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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Submitted March 17, 2009; Revised December 1, 2009; Accepted December 4, 2009

Monitoring Editor: Sean Munro

INTRODUCTION

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.

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).

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 to alter cell shape.

This article was published online ahead of print in MBC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–03–0217) on December 16, 2009.

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Abbreviations used: GEF, guanine nucleotide exchange factor, DSP dithiobis(succinimidylpropionate).
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; Pullkith 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 cytostatins, 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/Antibodies and Reagents

MATERIALS AND METHODS

Antibodies and Reagents

The 9E10 antibody against myc and mouse anti-FA (16812) 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 (B) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-ARF6 and mouse anti-ARNO were a kind gift from Slyvain Horvitz, 2000; Esteban et al., 2000; Kitano 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., 2000). These proteins can control the subcellular localization 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.

Expression Constructs

Recombinant adenoviruses for expressing full-length ARNO, ARNO E156K, or Dock180 under the control of the tetracycline responsive promoter have been described previously (Santy and Casanova, 2001; Santy et al., 2005). For the coiled-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 was 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 Pip3E 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-ElmoT625 were obtained from Kodi Ravichandran (University of Virginia). 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/Antibodies and Reagents

siRNA Knockdown

The siRNAs targeting human and dog GRASP (target sequence CTGTT- GAGATCGAGACTTAA), human and dog Pip3-E/IPCEF (target sequence CATCAGAAAAGTTGATT), human and dog CASP (target sequence CTTGCTGTCCTGGCAAAA), firefly luciferase, and a scrambled nontargeting control (siControl) were obtained from Dharmacon (Lafayette, CO). Fluorescently labeled myc-tagged Dock180 red control siRNA was obtained from Invitrogen (Carlsbad, CA). siRNAs were transfected into MCF-7 cells using LipofetRNA 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 × 105 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 × 106 MCF-7 cells were transfected with 30 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.

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% CO2. Cell culture media was purchased from Mediatech (Manassas, VA), and FBS was purchased from Hyclone (Rockford, IL).

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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 by 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 or flg-Dock180 for 18 h (MCF-7s) or 3 h (MDCKs). Alternatively, expression constructs were transfected into MDCK cells using Lipofectamine LTX according to the manufacturer’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 Na3VO4, 10 mM sodium pyrophosphate, 2 mg/ml aprotinin, 1 mg/ml leupeptin, 100 μg/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 cloned in frame into pGEX-2T to produce a construct expressing 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. MCF-7 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.
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 h 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, 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 1.](image1.png)

![Figure 2.](image2.png)
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 DSG, 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-coil 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...
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 resin. 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
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
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)P2, 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.
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 peripheral 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 by 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 IPCF, 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...
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). Thus, it is suggested 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 are consistent with the hypothesis 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.

ACKNOWLEDGMENTS

We thank Jim Casanova for reagents and suggestions. We thank Michael Teng for suggesting the DSP cross-linker. This work was supported by a grant from the American Heart Association, SDG-0730229N to L.C.S.

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ACKNOWLEDGMENTS

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