Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D

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Migration of epithelial cells is essential for tissue morphogenesis, wound healing, and metastasis of epithelial tumors. Here we show that ARNO, a guanine nucleotide exchange factor for ADP-ribosylation factor (ARF) GTPases, induces Madin-Darby canine kidney epithelial cells to develop broad lamellipodia, to separate from neighboring cells, and to exhibit a dramatic increase in migratory behavior. This transition requires ARNO catalytic activity, which we show leads to enhanced activation of endogenous ARF6, but not ARF1, using a novel pulldown assay. We further demonstrate that expression of ARNO leads to increased activation of endogenous Rac1, and that Rac activation is required for ARNO-induced cell motility. Finally, ARNO-induced activation of ARF6 also results in increased activation of phospholipase D (PLD), and inhibition of PLD activity also inhibits motility. However, inhibition of PLD does not prevent activation of Rac. Together, these data suggest that ARF6 activation stimulates two distinct signaling pathways, one leading to Rac activation, the other to changes in membrane phospholipid composition, and that both pathways are required for cell motility.

Introduction

Columnar epithelia grow as sheets of cells in which neighboring cells are attached to each other by junctional complexes, including tight junctions and adherens junctions. In tissues, epithelial cells are normally stationary and nonmigratory, however, during embryonic development, wound healing, or metastasis of tumors they may become migratory (Gumbiner, 1996; Schmitz et al., 2000). The adoption of a motile phenotype requires regulated changes in the actin cytoskeleton and in the attachment of cells to the substratum. Small GTPases of the Rho family are intimately involved in regulating these processes (for review see Schmitz et al., 2000). Recent work has demonstrated that members of the ADP-ribosylation factor (ARF)* family of small GTPases can also regulate assembly of the actin cytoskeleton (Radhakrishna et al., 1996; D'Souza-Schorey et al., 1997; Norman et al., 1998; Radhakrishna et al., 1999; Zhang et al., 1999; Boshans et al., 2000).

Like Rho GTPases, ARFs are members of the Ras superfamily and have been well characterized as regulators of vesicular transport. The six known ARF isoforms can be divided into three classes. Class 1 ARFs (ARFs 1–3) regulate trafficking in the secretory pathway and in endosomes. Very little is known about the role of class II ARFs (ARFs 4 and 5). The sole class III ARF, ARF6, functions in the endosomal plasma membrane system, and has been shown to regulate trafficking between these compartments (for review see Chavrier and Goud, 1999).

It is becoming increasingly clear that modulation of actin assembly is an important function of ARF proteins. For example, ARF1 has recently been shown to regulate the recruitment of paxillin into focal adhesions (Norman et al., 1998). Activation of ARF6 also results in reorganization of the cortical actin cytoskeleton (Radhakrishna et al., 1996; D'Souza-Schorey et al., 1997). Several studies have suggested an intimate connection between ARF6 and the Rho family of small GTPases; however, the precise relationship is poorly understood (D'Souza-Schorey et al., 1997; Radhakrishna et al., 1999; Zhang et al., 1999; Boshans et al., 2000).

Like all GTPases, ARFs are switches that are active when bound to GTP and inactive when bound to GDP. Interconversion of the two states requires the activity of several acces-

sory proteins. Guanine nucleotide exchange factors (GEFs) catalyze the release of bound GDP and its subsequent replacement by GTP, leading to ARF activation. Similarly, a return to the inactive state requires hydrolysis of the bound GTP to GDP, which is facilitated by the GTPase activating proteins (GAPs) (Donaldson and Jackson, 2000).

ARNO is a member of one subfamily of ARF-GEFs, which also includes cytohesin-1, GRP-1, and ARNO/cytohesin-4, all of which share a similar domain organization (for review see Jackson and Casanova, 2000). The NH_2-terminal 60 amino acids comprise a coiled coil domain that mediates homodimerization. A central catalytic domain, the Sec7 domain, is a conserved feature of all ARF-GEFs. Mutation of a key residue within this domain (E156K in ARNO) completely abrogates the exchange activity of all Sec7 family members tested to date (Beraud-Dufour et al., 1998). The COOH-terminal portion of the protein contains a PH domain and a polybasic domain that mediate the interaction of these proteins with membrane surfaces (Jackson and Casanova, 2000).

ARNO family members can catalyze exchange on multiple ARF isoforms in vitro. Therefore, it is likely that colocalization of these proteins with a specific ARF isoform is the primary determinant of their substrate specificity (Chardin et al., 1996; Frank et al., 1998a; Langille et al., 1999). Endogenous ARNO has been localized to the plasma membrane of BHK cells (Frank et al., 1998a), adrenal chromaffin cells (Caumont et al., 2000), and to the apical pole of kidney-proximal tubule epithelial cells (Maranda et al., 2001). Additionally, exogenous ARNO can be seen to colocalize with ARF6 at the plasma membrane (Frank et al., 1998a), is recruited to the plasma membrane in response to PI-$(3,4,5)P_3$ production (Venkateswarlu et al., 1998), and can stimulate actin cytoskeletal rearrangements (Frank et al., 1998b). Because of the relative enrichment of ARF6 at the plasma membrane and in the endosomal system (Peters et al., 1995), we and others have suggested that ARF6 is likely to be the primary substrate for ARNO in vivo (Frank et al., 1998a,b; Caumont et al., 2000). However, the ability of ARF1 to regulate focal adhesion assembly suggests that ARF1 may also have some role at the plasma membrane (Norman et al., 1998). No matter which ARF is involved, it is clear that ARNO is a key regulator of ARF function at the cell surface.

It has been shown recently that in polarized epithelial cells and tissues ARF6, like ARNO, is concentrated at or near the apical plasma membrane (Marshansky et al., 1997; Maranda et al., 2001). Additionally, ARF6 has been shown to selectively regulate apical endocytosis in MDCK cells (Altschuler et al., 1999). Therefore, we were interested in investigating the role of ARNO and ARF6 in modulating the actin cytoskeleton in epithelial cells.

We found that expression of ARNO in MDCK cells leads to a profound change in cell morphology and behavior. The cells rapidly form large fan-shaped lamellipodia and pull away from the tightly joined islands of cells that are characteristic of this cell line. Consistent with this morphological change, ARNO-expressing cells exhibit increased migratory activity in both a Boyden chamber migration assay and in a wound healing assay. We show that ARNO expression leads to activation of endogenous Rac1, and that Rac activity is required for acquisition of the migratory phenotype. This transition also requires activation of a known ARF effector, phospholipase D (PLD). However, PLD activity is not required for Rac activation, indicating that Rac and PLD lie on two distinct signaling pathways downstream of ARF, and function in concert to convert epithelial cells from a stationary to a migratory state. Therefore, ARF proteins may play a much more significant role in the regulation of cell motility than has previously been appreciated.

Results

Expression of ARNO induces migration of MDCK cells

To examine regulation of epithelial actin structure, we have taken advantage of a recombinant adenovirus system that expresses proteins under the control of a tetracycline-regulatable promoter (Altschuler et al., 1999). Infection of MDCK cells expressing the tetracycline-regulatable transactivator (T23 MDCK; Barth et al., 1997) with these adenoviruses results in rapid and robust expression of the encoded transgene. All controls are infected with the virus in the presence of 20 ng/ml doxycycline, which prevents transgene expression, to control for any possible effects of viral infection.

When grown at subconfluent density, MDCK cells normally form compact islands of cells with tight junctions and adherens junctions between adjacent cells. Cells shown in Fig. 1 A were infected for 3 h with adenovirus encoding myc-tagged wild-type ARNO in the presence of doxycycline, and appear identical to noninfected cells (data not shown). After 3 h of infection with the same virus in the absence of doxycycline, expression of ARNO is readily apparent (Fig. 1 B). Moreover, ARNO-expressing cells on the edges of the island form large, fan-shaped lamellae and begin to pull away from their neighbors. Intriguingly, ARNO-expressing cells located in the interior of the islands do not manifest this altered morphology and remain firmly attached to their neighbors (Fig. 1 B). Importantly, a point mutant of ARNO, E156K, which lacks nucleotide exchange activity, does not induce the formation of lamellipodia or separation from the cell cluster, even in cells at the margins (Fig. 1 C). Together, these data suggest that activation of ARF is required for the ARNO-induced changes in epithelial morphology and behavior.

