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Endogenous ARF6 Interacts with Rac1 upon Angiotensin II Stimulation to Regulate Membrane Ruffling and Cell Migration

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**Endogenous ARF6 Interacts with Rac1 upon Angiotensin II Stimulation to Regulate Membrane Ruffling and Cell Migration**

ARF6 and Rac1 are small GTPases known to regulate remodeling of the actin cytoskeleton. Here, we demonstrate that these monomeric G proteins are sequentially activated when HEK 293 cells expressing the angiotensin type 1 receptor (AT1R) are stimulated with angiotensin II (Ang II). After receptor activation, ARF6 and Rac1 transiently form a complex. Their association is, at least in part, direct and dependent on the nature of the nucleotide bound to both small G proteins. ARF6-GTP preferentially interacts with Rac1-GDP. AT1R expressing HEK293 cells ruffle, form membrane protrusions, and migrate in response to agonist treatment. ARF6, but not ARF1, depletion using small interfering RNAs recapitulates the ruffling and migratory phenotype observed after Ang II treatment. These results suggest that ARF6 depletion or Ang II treatment are functionally equivalent and point to a role for endogenous ARF6 as an inhibitor of Rac1 activity. Taken together, our findings reveal a novel function of endogenously expressed ARF6 and demonstrate that by interacting with Rac1, this small GTPase is a central regulator of the signaling pathways leading to actin remodeling.

INTRODUCTION

Reorganization of the actin cytoskeleton is an essential cellular response in various physiological and pathological conditions triggered by a broad variety of external stimuli such as hormones and growth factors. In mammalian cells, these stimuli promote the assembly of actin structures by activating signaling cascades regulated by small GTP-binding proteins of the Rho family. Like all GTPases, these cycle between an inactive (GDP-bound) and active (GTP-bound) state. Cycling between these two states is regulated by guanine-nucleotide exchange factors (GEFs), which facilitate the exchange of bound GDP for GTP, and GTPase-activating proteins (GAPs) that catalyze GTP hydrolysis (Geyer and Wittinghofer, 1997; Schmidt and Hall, 2002; Moon and Rossman et al., 2005). In addition, the activation of Rho-like GTPases is regulated by guanine-nucleotide dissociation inhibitors (GDIs), which retain the small G proteins in the cytosol (Olofsson, 1999). The best characterized members of the Rho family are RhoA, Rac1, and Cdc42. Although all promote actin reorganization, these small GTPases have distinct effects on cell shape and movement (Hall, 1998). For instance, Rho proteins have been classically associated with stress fiber formation (Ridley and Hall, 1992), Rac1 protein regulates ruffling and lamellipodia formation (Ridley et al., 1992), whereas Cdc42 is important for filopodia formation (Nobes and Hall, 1995).

Several studies have contributed to an understanding of the molecular mechanisms by which small GTP-binding proteins regulate actin remodeling, leading to membrane ruffling and cell migration after extracellular stimuli. The ADP-ribosylation factor 6 (ARF6), a small GTPase that regulates vesicular trafficking and the remodeling of membrane lipids, has also been shown to play an important role in actin rearrangement (reviewed in D’Souza-Schorey and Chavrier, 2006). It was recently demonstrated that aluminum fluoride and epidermal growth factor treatment can promote the relocation of ARF6 to the ruffling membranes (Fang et al., 2006). Interestingly, Radhakrishna et al. (1999) have suggested that this small GTP-binding protein is an important upstream regulator of Rac1-mediated ruffle formation because expression of a dominant negative mutant (ARF6T27N) prevents the aluminum fluoride–activated effect in Rac1-expressing cells. Similarly, Zhang et al. (1999) demonstrated that ARF6 was required for Rac1-mediated membrane ruffling in macrophages after growth factor stimulation. Recently, Nishiya et al. (2005) suggested that the localized formation of a complex including α4 integrin, pax-
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or ARF6 was synthesized as previously described (Houndolo et al., 2005) using the Silencer siRNA construction kit from Ambion (Austin, TX). The 21-nucleotide sequence from siRNA #1 (Houndolo et al., 2005) and #2 (Hashimoto et al., 2004) were previously characterized. To design ARF1-specific siRNA duplexes we chose a 21-nucleotide sequence corresponding to region 7–28 in the human ARF1 mRNA (5′-AAACATCTTCCGAACCTCTTC-3′). The scrambled siRNA targets a nonrelevant region in the human genome (5′-AACGAAGATGTCCAGGAT-3′).

