ADP-ribosylation Factor 1 Controls the Activation of the Phosphatidylinositol 3-Kinase Pathway to Regulate Epidermal Growth Factor-dependent Growth and Migration of Breast Cancer Cells

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Activation of intracellular signaling pathways by growth factors is one of the major causes of cancer development and progression. Recent studies have demonstrated that monomeric G proteins of the Ras family are key regulators of cell proliferation, migration, and invasion. Using an invasive breast cancer cell lines, we demonstrate that the ADP-ribosylation factor 1 (ARF1), a small GTPase classically associated with the Golgi, is an important regulator of the biological effects induced by epidermal growth factor. Here, we show that this ARF isoform is activated following epidermal growth factor stimulation and that, in MDA-MB-231 cells, ARF1 is found in dynamic plasma membrane ruffles. Inhibition of endogenous ARF1 expression results in the inhibition of breast cancer cell migration and proliferation. The underlying mechanism involves the activation of the phosphatidylinositol 3-kinase pathway. Our data demonstrate that depletion of ARF1 markedly impairs the recruitment of the phosphatidylinositol 3-kinase catalytic subunit (p110αε) to the plasma membrane, and the association of the regulatory subunit (p85αε) to the activated receptor. These results uncover a novel molecular mechanism by which ARF1 regulates breast cancer cell growth and invasion during cancer progression.

The epidermal growth factor receptor (EGFR) is considered a major oncogenic factor, and its presence in tumors is indicative of poor prognosis (1, 2). EGFRs not only modulate growth properties of transformed cells but are causally involved in survival signaling, cell migration, angiogenesis, and metastasis (3–5). EGFR belongs to the ErbB family of membrane-bound receptor tyrosine kinases (RTKs) that comprises four structurally related receptors, namely, the EGFR (HER-1 and HerB1), HER-2 (ErbB2 or Neu), HER-3/ErbB3, and HER-4/ErbB4 (6). With the exception of HER-2, which has no obvious ligand, these receptors bind to their ligands and become activated by homo-dimerization or hetero-dimerization and subsequent tyrosine autophosphorylation. The best-known ligands of EGFRs are EGF, transforming growth factor-α, and heparin-binding EGF-like growth factor (7).

EGFRs transmit extracellular mitogenic signals through the activation of a number of downstream signaling cascades. These include signaling modules that involve the phosphatidylinositol 3-kinase (PI3K) pathway (8). Although multiple forms of PI3K exist in higher eukaryotes, the class 1A enzymes are primarily responsible for production of D-3 phosphoinositides in response to ligand-dependent receptor stimulation and tyrosine-kinase activation. Over the past decade, it has become evident that the PI3K signaling pathway is one of the most highly mutated systems in human cancers, underscoring its central role in human carcinogenesis (8). Class 1A PI3Ks are obligate heterodimers in vivo, because the p110 catalytic subunits are labile and unstable as monomers (9). Dimerization of a p110 subunit with a p85 regulatory subunit maintains the enzyme in a low activity state in quiescent cells. Activation requires translocation of the normally cytosolic enzyme to a membrane and direct interaction with an activated receptor (8). The most commonly accepted mechanism for activation of class 1A PI3K by RTKs involves the binding of SH2 domains to phosphorylated YXXM motifs of activated receptors or their substrates (10, 11).

Enhanced migration is a fundamental characteristic of tumor cells. This process is believed to be involved in invasion and metastasis. As cells migrate, intracellular signaling cascades are activated to promote remodeling of the actin cytoskeleton to form membrane protrusions. Small GTPases of the Rho and ARF families have been characterized as key players regulating this process. These cycle between an inactive GDP-bound form and an active GTP-bound form through the action of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (12). ARF proteins consist of a family of six isoforms, and of these, ARF1 and ARF6 are the best characterized. ARF1 is classically associated with the Golgi to regulate vesicle trafficking, whereas ARF6 is present at the plasma membrane,
EGFR Activates ARF1 to Regulate Growth and Migration

where it is involved in receptor endocytosis and actin remodeling (13). Fifteen ARF-GEFs are encoded in the human genome (14). The large number of these regulatory proteins compared with the relatively small number of ARFs suggests that activation of these GTPases is under extensive regulatory control.

ARF6 is overexpressed in highly invasive breast cancer cells and plays an essential role during invasion (15, 16). Recently, GEP100 was identified as the ARF-GEF linking the EGFR to ARF6 activation in breast cancer cells (16). Furthermore, AMAP1 was reported to act as an effector of ARF6-GTP during invasion of glioblastomas and lung tumors (17). It was suggested that the regulation of invasion by ARF6 is dependent on the activation of the extracellular signal regulated kinase (Erk) (18). Collectively, these findings highlight the importance of ARF6 during cancer progression.