To investigate whether ARNO makes MDCK cells more migratory, we used a transwell migration assay. MDCK cells were infected with an adenovirus encoding either wild-type ARNO in the presence or absence of doxycycline, or with the catalytically inactive point mutant E156K. The cells were placed in the upper chamber of transwell filters that had been coated on the underside with fibronectin, and allowed to migrate for 16 h. As shown in Fig. 2, <2% of control cells migrated through the filter, whereas expression of ARNO led to an eightfold increase in the number of migrated MDCK cells. The catalytically inactive E156K mutant yielded results that were indistinguishable from controls, indicating that activation of ARF is a key element of this migratory behavior.

To more directly observe the effect of ARNO on the migratory behavior of MDCK cells, we used time lapse video
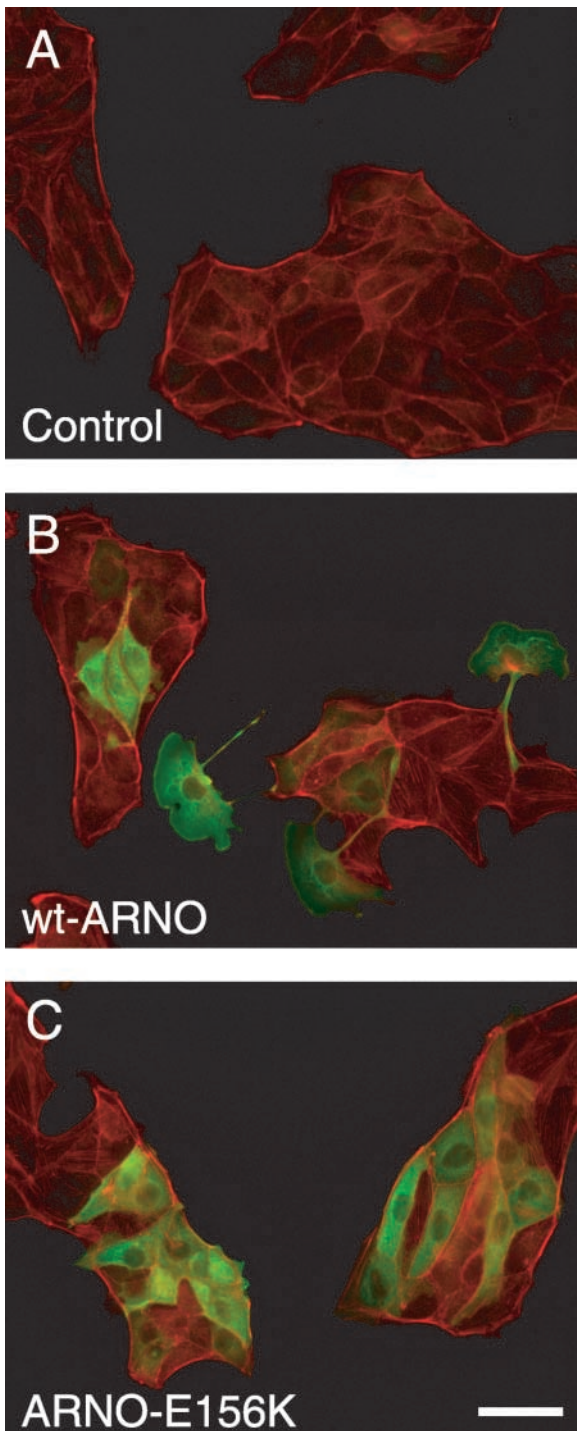
Figure 1. **Expression of catalytically active ARNO induces migratory morphology in MDCK cells.** MDCK cells were cultured on glass coverslips, infected with adenovirus encoding ARNO under the control of the tetracycline regulatable transactivator for 3 h, and processed for immunofluorescence microscopy as described in Materials and methods. Cells were stained with 9E10 monoclonal anti-myc (green) to label ARNO and rhodamine-conjugated phallodin to label filamentous actin. (A) Control cells infected in the presence of doxycycline to prevent transgene expression, (B) cells expressing myc-tagged wild-type (WT) ARNO, (C) cells expressing myc-tagged catalytically inactive ARNO E156K. Bar, 50 μm.
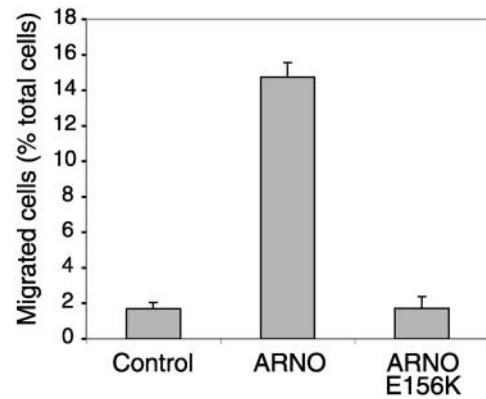

Figure 2. **Expression of ARNO enhances the migration of MDCK cells in a modified Boyden chamber migration assay.** MDCK cells were infected for 4 h with virus encoding wild-type ARNO, E156K ARNO in the presence (control) or absence of doxycycline. Cells were harvested and placed in transwell filter chambers coated on the underside with fibronectin (see Materials and methods). Cells were then allowed to migrate for 16 h and quantitated as described in Materials and methods. Data shown are mean ± standard deviation of triplicate samples and are representative of >10 experiments.

microscopy to examine cells in a wound-healing assay. When MDCK cells become confluent, they form a monolayer and adopt a typical columnar epithelial morphology. Disruption of the monolayer by dragging a Teflon cell scraper through the culture causes cells on the edge of the wound to rapidly spread and become flattened, rather than columnar. As these cells migrate into the wounded area, ranks of cells located further back from the wound edge are successively recruited to begin spreading. The leading wound edge migrates smoothly as a unit; the cells in this edge remain attached to adjacent cells and do not pull away from the monolayer (Fig. 3, A–C, and Video 1 [available at www.jcb.org/cgi/content/full/200104019/DC1]).

Monolayers of cells expressing ARNO have noticeably different morphology and behavior. The leading edge does not move forward as a smooth unit. These cells pull away from the wound edge and exhibit a distinct fan-shaped, leading edge and a trailing edge with a tail that often remains attached to the body of the monolayer (Fig. 3, D–F, and Video 2 [available at www.jcb.org/cgi/content/full/200104019/DC1]). This morphology is similar to that seen in islands (Fig. 1 B). Immunofluorescence microscopy of wounded monolayers revealed that these cells are indeed expressing ARNO (data not shown). We quantified the effects of ARNO expression on migration by measuring the area covered by the migrating monolayer over time (see Materials and methods). As shown in Fig. 3 J, expression of ARNO significantly increased the rate of migration of the monolayer into the wound.