Cell Culture and Transfection

HEK 293 cells stably expressing the AT,R-HA (Fessart et al., 2005) or AT-R-Flag were a gift from S. A. Laporte (McGill University). HEK 293 cells were maintained in minimal essential medium supplemented with 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal bovine serum at 37°C. Transfection of DNA plasmids and siRNAs were performed as previously described (Houndolo et al., 2005) using Lipofectamine 2000 according to the manufacturer’s instructions. However, in these experiments, cells were used 48 h after siRNA transfection. Hep2 cells were maintained in complete Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (Sigma), and penicillin and streptomycin (Sigma) at 37°C, 5% CO2. Cells were grown to 60–70% confluency before transfection by electroporation in HEBS buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 0.6 mM Na2HPO4, 0.25 mM glucose) using two 450-V, 125-μF pulses (Gene Pulsar II, Bio-Rad, Hercules, CA) and 1 μg of the relevant cDNA or 200 nM ARF6 siRNA. Cells were harvested and processed 48 h after transfection (Cant and Pitcher, 2005).

Western Blotting

All proteins were run on polyacrylamide gels (12%) and transferred onto nitrocellulose membranes. The membranes were blotted for relevant proteins using specific antibodies described in the following sections. Secondary antibodies were all FITC-conjugated, and fluorescence was detected using a Typhoon 9410 scanner (Amersham Biosciences, Baie D’Urfe, Quebec, Canada). Quantification of the digital images obtained was performed using ImageQuant 5.2 software (Amersham Biosciences).

Activation of ARF6 and Rac1

HEK 293 cells stably expressing the AT-R-HA (Fessart et al., 2005) or AT-R-Flag were a gift from S. A. Laporte (McGill University). The ARF1 and Rac1 cells were stably expressing Ang II (5 μM) at 37°C for the indicated times. Cells were lysed in 400 μl of ice-cold lysis buffer E (pH 7.4, 50 mM Tris HCl, 1% NP-40, 137 mM NaCl, 0.2% Triton X-100, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 mM Na2HPO4) containing 10% fetal calf serum (Sigma), and penicillin and streptomycin (Sigma) at 37°C, 5% CO2. Transfection of DNA plasmids and siRNAs were performed as previously described (Houndolo et al., 2005) using Lipofectamine 2000 according to the manufacturer’s instructions. Cell lysates were collected after siRNA transfection. Hep2 cells were maintained in complete Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (Sigma), and penicillin and streptomycin (Sigma) at 37°C, 5% CO2. Cells were grown to 60–70% confluency before transfection by electroporation in HEBS buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 0.6 mM Na2HPO4, 0.25 mM glucose) using two 450-V, 125-μF pulses (Gene Pulsar II, Bio-Rad, Hercules, CA) and 1 μg of the relevant cDNA or 200 nM ARF6 siRNA. Cells were harvested and processed 48 h after transfection (Cant and Pitcher, 2005).

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inhibitors (4°C for 1 h). Lysates were centrifuged at 10,000 rpm for 5 min, and equal concentrations of soluble protein were incubated with the monoclonal anti-ARF6 or polyclonal anti-Rac1 antibodies and protein G-PLUS agarose beads. The beads were washed, and bound proteins were eluted into 20 μl of SDS sample buffer containing 5% β-mercaptoethanol and heated to 95°C for 5 min. Proteins were resolved on 12% gels and detected by immunoblot analysis using specific antibodies (polyclonal anti-ARF6, polyclonal anti-Rac1).

RESULTS

Functional Interaction of ARF6 and Rac1

As early investigated whether ARF6 and Rac1 can associate in an endogenous setting, we examined the functional interaction of ARF6 and Rac1 in HEK 293 cells.