Several reports have suggested that ARF1 can also be present on plasma membranes and transmit signal from transmembrane proteins (17, 19–21). To better define the role of this ARF isoform in breast cancer development and progression, we examined whether ARF1 can regulate migration and proliferation of invasive cell lines. Our study demonstrates that ARF1 is localized to dynamic plasma membrane ruffles together with the EGFR, and this ARF isoform is activated following EGF stimulation. This small GTPase is critical for both cell migration and proliferation by directly regulating the activation of the PI3K pathway. These findings reveal an unexpected role for ARF1 in the regulation of cancer cell migration and proliferation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Biotinylated EGF complexed to Alexa-Fluor 488 streptavidin, anti-FLAG polyclonal antibodies, and Lipofectamine 2000™ were purchased from Invitrogen (Burlington, Ontario, Canada). EGF was purchased from Fitzgerald Industries International, Inc. (Concord, MA). Anti-GFP and Anti-ARF1 (raised against amino acids 174–180: SNQLRNQ of human ARF1 sequence) antibodies were obtained from Abcam (Cambridge, MA). Anti-phospho-tyrosine (Y99), anti-p85α, phospho-p85α (Y508), and p110α (N-20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Erk1/2, phospho-Erk1/2, phospho-Akt (Ser 473), Akt, EGFR, and phospho-EGFR (Y1148) antibodies were from Cell Signaling Technology® (Danvers, MA). Anti-HA (3F10) antibody was from Roche Applied Science (Laval, Quebec, Canada). Monoclonal anti-p115 antibody (7D1 (22)) was a gift from Dr. Dennis Shields (A. Einstein College of Medicine, New York, NY). Polyclonal anti-ARF6 antibody was a gift from Dr. Julie Donaldson (National Institutes of Health). LY294002 was from Cayman Chemicals. Mammary epithelial basal medium was from Cambrex Bio Science (Walkersville, MD). Alexa-Fluor 488 phalloidin and all secondary antibodies coupled to an Alexa-Fluor were from Molecular Probes (Eugene, OR). All others products were from Sigma-Aldrich.

DNA Plasmids and siRNAs—ARF1-FLAG was a gift from Dr. Jean-Luc Parent (University of Sherbrooke, Quebec, Canada). ARF1-GFP and ARF3-GFP plasmids were previously described (23). siRNA-insensitive ARF1 mutant (ARF1mut) was constructed by introducing silent mutations (aatattttgca), which contained an Ssp1 site into nucleotide sequence 7–18 (amino acids 3–6) of wild-type ARF1 (pBKΔ) by PCR. All constructs were analyzed by DNA sequencing (Sequencing service, IRIC, University of Montreal, Quebec, Canada). Double-stranded scrambled, ARF1, and ARF6 (#1) siRNAs, with 19-nucleotide duplex RNA and 2-nucleotide 3′ dTdT overhangs were previously described (24, 25). The 21-nucleotide sequence for the second siRNA targeting ARF1 (#2) was 5′-gaaatgcctactctcatgtgcc-3′ corresponding to region 50–71 of the human ARF1 mRNA. All siRNA were synthesized using the Silencer™ siRNA Construction Kit from Ambion (Austin, TX).

Cell Culture and Transfection—MDA-MB-231, SKBR3, and MCF10a cells were obtained from Dr. Sylvie Mader (University of Montreal, Quebec, Canada), and MDA-MB-435 were provided by Dr. Morag Park (McGill University, Quebec, Canada). Cells were maintained at 37 °C, 5% CO2, in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (MDA-MB-231, MDA-MB-435, and SKBR3 cells) or in mammary epithelium basal medium supplemented with 0.52 mg/ml bovine pituitary extract, 10 ng/ml recombinant EGF, 10 μg/ml insulin, 1 μg/ml hydrocortisone, 0.1 mg/ml gentamycin, and 0.1 μg/ml amphotericin B (MCF10a cells). Cells were transfected with DNA (48 h) and/or siRNA (72 h) using Lipofectamine™ 2000 according to the manufacturer’s instructions.

Western Blotting—Cells were harvested in 60 μl of TGH buffer (pH 7.3, 1% Triton X-100, 10% glycerol, 50 mM NaCl, 50 mM HEPES, 5 mM EDTA) complemented with protease inhibitors and 1 mM sodium orthovanadate. Cell lysates were solubilized (4°C, 1 h), and total soluble proteins were run on polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blotted for relevant proteins using specific primary antibodies (described for each experiment). Secondary antibodies were all fluorescein isothiocyanate-conjugated, and fluorescence was detected using a Typhoon 9410 scanner (Amersham Biosciences). Quantification of the digital images obtained was performed using ImageQuant 5.2 software.