We have demonstrated previously that phosphorylation of ARNO at a single serine residue (S392) reduces the affinity of ARNO for membranes and may be a mechanism for downregulation of ARNO activity (Santy et al., 1999). Therefore, we reasoned that a mutant of ARNO containing both the inactivating point mutation, E156K, and a mutation of the phosphorylated serine, S392A, might be an effective dominant inhibitory molecule. This mutant should be
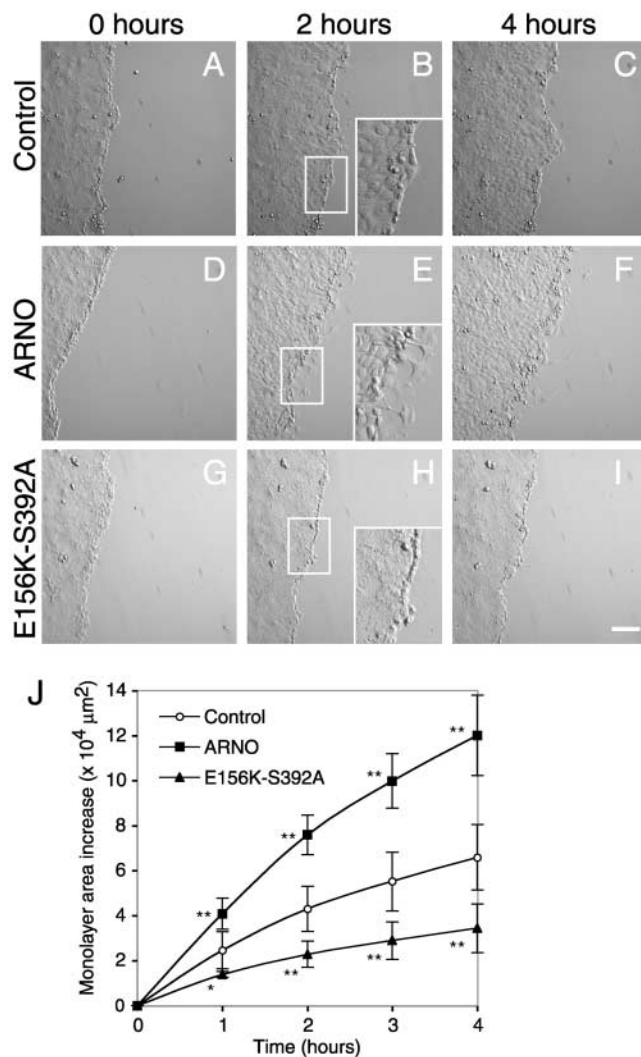
Figure 3. **Migration of MDCK cells in a wound healing assay is altered by expression of wild-type ARNO or the catalytically inactive mutant E156K-S392A.** Confluent MDCK monolayers were infected with recombinant adenovirus and wounded as described in Materials and methods. Control cells (A–C), wild-type ARNO–expressing cells (D–F), or E156K-S392A ARNO–expressing cells (G–I) were observed migrating into the wound by time lapse video microscopy. Frames shown were taken 0 h (A, D, and G), 2 h (B, E, and H), or 4 h (C, F, and I) after wounding. Video frames were recorded every minute for 4 h and videos are available at www.jcb.org/cgi/content/full/200104019/DC1. (J) ARNO alters the rate of migration into the wound. The surface area covered by the migrating monolayer was measured using the program OpenLab and the increase in monolayer area over time is shown. Data shown are mean ± standard deviation of eight (ARNO), six (control), and three (E156K-S392A) individual experiments. Data were evaluated using the Student's t test. Asterisk indicates $P < 0.05$; double asterisk indicates $P < 0.01$. Bar, 100 μm.

recruited to sites of ARNO action but remain membrane-bound, inhibiting recruitment of endogenous ARNO and preventing activation of ARFs. Indeed, we found that expression of this E156K-S392A mutant inhibited migration of MDCK monolayers in the wound healing assay. Very little spreading of cells is observed, and few if any ruffles form at the wound edge. Additionally, cells further back from the wound edge remain unspread and movement of the epithelial sheet into the wound is slowed (Fig. 3, G–J, and Video

3 [available at www.jcb.org/cgi/content/full/200104019/DC1]). These data suggest that activation of ARF proteins by ARNO is critically important for the migration of MDCK epithelial sheets into a wound.

ARNO selectively activates ARF6

Because ARNO is relatively promiscuous in its substrate specificity in vitro, we investigated the localization of endogenous MDCK ARF proteins in ARNO-expressing cells using immunofluorescence microscopy. Endogenous ARF6 was observed throughout the large lamellipodia and appeared to be concentrated at the leading edge of migrating cells, where it colocalized with ARNO (Fig. 4, C and D). ARF1, in contrast, could be seen in a perinuclear crescent resembling the Golgi and in other locations throughout the cell, but was barely detectable within the ruffle or at the leading edge (Fig. 4, A and B).

We also tested the ability of ARNO to activate endogenous MDCK ARF1 or ARF6 using a novel pull down assay. This assay is analogous to the widely used assays in which glutathione S-transferase (GST) fused to fragments of p21-activated kinase (PAK) are used to selectively precipitate GTP-bound Rac and Cdc42 from cells (Benard et al., 1999). Here, we took advantage of the GTP-dependent interaction of ARF with the GGAs (Golgi-localized, γ ear–containing ARF-binding proteins), a family of recently described ARF effector proteins (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000). To selectively precipitate GTP-bound ARFs, we used a GST fusion containing the VHS and ARF binding domains of GGA3 (GST-GGA). The specificity of this assay was tested using MDCK cells expressing GTP-binding state mutants of ARF1 and ARF6. As is shown in Fig. 4 E, only the mutationally activated, GTP-bound mutants of ARF1 (ARF1Q71L) and ARF6 (ARF6Q67L) bound to GST-GGA, whereas the GDP-bound dominant negative mutants (ARF1T31N, ARF6T27N) did not. To measure the activation of endogenous ARFs, GST-GGA was incubated with lysates of MDCK cells. The bound, active ARFs were isolated and visualized by Western blotting with ARF1- and ARF6-specific antibodies. The amount of GTP-ARF was compared with the total amount of ARF present in the initial lysate and quantitated by densitometry.

After adenovirus-mediated expression of ARNO for only 2–3 h, which is sufficient to induce the ARNO-specific morphological changes described above, the activation of endogenous ARF1 and ARF6 in the same sample was determined and compared with activation levels in control cells (Fig. 4 F). Although the extent of ARF activation by ARNO varied between experiments, pairwise comparison of ARF6 versus ARF1 isolated from the same samples in nine experiments demonstrates that ARNO expression activates ARF6 (2.1 ± 0.84-fold) selectively over ARF1 (1.0 ± 0.27-fold; paired t test ARF6 versus ARF1; $P = 0.001$). Although it is possible that activation of a small pool of peripheral ARF1 by ARNO is responsible for the modulation of morphology, these data are most consistent with the idea that ARNO induces the observed changes by enhancing levels of GTP-ARF6.