To delineate the molecular mechanisms by which the AT1R, a G protein–coupled receptor, promotes cytoskeleton reorganization, we examined the activation profile of ARF6 and Rac1 after agonist stimulation of HEK 293 cells stably expressing this receptor. Activation of endogenous ARF6 is rapid and transient with maximal levels of ARF6-GTP detected after 2 min of agonist-stimulation (Figure 1). Consistently, we observed that the amount of ARF6-GTP was significantly lower after 60 min of Ang II stimulation compared with that observed in unstimulated cells (0 min). Conversely, the activation of endogenous Rac1 was much slower than ARF6. GTP loading of Rac1 was found to increase gradually with time. Maximal activation was observed after 60 min of agonist treatment (Figure 1) and remained stable for at least 3 h (data not shown). Overexpression of Rac1-myc (1.6-fold/endoenous) markedly altered actin and stress fiber organization. In these conditions, GTP-loading on Rac1-myc was maximal 2 min after Ang II stimulation and remained sustained for at least 60 min. In addition, Rac1 overexpression led to an increased fold activation of Rac1 after Ang II stimulation (4-fold/endoenous) compared with 1.5-fold/basal for endogenous proteins (Supplementary Figure 1). **p < 0.01.

Preclinical studies have suggested a role for ARF6 in regulating Rac1 activity (D’Souza-Schorey et al., 1997; Radhakrishna et al., 1999; Zhang et al., 1999). In an attempt to determine if, in our model system, ARF6 is required for Rac1 activation, we initially investigated whether ARF6 and Rac can associate in an agonist-dependent manner.
Ang II Stimulation Promotes the Association of ARF6 and Rac1 in HEK 293 Cells

The molecular mechanisms by which ARF6 regulates Rac1 activity after stimulation of a G protein-coupled receptor remain unclear. A previous study has reported the colocalization of ARF6 and Rac1 in a perinuclear recycling compartment in HeLa cells and the subsequent translocation of both GTPases to the plasma membrane in response to aluminum fluoride treatment (Radhakrishna et al., 1999), suggesting a G protein–dependent relocalization of ARF6 and Rac1. We therefore sought to examine the localization of both small GTP-binding proteins before and after Ang II treatment in our AT1R expressing HEK 293 cells. To perform these experiments, we coexpressed ARF6-HA together with Rac1-myc and examined their distribution using confocal microscopy. Before agonist stimulation, both monomeric G proteins were present at the plasma membrane colocalized with actin. On Ang II treatment, remodeling of the actin cytoskeleton was observed; activation of the AT1R led to the formation of membrane protrusions and ruffles, which appeared between 10 and 15 min after Ang II treatment. Sixty minutes after treatment, both GTPases remained present at the site of ruffling. Colocalization of ARF6 and Rac1 in these protrusions is consistent with a potential role for these GTPases in this Ang II–dependent remodeling event (Figure 2A).

Using coimmunoprecipitation experiments, we subsequently investigated whether endogenous ARF6 and Rac1 could be found in complex upon agonist stimulation. Figure 2, B and C, illustrates that stimulation of the AT1R promotes the transient association of ARF6 and Rac1 in a time-dependent manner, with maximal association being observed after 15 min of agonist treatment. Quantification of the data reveals that agonist stimulation promotes a 3.3-fold enhancement of ARF6/Rac1 complex formation (Figure 2C). In addition, the association of the two GTPases could also be observed when endogenous Rac1 was immunoprecipitated. In these conditions, the maximal association between Rac1 and ARF6 also occurred after 15 min of Ang II treatment (Supplementary Figure 2).

The Association between ARF6 and Rac1 Is Direct and Dependent on the Activation State of Both Proteins

