuss *et al.*, 2006; Dyer *et al.*, 2007). These data suggest that the cytohesins have functions that go beyond activation of ARF6. Knockdown experiments in cells have demonstrated that obvious trafficking defects are only distinguishable when pairs of ARFs are knocked down (Volpicelli-Daley *et al.*, 2005). Activation of ARF1, ARF6, or both might therefore be required for ARNO-induced Rac activation.

Cell biological studies have identified important roles for ARF6 in endocytosis, recycling, cytokinesis, and regulation of the actin cytoskeleton. It is therefore somewhat surprising that an ARF6 mouse knockout model survives until midgestation, which suggests that other proteins can assume many ARF6 functions (Suzuki *et al.*, 2006). A knockout of the cytohesins in mice has not been reported so it is not possible to compare cytohesin and ARF6 mutant phenotypes as can be done in *Drosophila*. In the ARF6 knockout mice, liver cells do not migrate properly after exiting the early hepatic epithelium. This leads to a failure of hepatic cord formation and hepatic apoptosis (Suzuki *et al.*, 2006). ARF6-regulated cell migration therefore stands out as one of the critical functions of ARF6 during development. Intriguingly, this early migration requires HGF and overexpression of ARNO in MDCK cells mimics the effect of HGF on these cells (Santy and Casanova, 2001).

Recent experiments have demonstrated that active ARF6 can bind to cytohesin PH domains and recruit cytohesins to the plasma membrane (Cohen *et al.*, 2007). These experiments led to the proposition of a model whereby ARF6 recruits a cytohesin that then activates ARF1 and ARF1 interacts with additional effectors to produce downstream actions (Cohen *et al.*, 2007). This model is supported observations of the activation of ARF1 and ARF6 during phagocytosis (Beemiller *et al.*, 2006). This study found that initially ARF6 is activated and that subsequently ARF1 is activated and that both ARFs are required for phagocytosis (Beemiller *et al.*, 2006). In the context of ARNO-induced migration and Rac activation, this model suggests that ARF6 activation by ARNO could act as a positive feedback to stabilize ARNO-containing complexes at the plasma membrane, whereas ARF1 activated by ARNO might interact with additional effectors to promote Rac activation. Further studies will be necessary to tease out the roles of the various ARFs during the regulation of motility.

The Role of Multiple Scaffold Proteins during ARNO-induced Rac Activation

Rac activation in ARNO-expressing cells is impaired by knockdown of either GRASP or IPCEF, suggesting that both of these proteins are necessary for this signaling cascade. Although both proteins bind to ARNO's coiled-coil domain, the precise binding sites of these two proteins are unknown so it is unclear if they overlap. Additionally ARNO exists in cells as a dimer (DiNitto *et al.*, 2007). Therefore even if the binding sites for the two proteins overlap, the ARNO dimer could be bound to both proteins at the same time. One possible model for the activation of ARF6 and Rac by ARNO is depicted in Figure 9. In this scenario one of the scaffolds would recruit and anchor ARNO at a particular subcellular location so that it is available to interact with Dock180. The other scaffold would recruit ARNO into the Dock180 complex to promote ARF-to-Rac cross-talk. One or both of these interactions could be regulated by upstream signals.

IPCEF contains a PH domain and the cytohesin-interacting domain. When IPCEF and ARNO are cooverexpressed

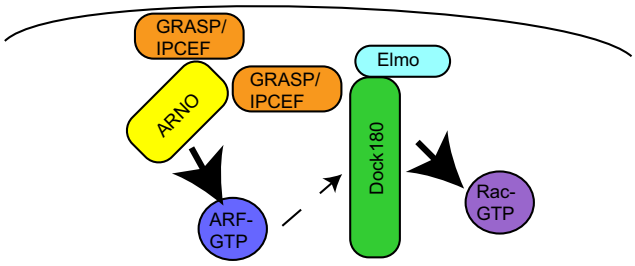

Figure 9. Model for the duel role of GRASP and IPCEF in promoting ARF-to-Rac cross-talk. See *Discussion* for details.

they are coordinately recruited to the plasma membrane in a PI-3-kinase–dependent manner after treatment of the cells with growth factors. Additionally binding of IPCEF to the coiled-coil domain of ARNO enhances ARNO's GEF activity (Venkateswarlu, 2003). A number of growth factors, including HGF, PDGF, NGF, EGF, CSF, and insulin have been shown to enhance membrane ruffling and migration in an ARF6-dependent manner (Venkateswarlu *et al.*, 1998a,b; Zhang *et al.*, 1999; Palacios and D'Souza-Schorey, 2003; Hall *et al.*, 2008). These growth factors act via receptor tyrosine kinases and activate PI-3-kinase. Therefore IPCEF is well suited to recruit ARNO to a particular subcellular location and anchor it there in response to growth factor signaling.

GRASP/Tamalin contains multiple protein–protein interaction domains. In neuronal tissues Tamalin is present in several large multiprotein complexes (Kitano *et al.*, 2002, 2003). Tamalin complex formation regulates trafficking of the group 1 metabotropic glutamate receptors (Kitano *et al.*, 2002, 2003). Although GRASP/Tamalin is highly expressed in the nervous system, it is also present at lower levels in a variety of tissues (Nevrivy *et al.*, 2000). Significantly Tamalin is required for ARF6-dependent Rac activation downstream of the TrkC receptor (Esteban *et al.*, 2006). These data suggest that GRASP/Tamalin is the likely candidate for building a larger complex that contains both ARNO and Dock180 and promotes ARF-to-Rac signaling.

Interestingly a different Rac GEF, Kalirin, has been reported to promote ARF6-dependent Rac activation downstream of EFA6, another ARF6-GEF (Koo *et al.*, 2007). Therefore different ARF-GEFs might not only regulate ARFs at different subcellular locations, they might also couple ARF to different signaling pathways to achieve similar outputs. Clearly much work remains to delineate signaling networks involving ARF6.

The data presented here demonstrate that IPCEF and GRASP promote ARF-to-Rac signaling downstream of ARNO by assembling a multiprotein complex containing ARNO and Dock180. Scaffold proteins assemble complexes containing multiple members of a signaling cascade. The ability of scaffold proteins to regulate and modulate signaling pathways is well recognized for other signaling cascades, particularly for the MAPK cascade. A variety of scaffolds including, KSR, MORG1, MP1, and JIPs can alter the kinetics, location, and output of MAPK modules (Morrison and Davis, 2003; Kolch, 2005; Dard and Peter, 2006; Pullikuth and Catling, 2007). Our data suggest that similar principles could apply to signaling pathways involving ARF6.

Although there are six mammalian ARFs, there are 15 Sec7 domain GEFs. These GEFs can be divided into five families, and at least three of these families are able to activate ARF6. These data strongly suggest that different GEFs are optimized to regulate ARFs during different processes and at different subcellular locations. Scaffold proteins are likely to play a central role in localizing these GEFs and biasing downstream outputs toward particular pathways.

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