To further investigate the ability of ARF6 to regulate migration, we observed the effect of the dominant negative
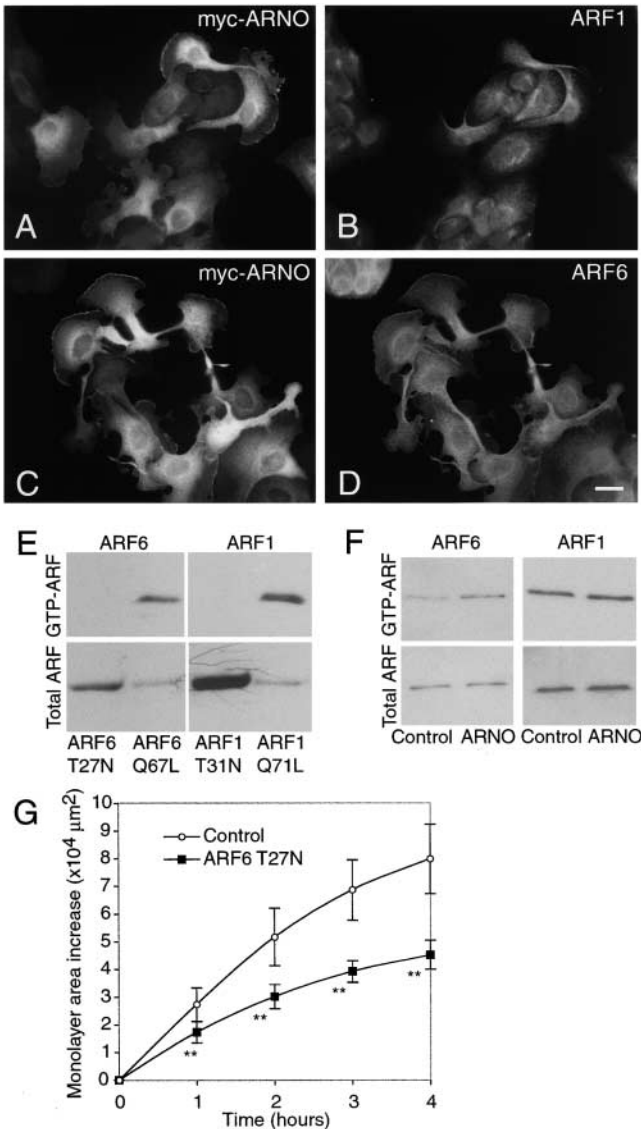
Figure 4. **Association of ARNO with endogenous ARF6 in MDCK cells.** (A–D) Cells were infected with adenovirus encoding myc-tagged ARNO for 3 h. Cells were labeled with 9E10 monoclonal anti-myc (A and C) and rabbit polyclonal antisera specific for either ARF1 (B) or ARF6 (D). E,F: Affinity precipitation of active ARFs using GST-GGA. To demonstrate the specificity of GST-GGA for ARF-GTP, MDCK cells were infected with adenovirus encoding HA-tagged constitutively active (ARF6Q67L, ARF1Q71L) or dominant negative (ARF6T27N, ARF1T31N) ARF mutants (E). Cells were lysed and GTP-bound ARF was isolated as described in Materials and methods. To measure activation of endogenous ARFs (F), cells were infected with adenovirus encoding wild-type ARNO in the presence (control) or absence (ARNO) of doxycycline. Cells were lysed and precipitated with GST-GGA as in E. Arfs were quantitated by Western blotting with monoclonal anti-HA (E) or ARF1- and ARF6-specific antibodies (F). Data shown in F are representative of nine experiments. (G) ARF6 T27N inhibits migration into the wound. Confluent monolayers of MDCK cells were infected with adenovirus encoding ARF6 T27N and incubated overnight in the presence of 20 ng/ml doxycycline (Control) or 0.2 ng/ml doxycycline (ARF6 T27N) and wound healing was performed as in the legend to Fig. 3. Data shown are mean ± standard deviation of four (control) or six (ARF6 T27N) individual experiments. Data were evaluated using the Student's t test. Double asterisk indicates $P < 0.01$. Bar, 20 μm.

ARF6 mutant, T27N, on migration of MDCK cells. Monolayers of MDCK cells were infected with adenovirus encoding ARF6 T27N and incubated overnight in the presence of 0.2 ng/ml dox before migration was tested using the wound healing assay. Under these conditions, the dominant negative ARF6 is overexpressed to levels less than fivefold over the endogenous protein (Altschuler et al., 1999, and data not shown). Expression of ARF6 T27N significantly inhibited the migration of MDCK cell monolayers in the wound healing assay (Fig. 4 G). These data further support the idea that activation of ARF6 enhances migration of MDCK cells.

Stimulation of migration by ARNO requires activation of Rac

The large lamellipodia induced by ARNO are similar in morphology to those induced by activation of Rac. Additionally Rac activity has been shown to be required for wound healing in monolayers of MDCK cells (Fenteany et al., 2000) or fibroblasts (Nobes and Hall, 1999). To assess the activation state of endogenous Rac, we used a GST fusion to the p21 binding domain of PAK in a pulldown assay. Lysates of control cells, cells expressing wild-type ARNO, or cells expressing ARNO E156K were incubated with GST-PAK and the relative amounts of Rac-GTP in each lysate were analyzed by Western blotting. As shown in Fig. 5, cells expressing wild-type ARNO contained significantly more active Rac than the control cells (average activation 2.2-fold, paired t test; $P = 0.008$, $n = 11$). In contrast, cells expressing the E156K mutant contained levels of Rac-GTP similar to controls (Fig. 5, average activation 0.84-fold, paired t test; $P = 0.19$, $n = 6$). Because ARNO is selective for ARFs and is unable to activate Rac directly in vitro (Chardin et al., 1996; Casanova, J.E., unpublished data), these observations suggest that ARF activation by ARNO leads to the downstream activation of Rac.

To investigate whether Rac activation is required for ARNO-induced migratory effects, we examined the ability of a dominant negative mutant of Rac (N17) to block these changes. In contrast to control cells expressing ARNO alone (Fig. 6, A and B), MDCK cells expressing both N17-Rac and ARNO failed to extend lamellipodia and remained firmly attached to their neighbors (Fig. 6, C and D). Additionally, coexpression of N17-Rac with ARNO blocks the migratory morphology seen in cells expressing ARNO alone during wound healing (data not shown). These data suggest that activation of Rac downstream of ARF is indeed necessary for ARNO-induced cell migration.

Activation of PLD correlates with ARNO-induced cell motility

To explore signal transduction pathways leading from ARNO to Rac activation and motility, we investigated the role of one well-characterized downstream ARF effector, PLD, which converts phosphatidylcholine into phosphatidic acid (PA) (Exton, 1997). As shown in Fig. 7, expression of ARNO in MDCK cells increased PLD activity 2.6-fold. This increase was dependent upon ARNO catalytic activity, as the E156K point mutant did not stimulate PLD activity (Fig. 7).
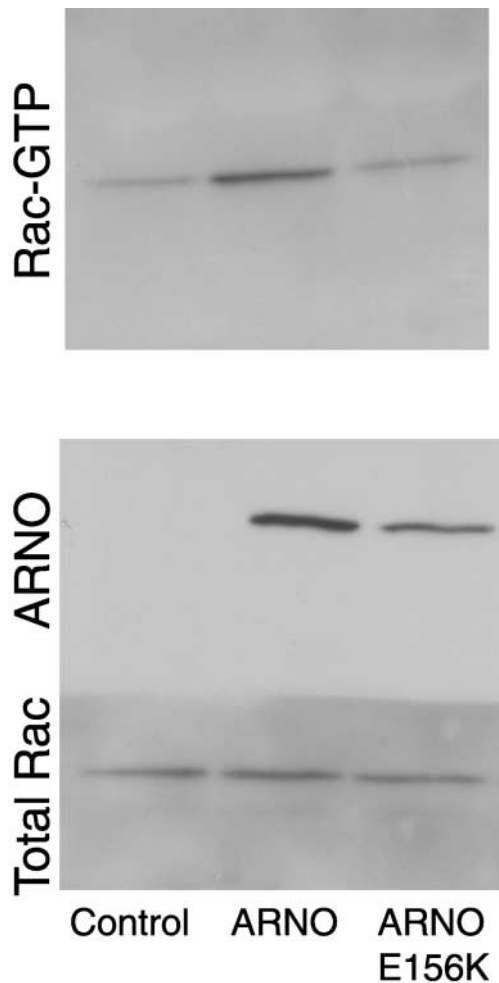
Figure 5. **Expression of ARNO leads to activation of endogenous Rac.** MDCK cells were infected with adenovirus encoding ARNO in the presence (control) or absence (ARNO) of doxycycline or with virus encoding ARNO E156K. Cells were lysed and GTP-Rac was isolated by incubation with a GST fusion containing the PBD domain of PAK as described in Materials and methods. Rac-GTP (top) and total Rac (bottom) were quantitated by Western blot. Data are representative of 11 (ARNO) or 6 (E156K) experiments.

Next, we investigated the effect of PLD inhibition on the morphology of ARNO-expressing cells. PLD can utilize primary alcohols in the place of H_2O during the hydrolysis of phosphatidylcholine, producing a phosphatidylalcohol, rather than phosphatidic acid (Exton, 1997). The production of this alternate product disrupts further signaling downstream of PLD that requires PA. Secondary and tertiary alcohols cannot be used by PLD and therefore can be used to control for other nonspecific effects of alcohol on cells. We analyzed the morphology of ARNO-expressing cells upon treatment with 0.5% 1-butanol (Fig. 8, C and D) or, as a control, tert-butanol (Fig. 8, E and F). Treatment with 1-butanol inhibits formation of the large fan-shaped lamellipodia characteristic of ARNO-expressing cells. The only ruffle-like structures seen in these cells are very small protrusions seen at the edges of ARNO-expressing cells on the margins of the islands. Additionally, these cells remain firmly attached to the island. In contrast, ARNO-expressing cells treated with tert-butanol display large lamellipodia and

separate from their neighbors, similarly to cells in the absence of butanol (Fig. 8, E and F).