It is likely that, in cells, the formation of a complex between ARF6 and Rac1 is highly regulated by the recruitment of regulatory/scaffold proteins such as exchange factors and/or GTPase-activating proteins. However, the possibility exists that the two small G proteins directly interact after receptor activation. The ability of ARF6 to bind directly to Rac1 was assessed in vitro using purified proteins. Pull-down assays revealed a direct and specific interaction between GST-Rac1 and ARF6 (Figure 3A). In contrast, GST-RhoA and GST-Cdc42 did not bind purified ARF6. To our knowledge, these experiments represent the first demonstration of a direct interaction between two small GTP-binding proteins. Because ARF6 and Rac1 both cycle between an inactive (GDP) and an active (GTP) state, we next examined the nucleotide specificity of ARF6/Rac1 complex formation.
Preloading GST-Rac1 with GTP\textsuperscript{S} markedly impaired its ability to interact with soluble ARF6, suggesting that ARF6 binds the inactive (GDP-bound) form of Rac1 (Figure 3B). Conversely, preloading recombinant ARF6 with GTP\textsuperscript{S} increased its ability to interact with Rac1, suggesting that the activated form of ARF6 binds preferentially to Rac1 (Figure 3C). To further characterize the specificity of this interaction, we examined whether GST-Rac1 could also directly bind ARF1, another ARF isoform. As depicted in Figure 3D, Rac1 can also directly bind ARF1 in a GST pulldown assay. However, the interaction of the two small GTPases does not appear to be dependent on the nature of the nucleotide bound to ARF1. Several groups have identified interacting partners common to both ARF6 and Rac1 (D’Souza-Schorey et al., 1997; Di Cesare et al., 2000; Tarricone et al., 2001) and have proposed models for cross-talk between these two small GTPases. Our findings raise the possibility of an alternative mechanism whereby ARF6 could influence the activity of Rac1, via direct association.

To confirm that, in cells, activated ARF6 preferentially interacts with inactive Rac1, we also examined the ability of Rac1 and ARF6 mutants to coimmunoprecipitate. Consistent with the results obtained using purified proteins, the fast cycling mutant of ARF6, ARF6 T157A, and the dominant negative form of Rac1, Rac1 T17N, represented the strongest interacting partners (Figure 3E). The observation that an association between wild type ARF6 and Rac1 T17N could also be detected in these cells (Figure 3E) suggests the possibility that ARF6 is, at least in part, in an active conformation when expressed in HEK 293 cells.

Depletion of ARF6 in HEK 293 Cells Leads to Enhanced Basal Rac1 Activation

Several studies, using mutants mimicking the inactive or active states of ARF6, have been useful in demonstrating a role for this small G protein in Rac1 activation (Donaldson, 2003). However, a recent report has pointed out that ARF6 mutants, in particular ARF6 T27N, which mimics the GDP-bound form of the GTPase, may not recapitulate the effects observed with endogenous inactive ARF6 proteins in a cellular setting (Macia et al., 2004). To study the role endogenous ARF6 plays in regulating Rac1 activity after Ang II treatment, we used RNA interference (siRNA) strategies to silence the expression of ARF6 in our AT,R-expressing HEK293 cells. Surprisingly, depletion of this small GTPase dramatically altered the pattern of basal Rac1 activation (Figure 4A). Transfection of siRNA directed against ARF6 led to a marked activation of Rac1 that was not further increased by a 60-min Ang II treatment, which we have shown results in maximal Rac1 activation. To verify that the increased Rac1 activity observed was a specific effect resulting from the depletion of ARF6, and therefore independent of the nature of the siRNA, we compared the effect of our siRNA to another one designed against a different portion of the ARF6 sequence (Hashimoto et al., 2004) or an irrelevant protein (GAPDH). As illustrated in Figure 4A, depletion of

![Figure 3](https://example.com/figure3)

**Figure 3.** Activated ARF6 binds directly to the GDP-bound form of Rac1. (A) Equal amounts of GST, GST-Rac1 (ΔCAAX), GST-RhoA, or GST-Cdc42 fusion proteins (Coomassie staining) were incubated with purified ARF6. Using a GST pulldown assay, interacting ARF6 was precipitated and detected by Western blot analysis using an anti-ARF6 antibody. These results are representative of three independent experiments, and total input represents 6% of the total protein present in the sample. (B) The GST-Rac1 (ΔCAAX) fusion protein coupled to glutathione-Sepharose 4B beads was preloaded with either GDP\textsuperscript{S} or GTP\textsuperscript{S}. The nucleotide-bound proteins were incubated with purified ARF6. Interacting proteins were precipitated by a GST pulldown assay, and amounts of associated ARF6 were detected by Western blot analysis using a specific anti-ARF6 antibody. These results are representative of three independent experiments, and the total input represents 6% of the total protein present in the sample. (C) GST-Rac1 (ΔCAAX) coupled to glutathione-Sepharose 4B beads was incubated with GDP\textsuperscript{S}- or GTP\textsuperscript{S}-bound purified ARF6. The GST-Rac1 was precipitated and interacting ARF6-GDP\textsuperscript{S} or ARF6-GTP\textsuperscript{S} detected by Western blot analysis. These results are representative of four independent experiments. Total input represents 6% of the total protein present in the sample. (D) GST-Rac1 (ΔCAAX) coupled to glutathione-Sepharose 4B beads was incubated with GDP\textsuperscript{S}- or GTP\textsuperscript{S}-bound purified ARF1. The GST-Rac1 was precipitated and interacting ARF1-GDP\textsuperscript{S} or ARF1-GTP\textsuperscript{S} was detected by Western blot analysis. These results are representative of six independent experiments. Total input represents 30% of the total protein present in the sample. (E) HEK 293 cells stably expressing AT,R-Flag were transfected with Rac1-myc or Rac1 T17N-myc and either ARF6-HA, ARF6 T157A-HA or empty vector. Using an anti-ARF6 antibody coupled to agarose beads, HA-tagged proteins were immunoprecipitated from the cell lysates, and interacting Rac1 (wild type and mutants) was detected by Western blot analysis using an anti-myc antibody. These results are representative of five independent experiments, and the total input represents 4% of the total protein present in the sample.

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ARF6 by a siRNA designed by Hashimoto et al. (2004) resulted in a similar increase of endogenous Rac1 activity. In contrast, transfection of an siRNA directed against GAPDH or a scrambled siRNA (data not shown) did not significantly affect ARF6 expression or basal Rac1 activation. These results support the hypothesis that, in unstimulated cells, ARF6 is responsible for maintaining Rac1 in an inactive state.

Depletion of ARF6 Leads to Spontaneous Membrane Ruffling of HEK 293 Cells

The principal cellular event associated with Rac1 activation, in HEK 293 cells, is remodelling of cortical actin leading to membrane ruffling as shown in Figure 2A. We thus compared the effects of Rac1 activation mediated by either depletion of ARF6 or activation of the AT1R on this cellular response. Stimulation of AT1R expressing HEK 293 cells with Ang II results in the formation of actin-rich membrane protrusions, which appear 10–15 min after agonist treatment and remain present for at least 60 min (Figure 2A). Transfection of ARF6 siRNA, which as shown in Figure 4 leads to Rac1 activation, promotes agonist-independent membrane ruffling (Figure 2B). This phenomenon can be observed as soon as 24 h after transfection. To confirm that this spontaneous ruffling is dependent upon Rac1 activation, we co-transfected the dominant negative form of Rac1 together with the ARF6 siRNA. Rac1T17N expression abolished the spontaneous membrane ruffling induced by ARF6 depletion (Figure 2B). In these cells, membrane ruffling can be spontaneously initiated by the expression of a constitutively active mutant form of Rac1, Rac1Q61L-myc (Figure 5C). The reorganization of cortical actin in ARF6-depleted cells was very similar to that observed in Ang II–stimulated control cells. Indeed, in these two conditions, formation of protrusions as well as ruffles were observed. This is in contrast to actin remodeling induced by overexpression of Rac1Q61L, which results exclusively in membrane ruffle formation. These findings may suggest an impaired ability of the Rac1Q61L mutant to interact with its full range of effectors compared with endogenous activated Rac1. The ability of both AT1R activation and ARF6 siRNA transfection to induce membrane ruffling suggests similar functional capabilities for Rac1 activated via these two different stimuli and demonstrates that modulating ARF6 expression levels has profound functional consequences in a cellular setting. In contrast, depletion of ARF1 did not initiate spontaneous

Figure 4. Depletion of ARF6 increases basal Rac1 activation. (A) HEK293 cells stably expressing HA-AT1R were transiently transfected with siRNA targeting ARF6 (#1 or #2, 60 nM), or GAPDH (60 nM). Cells were treated with Ang II for 60 min, and activated Rac1 was captured using GST-PAK(CRIB) coupled to glutathione-Sepharose 4B beads in a GST pulldown assay. Endogenous levels of activated and total Rac1/ARF6 (4% of total input) were detected by Western blotting using specific antibodies. These results are representative of three independent experiments. (B) Quantification of the inhibition of ARF6 expression by transfection of different siRNA. Data are the mean ± SEM of three to eight independent experiments. ***p < 0.001.