Activation of Rac by ARNO does not require PLD activity

Because acquisition of the migratory phenotype required activation of both Rac1 and PLD, we examined whether activation of Rac was dependent on PLD and production of PA. MDCK cells were infected with adenovirus encoding ARNO for 2 h. Further expression was stopped with the addition of doxycycline, and 0.5% 1-butanol or tert-butanol was added to the incubation medium. After an additional 1 h of incubation, the activation state of Rac was determined. As can be seen in Fig. 9, addition of butanol had no detectable effect on the activation of Rac by ARNO under these conditions. It is possible that during the initial expression period enough PA was generated by the action of ARF on PLD to stimulate the activation of Rac, and that the subsequent addition of butanol did not sufficiently reduce PA levels in the cells. We were unable to test this possibility, as inclusion of 1-Butanol during the entire infection period severely inhibited expression of exogenous ARNO from the adenovirus construct (Santy, L.C., unpublished data). Nevertheless, as far as we were able to determine, PA production by PLD is not necessary for Rac activation, although it is required for ARNO's morphological and migratory effects.

Discussion

The migration of epithelial cells is central to several important biological processes, including tissue morphogenesis, wound healing, and metastasis (Gumbiner, 1996). GTPases of the Rho family have been implicated as regulators of the cytoskeletal reorganization associated with cell motility (Schmitz et al., 2000). More recently, it has become clear that members of the ARF GTPase family also function in the regulation of cytoskeletal assembly, and may integrate with Rho proteins in this process (Radhakrishna et al., 1996, 1999; D'Souza-Schorey et al., 1997; Zhang et al., 1999; Boshans et al., 2000). In this study, we demonstrate that activation of ARF through expression of the GEF ARNO produces profound changes in the shape and migratory potential of MDCK epithelial cells. Expression of ARNO in subconfluent MDCK cultures causes cells at the edge of islands to form large, fan-shaped lamellipodia and to become significantly more migratory than nonexpressing cells. The adoption of this motile phenotype is the result of ARNO-mediated activation of ARF6 and the subsequent activation of both PLD and Rac.

Induction of the migratory phenotype by ARNO

The ability of ARNO to induce a migratory phenotype is not shared among all cells in the epithelial population. Only MDCK cells with a free edge, either those at the edge of an island of cells or those with a side exposed by wounding, exhibit broad lamellipodia upon ARNO expression. As tight junctions serve as barriers to the lateral diffusion of both proteins and lipids in the plane of the membrane, cells with a free edge (i.e., lacking junctions) cannot prevent the intermixing
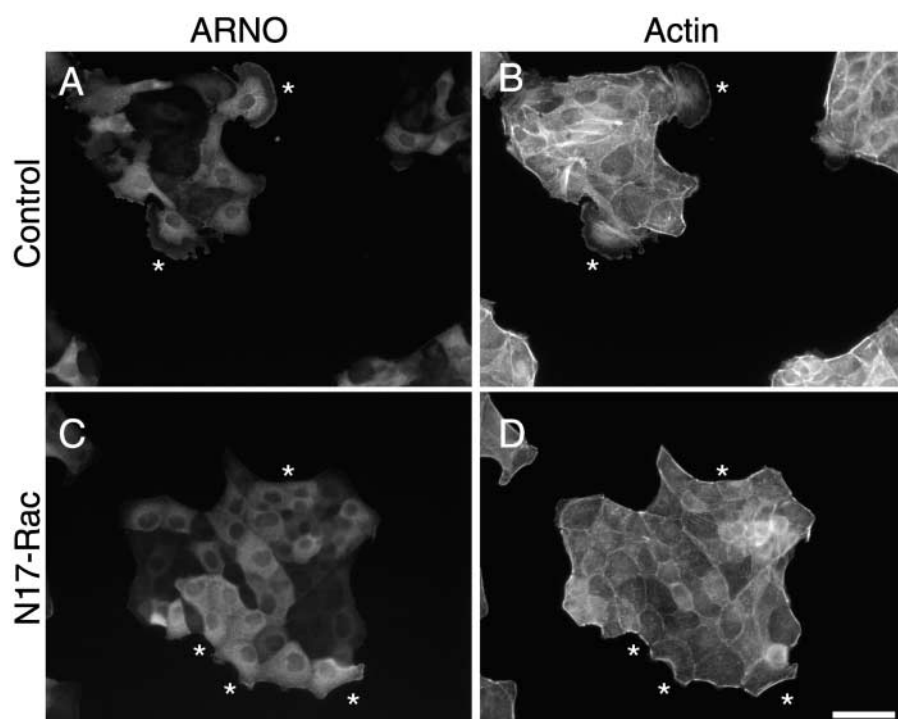
Figure 6. **N17 Rac expression blocks morphological changes induced by ARNO.** MDCK cells expressing N17 Rac under the control of the tetracycline regulatable transactivator were either induced to express for 18 h (C and D) or removed from doxycycline immediately before (A and B) being infected with ARNO encoding adenovirus. Cells were incubated for 3 h and then fixed and processed for immunofluorescence microscopy. Cells were stained with monoclonal anti-ARNO (A and C) and rhodamine phallodin (B and D). Asterisks indicate ARNO-expressing cells. Bar, 50 μm.

of apical and basolateral membrane constituents (Gumbiner, 1996). This leads to the creation of a novel membrane/cytoskeletal domain at the free edge of epithelial cells, where, for example, adhesive interactions would occur predominantly through integrins as opposed to cadherins. Recruitment of ARNO into this location may bring together all the components necessary for induction of the migratory phenotype.

Interaction of ARNO with membranes is thought to be primarily mediated by binding of its PH domain to phospho-

Figure 7. **ARNO expression increases PLD activity in MDCK cells.** Control cells, cells expressing wild-type ARNO, or ARNO E156K were assayed for PLD activity by incorporation of labeled lipid into phosphatidylbutanol as described in Materials and methods. Data shown are mean ± standard deviation of triplicate samples and are representative of >10 experiments.

inositides (Chardin et al., 1996; Klarlund et al., 2000). Accumulation of phosphoinositides at the free edge could therefore lead to preferential recruitment of ARNO to this location. Indeed, a recent study of wound healing in MDCK monolayers demonstrated a requirement for polyphosphoinositides in this process (Fenteany et al., 2000). However, the subcellular localization of lipid binding sites may not entirely explain localization of ARNO to the free edge, as it has recently been shown that the coiled coil domain mediates the interaction of ARNO family members with GRASP, a retinoic acid–induced scaffolding protein (Nevrivy et al., 2000). Therefore, ARNO may be concentrated at the free edge at least in part by interaction with a localized interacting protein.

Because ARNO is relatively promiscuous in vitro, it is likely that its specificity in vivo depends largely on its intracellular colocalization with specific ARF isoforms (Chardin et al., 1996; Frank et al., 1998a). In this study, we demonstrate that endogenous ARF6 is colocalized with ARNO in the ARNO-induced lamellipodia, whereas endogenous ARF1 is barely detectable in this location. Additionally, we examined the activation of endogenous ARFs using a novel pulldown assay, and demonstrate that exogenous ARNO stimulates a specific activation of ARF6 relative to ARF1. Finally, we demonstrate that expression of dominant negative ARF6 T27N can inhibit migration of MDCK cells. These findings are consistent with the previously demonstrated peripheral localization of both ARNO and ARF6, with the relative paucity of ARF1 at the plasma membrane, and with the well-documented effects of ARF6 activation on the cortical actin cytoskeleton (Chavrier and Goud, 1999; Jackson and Casanova, 2000).