Figure 5. Ang II stimulation or ARF6 depletion promotes membrane ruffling in HEK293 cells. (A) Cells stably expressing the HA-AT1R were stimulated with Ang II for the indicated times, fixed, and stained for the distribution of actin using phalloidin coupled to Alexa-488. (B) HA-AT1R stably expressing cells were transfected with siRNA directed against ARF6 (#1, 60 nM) or with both the siRNA for ARF6 and the inactive form of Rac1 (T17N), and staining of actin was performed as in A. (C and D) Cells were transfected with a constitutively active mutant form of Rac1 (Q61L; C) or an siRNA directed against ARF1 (25 nM; D), and actin staining was performed as in A and B. Scale bar, 10 μm. This figure is representative of more than 30 cells observed in three to six independent experiments.
ruffling (Figure 5D) or alter the Ang II–dependent ruffling response (Supplementary Figure 3A).

To determine whether this phenomenon can also be observed in other cell types, we depleted ARF6 from Hep2 cells, a cell line that we have previously used to study receptor-mediated cytoskeletal reorganization (Cant and Pitcher, 2005). As depicted in Figure 6, ruffling can be initiated by activation of transfected muscarinic M1 receptor (M1MR), but also by overexpression of Rac1(Q61L)-myc. As in HEK 293 cells, inhibition of ARF6 expression resulted in spontaneous membrane ruffling, suggesting that the ability of endogenous ARF6 to inhibit endogenous Rac1 activity is not unique to HEK 293 cells (Figure 6D). Similarly, spontaneous ruffling induced by ARF6 depletion was abolished when a dominant negative form of Rac1 was overexpressed (Figure 6E). In these cells, the number of ruffling cells was proportional to the inhibition of ARF6 expression (Figure 6, F and G).

Remodelling of the Actin Cytoskeleton Induced by ARF6 Depletion Promotes Cell Migration

The formation of actin-rich protrusions is one of the first steps required for cell migration. We therefore investigated the role of ARF6-mediated Rac1 activation in this important cellular process. We seeded HEK 293 cells into collagen-coated Boyden chambers and analyzed migratory phenotypes under different conditions. As illustrated in Figure 7, Ang II stimulation resulted in a 2.1-fold increase of cell motility as previously reported (Barnes et al., 2005). Notably, siRNA-mediated depletion of ARF6 also stimulated cell migration to a similar extent (2.1-fold). Agonist treatment had no additional effect on the migratory phenotype of ARF6-depleted cells. In contrast, depletion of ARF1 did not alter basal or Ang II-stimulated migration of HEK 293 cells. These results support the hypothesis that Rac1 activated, via ARF6 depletion or AT1R activation, is functionally equivalent. In addition, our findings highlight the importance of ARF6-mediated Rac1 activation in the process of AngII-dependent cell migration. Expression of a constitutively active mutant form of Rac1 (Rac1Q61L-myc), effective in promoting membrane ruffling, but not surface protrusion, had no effect on cell migration (data not shown). These results further suggest that Rac1Q61L expression is not functionally equivalent to activated endogenous Rac1.
The Rac1 Guanine-Nucleotide Exchange Factor β-PIX Is Relocalized to the Plasma Membrane in ARF6-depleted Cells

It is generally accepted that relocalization of small GTPases is necessary for their activation, although the mechanisms that control targeting of Rho GTPases in general are poorly understood. It was recently demonstrated that Rac1 binds to β-PIX (p21-activated kinases [PAK]-interacting exchange factor) and that this interaction is necessary and sufficient for Rac1 recruitment to membrane ruffles, providing a model for the intracellular targeting and localized activation of Rac1 (ten Klooster et al., 2006). To begin to address why Rac1 is found basally activated in ARF6-depleted cells, we examined the localization of the Rac/Cdc42 GEF β-PIX. As illustrated in Figure 8A, β-PIX is present mainly in the cytosol when overexpressed in HEK 293 cells. Ang II stimulation promotes the relocalization of β-PIX to the membrane ruffles, where it is found colocalized with actin. The agonist-dependent relocalization of β-PIX is consistent with a role for this protein in mediating receptor-dependent Rac1 activation. In ARF6-depleted cells, β-PIX is found at the plasma membrane (Figure 8C). Transfection of a control scrambled siRNA has no effect on the localization of β-PIX in basal and agonist-stimulated conditions (Figure 8B). Similar results can be obtained using a biochemical approach. Ang II treatment as well as depletion of ARF6 promotes the recruitment of endogenous β-PIX to the membrane fraction (Figure 8D). That ARF6 depletion promotes membrane recruitment of β-PIX in a similar manner to AT1R activation provides a potential explanation of how ARF6 depletion may regulate Rac1 activity.

DISCUSSION

In this study, we show that stimulation of a G protein-coupled receptor (AT1R) leads to the activation of endogenous ARF6 and Rac1 in HEK 293 cells, promoting the formation of actin-rich membrane protrusions and cell migration. We also demonstrate that upon Ang II treatment, ARF6 and Rac1 are relocalized to the edge of the membrane protrusions, where they transiently associate. In vitro assays suggest that this interaction can be direct and is regulated by the nature of the nucleotide bound to both ARF6 and Rac1. Our experiments demonstrate that ARF6, when bound to GTP, preferentially interacts with Rac1-GDP, suggesting that once Rac1 becomes loaded with GTP, the two small G proteins dissociate. In cells, it is likely that the signaling complex necessary to promote migration involves other signaling partners/regulators/effectors of both small GTPases. For example, we and others have shown that GIT proteins, characterized as ARF GAPs, and β-PIX proteins, a Rac GEF are tightly associated (Bagrodia et al., 1999; Premont et al., 2004). One would therefore expect these proteins to associate with ARF6 and Rac1 to promote signal transduction in a cellular context.

In our experiments, the maximal activation of endogenous ARF6 after Ang II stimulation occurs very rapidly (2 min) and can return to levels lower than basal in ruffling cells...
exposed to agonist for 60 min. These data suggest that under basal (unstimulated) conditions, a certain proportion of ARF6 is already GTP bound. Interestingly, activation of endogenous Rac1 is much slower (maximal at 60 min) and remains sustained for several hours. The activation profile of overexpressed Rac1, in contrast to ARF6, significantly differs from its endogenous counterpart, being much faster. This represents an important consideration when performing experiments with overexpressed proteins. For this reason, we have largely focused our effort on examining the role of endogenously expressed proteins. However, because of a lack of commercially available antibodies that recognize endogenous levels of these small GTPases by microscopy, we were unable to localize endogenous ARF6 and Rac1 in cells. To visualize their distribution, we had to express tagged versions of the wild-type proteins. In these conditions, both proteins were relocalized to the membrane ruffles upon Ang II stimulation.

Stimulation of the AT₁R not only promotes relocalization of the GTPases to the ruffling membrane but also their association. We have observed that 2 min after Ang II treatment, ARF6 and Rac1 coimmunoprecipitate. This interaction is transient and maximal at 15 min. At this specific time point, ARF6 has been maximally activated and Rac1 is in the process of being maximally activated. Using confocal microscopy, we were able to visualize morphological changes in the actin cytoskeleton after 2 min of agonist stimulation, indicating that early after receptor activation, when ARF6 is maximally activated, specific proteins and signaling cascades are activated to initiate actin reorganization. In our cells, formation of membrane protrusions and ruffling appeared 10–15 min after Ang II treatment (depending on the cell), coinciding with the time of maximal ARF6/Rac1 association and suggesting that Rac1 does not need to be fully activated to induce this morphological effect. Although the peak of interaction was observed at 15 min, association of the two proteins to a level comparable to what is observed at 10, 30, and 60 min is sufficient to promote ruffling. Because the activation/inactivation process of ARF6 after stimulation of a G protein–coupled receptor involves a yet to be characterized complex cascade of events, we suspect that relocalization of proteins and the assembly of signaling complexes is important for the interaction of both small GTPases. This argument is supported by the findings of Fang et al. (2006), who showed that GTP hydrolysis is required for the ARF6-dependent membrane remodeling.

Because ARF6 and Rac1 were found to associate in cells, we hypothesized that depletion of ARF6 would prevent the transmission of the signal that leads to activation of Rac1. Previous studies had demonstrated the coordinated action of ARF6 and Rac1 during the remodeling of the actin cytoskeleton, leading to membrane ruffling and cell migration. To allow the loading of GTP on Rac1, β-PIX is relocalized to the plasma membrane. In ARF6-depleted cells, β-PIX is found principally at the plasma membrane, and Rac1 is largely GTP bound. This results in spontaneous ruffling and migration of these cells.