ARF activation and downstream signaling

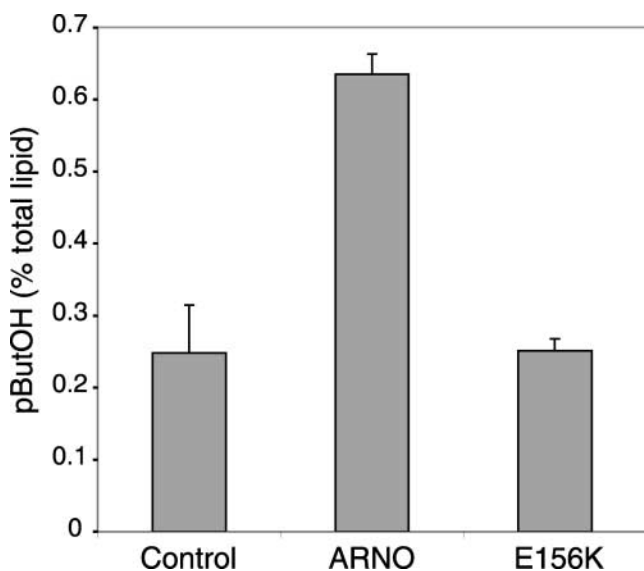Our data demonstrate that activation of ARF6 by ARNO in MDCK cells leads to the adoption of a migratory pheno-

Figure 8. **Inhibition of PLD activity prevents morphological changes induced by ARNO.** MDCK cells were infected with ARNO-encoding adenovirus for 90 min. Further expression was prevented by the addition of 20 ng/ml doxycycline. Cells were incubated for another 90 min in the absence of butanol (A and B) or in the presence of 0.5% 1-butanol (C and D) or 0.5% tert-butanol (E and F). Cells were then fixed and stained with 9E10 monoclonal anti-myc (B, D, and F) to label ARNO and rhodamine-phalloidin (A, C, and E) to label filamentous actin. Asterisks indicate ARNO-expressing cells. Bar, 50 μm.
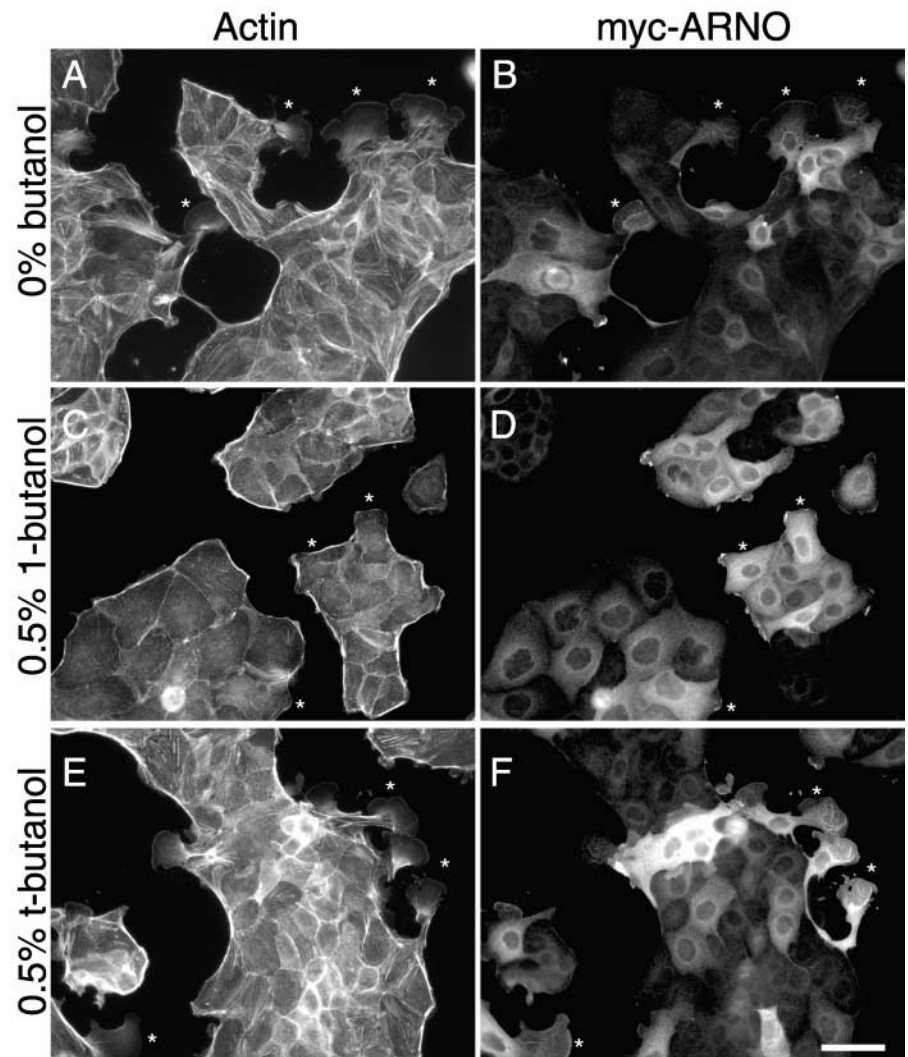
type. Several possibilities may explain how the known direct effects of ARF6 activation lead to this altered morphology and behavior.

ARF6 was originally characterized as a regulator of the plasma membrane endocytosis and recycling system (for review see Chavrier and Goud, 1999). In migrating cells, recycling pathways are polarized towards the leading edge, which may serve to efficiently recycle molecules involved in actin reorganization and substrate attachment (i.e., integrins) to the rapidly expanding leading edge (Nabi, 1999). Recruitment of ARNO and subsequent activation of ARF6 at the free edge of cells may polarize the recycling process, leading to an enhanced accumulation of actin-modifying proteins, matrix attachment proteins or signaling molecules at the free edge.

Additionally, activation of ARFs can lead to significant changes in the lipid composition of the local membrane. Specifically, at the plasma membrane, ARFs are known to directly activate two lipid-modifying enzymes, PLD and phosphatidylinositol-4-phosphate 5-kinase (Exton, 1997; Honda et al., 1999). PLD cleaves phosphatidyl choline into PA, thereby changing the charge of the bilayer surface and removing a bulky head group. PA can be further metabolized to produce diacylglycerol or lysoPA, two molecules with well known signal transduction properties (Exton, 1997). Addi-

tionally, PA is known to be a cofactor in the activation of a PI-4P-5 kinase isoform (PI-4P-5Kα) that is also allosterically activated by ARF (Honda et al., 1999). In this study, we show that expression of ARNO leads to increased production of PA by PLD, and that inhibition of PLD activity with 1-butanol prevents formation of lamellipodia and cell migration. Although 1-butanol might be inhibiting another process that is required for migration, at present no specific inhibitors of PLD exist. The fact that t-butanol has no effect on ARNO-induced morphology suggests that butanol is indeed inhibiting these changes via inhibition of PLD. Whether this is due to inhibition of PA production, PA-dependent phosphatidylinositol 4,5 bisphosphate synthesis, or the production of other PA metabolites, remains to be determined.

Rac activation by ARF

Numerous studies have established a requirement for Rac activation in cell motility. In the context of a wound-healing assay, microinjection of dominant negative Rac1 constructs into either MDCK cells (Fenteany et al., 2000) or rat embryo fibroblasts (Nobes and Hall, 1999) has been shown to inhibit both the formation of lamellipodia and migration of cells into the wound. Here, we demonstrate that expression
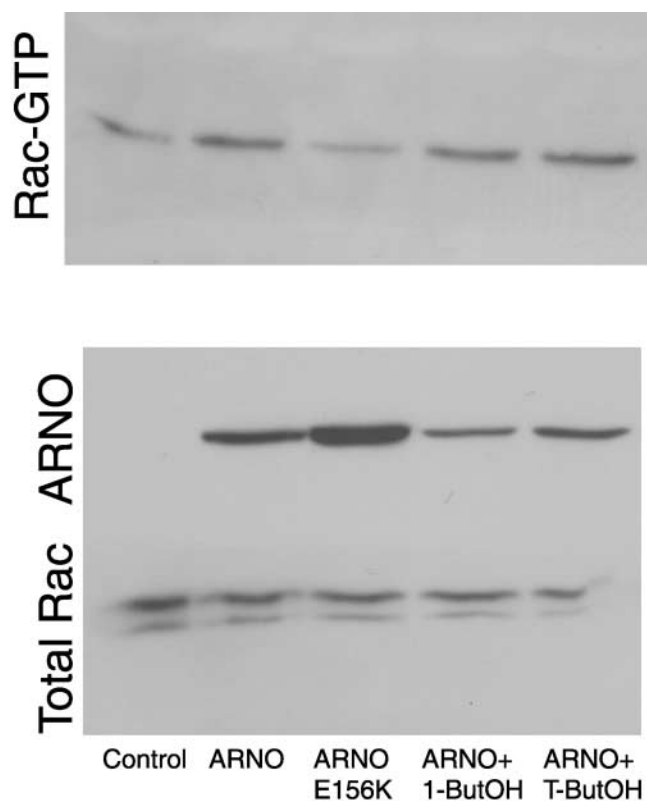
Figure 9. Inhibition of PLD does not prevent activation of Rac by ARNO. MDCK cells were infected with adenovirus encoding ARNO for 2 h. Cells were incubated for an additional hour in the presence or absence of 0.5% 1-butanol or tert-butanol. Cells were then lysed and GTP-Rac isolated as described in Materials and methods. Data are representative of four separate experiments.

of ARNO in MDCK cells leads to increased activation of endogenous Rac1, and that dominant negative N17Rac prevents induction of the migratory phenotype by ARNO. Taken together, these data suggest that Rac1 functions downstream of ARF6 in a GTPase cascade. To our knowledge, this is the first direct demonstration of endogenous Rac activation by a pathway involving ARF.

How does ARF6 activation lead to activation of Rac? Several studies have suggested that Rac1 is enriched in membranes of the endosomal system and that this endosomal pool of Rac can be translocated to the plasma membrane as a result of ARF6 activation (Radhakrishna et al., 1999; Boshans et al., 2000). Additionally, these studies demonstrated that dominant negative ARF6 T27N can block ruffling induced by Rac. However, another study demonstrated that dominant negative ARF6 could block ruffling induced by a Rac construct that was constitutively localized to the plasma membrane, suggesting a role for ARF6 independent of the control of Rac localization (Zhang et al., 1999). None of these studies directly examined the activation state of endogenous Rac; therefore, the precise role of ARF6-mediated localization in the activation of Rac has remained unclear.

A second possibility is that ARF-induced changes in the membrane phospholipid composition create a local environment conducive to Rac activation. In opposition to this model, we found that inhibition of PLD-mediated PA production does not prevent activation of Rac. Under the same experimental conditions, morphological changes induced by ARNO are strongly inhibited. Taken together, these data suggest that ARF-mediated Rac activation occurs independently of ARF-mediated lipid modification, but that both are required to regulate actin structure and cell motility. Consistent with this model, whereas activation of Rac is required for spreading and ruffling of MDCK cells in response to hepatocyte growth factor, injection of constitutively active Rac into MDCK cells induces membrane ruffling but not motility (Ridley et al., 1995). These observations combine to suggest that signals in addition to Rac activation are needed to convert MDCK cells to a migratory state and that ARF-mediated activation of PLD can provide at least some of these signals.

Another potential mechanism for the activation of Rac downstream of ARNO is the recruitment of a Rac-GEF in response to ARF-GTP. Recently, multiple proteins containing ARF GTPase–activating (GAP) domains have been identified. These proteins possess other domains involved in protein–protein interactions, such as ankyrin repeats, PH domains, proline-rich domains, or SH3 domains, suggesting that they may participate in the formation of large, multiprotein complexes (Donaldson and Jackson, 2000). The subfamily of ARF-GAPs, consisting of Git1, p95Pkl, and p95APP1, have been shown to associate directly with the Rac exchange factor PIX (also known as COOL) (Bagrodia et al., 1999; Turner et al., 1999; Di Cesare et al., 2000; Zhao et al., 2000). All three of these proteins are localized to focal adhesions and bind to paxillin, further implicating this family of proteins in the regulation of cortical actin organization, cell adhesion, and migration (Turner et al., 1999; Di Cesare et al., 2000; Zhao et al., 2000). Moreover, overexpression of both GIT1 and APP1 cause a loss of paxillin from focal adhesions (Di Cesare et al., 2000; Zhao et al., 2000).

These observations suggest one possible model for the activation of Rac downstream of ARF-GTP in which binding of ARF-GTP to GIT, Pkl, or APP1 leads to the assembly of a complex containing GIT–Pkl–APP and PIX and the subsequent local activation of Rac. Consistent with the possible existence of such a complex, GIT1, PIX, and paxillin colocalize with ARNO in the broad lamellipodia seen in migratory MDCK cells (data not shown). Whether the assembly of this complex is directly modulated by ARF remains to be determined.

In this scenario, the GIT family proteins would be acting not only as GAPs to terminate further ARF signaling, but also as effectors downstream of ARF-GTP to enhance Rac activation. Recent evidence indicates that the distinction between G-protein GAPs and effectors is less clear cut than originally conceived. For example, phospholipase C, adenylyl cyclase, and p115-RhoGEF are effector proteins for heterotrimeric G-proteins that have also been shown to possess GAP activity towards these same G-proteins (Montell, 2000). This combination of GAP and effector activity appears likely to apply to the ARF GAPs as well.

The role of Rac in the regulation of epithelial structure and motility is complex. Rac activation is required for the formation and maintenance of cell–cell junctions in epithelial monolayers (Hordijk et al., 1997; Takaishi et al., 1997;

Jou and Nelson, 1998). Along the same lines, in MDCK cells that have adopted a mesenchymal phenotype in response to coexpression of oncogenic Ras, expression of the Rac exchange factor Tiam1 induces a reversion to an epithelial phenotype (Hordijk et al., 1997; Zondag et al., 2000). In established epithelia, on the other hand, activation of Rac is required for the adoption of a motile state in response to growth factors such as HGF or in response to wounding (Ridley et al., 1995; Gimond et al., 1999; Fenteany et al., 2000). These data combine to suggest that the location and precise levels of active Rac may play a critical role in the structure and motility of epithelial cells.

Although the acquisition of motility by epithelial cells requires the activation of Rac, activated Rac alone is not sufficient to induce motility in these cells (Ridley et al., 1995; Jou and Nelson, 1998; Gimond et al., 1999). This has led to the suggestion that activation of another signaling pathway(s) is required for this transition (Ridley et al., 1995; Gimond et al., 1999; Zondag et al., 2000). We have found that activation of ARF via expression of the exchange factor ARNO is sufficient to induce motility in MDCK cells. This transformation requires downstream activation of both Rac and PLD, which presumably then function in concert to regulate actin structure and cell motility. Activation of ARF proteins provides one mechanism whereby these cells can engineer a localized production of active Rac associated with phospholipid modifications, thereby enhancing their migratory potential.

Materials and methods

Reagents, antibodies, and DNA constructs
The following antibodies were used: mouse anti-myc 9E10, rabbit anti-HA (BabCo), mouse anti-Rac (clone 102; BD Transduction Laboratories), polyclonal anti-ARNO (Frank et al., 1998a). Monoclonal mouse anti-ARNO (6G11). Monoclonal mouse anti-ARF6 8A6-2 (used for Western blots) and rabbit anti-ARF1 (used for immunofluorescence) were gifts from Sylvain Bourgoin (Laval University, Ste-Foy, Canada), rabbit anti-ARF6 (for immunofluorescence) was obtained from Victor Hsu (Harvard Medical School, Boston, MA) and rabbit anti-ARF1 (for Western blot) was obtained from Paul Melancon (University of Alberta, Edmonton, Canada). Cy2-donkey anti–mouse, Cy2-donkey anti–rabbit, Texas red goat anti–mouse, and Texas red donkey anti–rabbit were obtained from Jackson ImmunoResearch. Rhodamine-conjugated phalloidin was from Molecular Probes. Horseradish peroxidse–coupled sheep anti–mouse and goat anti–rabbit secondary antibodies (Amersham Pharmacia Biotech) were used for Western blotting.