Figure 9. Schematic diagram depicting a potential role for ARF6 and Rac1 in Ang II–stimulated membrane ruffling and cell migration. Under basal conditions, ARF6 and Rac1 are mainly found in their inactive state, associated with the plasma membrane. β-PIX, the Rac/Cdc42 guanine-nucleotide exchange factor, is mostly present in the cytosol. On AT₁R stimulation, ARF6 and Rac1 are subsequently activated and transiently associate to promote remodeling of the actin cytoskeleton, leading to membrane ruffling and cell migration. To allow the loading of GTP on Rac1, β-PIX is relocalized to the plasma membrane. In ARF6-depleted cells, β-PIX is found principally at the plasma membrane, and Rac1 is largely GTP bound. This results in spontaneous ruffling and migration of these cells.
localization of the Rac1 GEF, β-PiX. It was recently reported that the interaction with β-PiX was necessary and sufficient for Rac1 recruitment to membrane ruffles and to focal adhesions (ten Klooster et al., 2006). We therefore hypothesized that the depletion of ARF6 might allow the relocation of β-PiX to the plasma membrane and the activation of Rac1. This is indeed what we observed. In ARF6-depleted cells, β-PiX is found mainly in the membrane ruffles. The molecular mechanism by which the endogenous expression of ARF6 prevents the translocation of β-PiX to the plasma membrane in normal conditions remains however to be defined. Figure 9 represents a model of the sequence of events that may occur in basal, Ang II–stimulated and ARF6-depleted cells. Before agonist-stimulation, ARF6 and Rac1 are largely found in a GDP-bound state because their exchange factors (ARNO and β-PiX) are mainly cytosolic. Stimulation of the AT,R results in the activation of ARF6, which we have previously suggested occurs via the agonist-dependent recruitment of a β-arrestin/ARNO complex (Claing et al., 2001). This study indicates that ARF6-GTP can directly interact with Rac1-GDP and that Ang II treatment ultimately leads to Rac1 activation and actin remodelling. We suspect that ARF6 could result in the activation of Rac1, via recruitment of its GEF β-PiX, which directly interacts with GIT1, an ARF GAP. In ARF6-depleted cells, we have observed that β-PiX is mainly localized to the plasma membrane and that Rac1 is mostly bound to GTP, resulting in cell ruffling and migration.

Taken together, our results suggest that changes in ARF6 expression may have important cellular consequences. ARF6 may exhibit differential effects in cells with variable basal levels of Rac1 activity. It is possible that noninvasive cells could acquire spontaneously a migratory phenotype when ARF6 expression is reduced, suggesting that ARF6-dependent regulation of Rac1 activity may be of pathological importance. However, in other cell types and experimental conditions, the function of endogenous ARF6 might be different. It was reported previously that ARF6 depletion blocks the invasive activity of breast cancer cells (Hashimoto et al., 2004). In these cells, the signaling events regulating Rac1 activity might involve different proteins and be regulated through distinct molecular mechanisms.

How exactly ARF6 functions in cells remains an important biological question. This GTPase is well known for its role in vesicle trafficking and remodeling of the membrane lipids (reviewed in D’Souza-Schorey and Chavrier, 2006). We have previously shown that depletion of ARF6 in AT,R-expressing HEK 293 cells leads to the inhibition of the endocytosis of a variety of G protein–coupled receptors, namely the AT,R (Houndolo et al., 2005). However, the spontaneous activation of Rac1 leading to membrane ruffling that we observed in ARF6-depleted cells is not linked to the agonist-promoted block of receptor internalization. Indeed, unstimulated, untransfected Hep2 cells still spontaneously ruffle when transfected with siRNA targeted against ARF6. In normal conditions, however, it is likely that the processes of endocytosis, lipid remodeling and actin rearrangement are intimately related to regulate agonist-promoted cellular responses.

Our data demonstrate that an imbalance between ARF6 and Rac1 activity/expression levels can have profound cellular consequences. A change in the expression or in the activation mechanisms of ARF6 may thus contribute to the development of new cell phenotype, which may lead to important pathological conditions.

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