Recently, it has been demonstrated that ARNO and its relatives each exist in two splice isoforms which produce PH domains with differing affinities for PI-(3,4,5)P_3 and PI-(4,5)P_2 (Klarlund et al., 2000). In this study, we used the isoform of ARNO (GGG, tri-gly), which has equivalent affinities for the two inositol lipid moieties.

The construct pGST-GGA3$_{VHS-GAT}$ (Dell'Angelica et al., 2000) encoding the VHS and ARF binding domains of GGA3 fused to GST was obtained from Paul Randazzo and Juan Bonifacino (National Institutes of Health, Bethesda, MD).

Oleic acid (9,10-^{3}H[N]) (5 mCi/ml, 5–15 Ci/mmol) was obtained from NEN Life Science Products. Fibronectin was purchased from Becton Dickinson. Tissue culture reagents were purchased from GIBCO BRL.

Tissue culture
The T23 line of MDCK cells, which expresses the tetracycline-repressible transactivator (Barth et al., 1997), was maintained in DME with 4.5 g/L glucose, 10% FBS, and antibiotics in a 37°C, 5% CO_2 incubator. A clone of these cells expressing N17 Rac under control of the tetracycline-responsive promoter (Jou and Nelson, 1998) was grown in the same manner, with the addition of 20 ng/ml doxycycline to repress transgene expression.

Transient transfections were performed using the fugene 6 reagent (Roche) according to the manufacturer's instructions.

For immunofluorescence, cells (5×10^4 cells) were plated on glass coverslips in 35-mm dishes 38–44 h before the experiment. Monolayers for wound healing experiments were formed by plating 10^6 cells in 35-mm dishes 40–48 h before wounding. For Rac and ARF (5×10^5 cells) GTPase activation assays and for PLD assays (2.5×10^5 cells) cells were plated in 35-mm wells 18 h before the experiment. Finally, for transwell migration assays 2.5×10^6 cells were plated in 60-mm dishes 18 h before the initiation of the experiment.

Adenovirus-mediated expression
The recombinant adenoviruses used to express exogenous myc-tagged ARNO proteins under the control of the tetracycline regulatable transactivator have been described previously (Frank et al., 1998b; Altschuler et al., 1999). For morphological and GTPase activation experiments, cells were infected in serum free DME for 2.5–3 h. For wound healing assays, monolayers of cells were infected for 1.5 h in Hank's Balanced Salt Solution (HBSS$^-$) without divalent cations (GIBCO BRL) in the presence of 20 ng/ml doxycycline to repress transgene expression. Monolayers were recovered overnight in DME with 10% FBS and either 20 ng/ml doxycycline (ARNO-expressing cells) or 0.2 ng/ml doxycycline (ARF6 T27N cells). The doxycycline was removed by washing with fresh DME and monolayers were incubated for 2–3 h to allow expression of the transgene before wounding. In all cases control cells were infected in the same manner, but were continuously maintained in 20 ng/ml doxycycline to prevent transgene expression.

Immunofluorescence microscopy
Cells were infected with recombinant adenovirus as described above, then fixed with 4% paraformaldehyde in PBS, and processed for indirect immunofluorescence as described previously (Frank et al., 1998b). Cells were observed and photographed using a microscope (Eclipse E800; Nikon) equipped with a Diagnostic Instruments spot digital camera. Images were recorded using Adobe Photoshop®.

Transwell migration assay
Migration was assessed using modified Boyden chambers containing Transwell filters (6.5-mm diameter, 8-μm pores; Costar) coated on the underside with 5 μg/cm² fibronectin. T23 cells were infected with recombinant adenovirus in HBSS$^-$ for 2 h as described above. Cells were then allowed to recover for 2 h in DME with 10% FBS, then were harvested by incubation in PBS with 1 mM EGTA, 4 mM EDTA, and then resuspended in Optimem (GIBCO BRL), 10 mM Hepes, pH 7.4, and 0.1% heat-denatured BSA. Cells (2×10^5) were added to the upper chamber of the Transwell filter, with the same buffer located in the lower chamber. After migration for 16 h, cells remaining on the upper surface of the filter were removed with a cotton swab. Cells on the lower side were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet. The dye was extracted into 1% sodium deoxycholate and quantitated by measuring the optical density at 590 nm.

Wound healing
Monolayers of cells were infected with recombinant adenovirus and induced to express as described above. Monolayers were wounded by scraping with a teflon cell scraper, rinsed several times with media to remove dislodged cells, and placed back into DME with 10% FBS containing 2 ng/ml doxycycline to limit further transgene expression. Cells were incubated at 37°C under 5% CO_2 while migration of the monolayer into the cleared wound area was recorded using a PDMI-2 stage incubator (Harvard Instruments) on a microscope (IX-70; Olympus) outfitted with Hoffman optics. Digital images were collected every minute for 4 h using a Hamamatsu Orca camera. The area covered by the monolayer was traced and measured using OpenLab 2.0 software (Improvision).

G-protein pull down assays
Activation levels of endogenous Rac were assayed in cells infected with ARNO-expressing adenoviruses as described above. Endogenous GTP-Rac was isolated by incubation with a GST fusion to the PBD domain of PAK and quantitated as described previously (Criss et al., 2001).

Activation of endogenous ARFs by ARNO expression was assayed using a novel pulldown assay. Cells were infected with ARNO-expressing adenoviruses in serum-free DME in the presence or absence of doxycycline as described above for 2 h. 20 ng/ml doxycycline was then added and the cells were incubated for an additional hour. Extensive ruffling and migration of the ARNO-expressing cells was apparent at this point. Cells were

then lysed at 4°C in 0.65 ml of 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl$_2$, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol with 0.1 mM PMSF and 1 µg/ml each pepstatin, leupeptin, and antipain. Lysates were clarified by centrifugation at 16 g for 2 min in the presence of CL-4B Sepharose beads (Amersham Pharmacia Biotech). 0.5 ml of the clarified lysate was incubated with 40 µg of GST-GGA3 bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 30 min. The beads were then washed three times with 50 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl$_2$, 1% NP-40, 10% glycerol with 0.1 mM PMSF, and 1 µg/ml each of pepstatin, leupeptin, and antipain. Bound proteins were eluted into 60 µl SDS-PAGE sample buffer. This sample was divided (15 µl for ARF1 and 40 µl for ARF6) and assayed for the presence of endogenous ARF1 and ARF6 by Western blotting with ARF1- and ARF6-specific antibodies. Total levels of each ARF in the starting lysates were assayed by Western blotting of 3% of the clarified lysate.

PLD assay
Cellular lipids in T23 MDCK cells were labeled by incubation in Optimem, 0.5% FBS, and 1 µCi/ml ^{3}H-oleic acid for 18–24 h. Cells were then infected with adenovirus for 3 h in serum-free Optimem. Butanol was added to 0.3% and incubation continued for an additional 30 min. Cellular lipids were isolated by extraction with chloroform/methanol and separated by TLC on Silca gel plates with H$_2$O-saturated ethyl acetate/isooctane/acetic acid (55:25:10). Phosphatidylbutanol was identified by comigration with an unlabeled standard and quantitated by liquid scintillation counting.

Online supplemental material
Time lapse videos of the wound healing assays shown in Fig. 3 are provided as supplemental videos. Three videos are provided showing migration of the monolayer into the cleared wound area of control cells (Video 1), ARNO-expressing cells (Video 2), and cells expressing the E156K-S392A mutant of ARNO (Video 3). The videos were recorded as described above. A frame was recorded every minute for 4 h and QuickTime movies were produced using the program OpenLab. Supplementary videos are available at www.jcb.org/cgi/content/full/200104019/DC1.

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