Real-time PCR—For real-time reverse transcription-PCR, 5 μg of total mRNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems, Foster City, CA). Gene expression level was determined using primers and probe sets from Applied Biosystems (ABI Gene Expression Assays). PCR reactions for 384-well plate formats were performed using 2 μl of cDNA samples (20–50 ng), 5 μl of the TaqMan Universal PCR Master Mix (Applied Biosystems), 0.5 μl of the TaqMan® Gene Expression Assays (20×), and 2.5 μl of water in a total volume of 10 μl. The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) was used to detect the amplification level and was programmed to an initial step of 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. All reactions were run in triplicate, and the average values were used for quantification. Human glyceraldehyde-3-phosphate dehydrogenase, β-actin, and 18 S ribosomal RNA were used as endogenous controls. Primers for ARFs were the following: ARF1, gccactttcagacacacca (forward) and ctcgctcagctctcgtg (reverse); ARF3, cgcagctgatagcagga (forward)
and gctctctctgtgccagagtt (reverse); ARF4, catgggctactatatctct (forward) and gcagctcactcaacct (reverse); ARF5, ttctcgggaagaagcagagtt (forward) and aagctctgaaggggatgg (reverse); and ARF6, gactgcaagcgtacagaga (forward) and accagatcctgctaat (reverse).

Activation of ARF1 and ARF6—Cells were plated into 6-well dishes and serum-starved for 24 h. Cells were stimulated with EGF (10 ng/ml) at 37 °C for the indicated times, and activation of ARF1 and ARF6 was measured as described previously (25). Briefly, cells were lysed in 60 μl of ice-cold lysis buffer E. Samples were incubated for 30 min (4 °C) and spun for 10 min at 10,000 rpm. GST-GGA3 (1–316) (26) coupled to glutathione-Sepharose 4B was added to each tube, and the samples were rotated at 4 °C for 1 h. Proteins were eluted in 25 μl of SDS-sample buffer by heating to 95 °C for 5 min. Detection of ARF1-GTP and ARF6-GTP was performed by immunoblot analysis using an anti-ARF1 or anti-ARF6 antibody.

Confocal Microscopy—MDA-MB-231 cells transiently transfected with ARF1-FLAG or ARF6-HA constructs were serum-starved for 1 h, stimulated with EGF (10 ng/ml) as indicated, and fixed using paraformaldehyde (4%) for 15 min at room temperature. Overexpressed ARF1-FLAG or ARF6-HA were detected using a polyclonal anti-FLAG antibody and a secondary antibody coupled to Alexa-Fluor 568 or a monoclonal anti-HA antibody and an anti-mouse antibody coupled to Alexa-Fluor 568, respectively. In some experiments, cells were subsequently incubated with Phalloidin-488 for 1 h. In a second set of experiments, the secondary antibody used to detect ARF1-FLAG was coupled to Alexa-Fluor 488. These cells were subsequently incubated with a p115 antibody (monoclonal) and a secondary antibody coupled to Alexa-Fluor 568. For labeling of the EGFR, cells were incubated with a primary antibody coupled to Alexa-Fluor 488, and images were acquired on the confocal microscope. For each image, 10 areas of the plasma membrane were assessed using the MetaMorph software.

Migration Assay—Cells were transfected with siRNA (72 h) or plasmid DNA (48 h), and serum-starved 24 h before the assay as in a previous study (25). Briefly, cells were trypsinized and seeded into Boyden chambers (24-well inserts with 8-μm pore, coated with collagen). One hour after plating, cells were stimulated with EGF (10 ng/ml). After 6 h, cells were fixed using paraformaldehyde and incubated with crystal violet. Membranes were washed (dH2O), cells present in the upper chamber were removed, and migrated cells were quantified in the lower chamber. For some experiments, cells were pretreated with PD98059 or LY294002 (15 μM) for 30 min before EGF (10 ng/ml) stimulation.

RESULTS

EGF Stimulation Promotes the Transient Activation of ARF1 and ARF6—To determine whether stimulation of endogenously expressed EGFR led to the activation of ARF1 and/or ARF6 in invasive breast cancer cells, we first examined levels of scrambled or ARF1 siRNA (25 nM for 72 h), and serum-starved for 24 h. Three scratches were performed using a micropipette tip to wound confluent cells. Cells were subsequently treated with EGF and fixed after 6 h using paraformaldehyde and stained using crystal violet (0.1% in 20% MeOH, overnight). Pictures of five different fields were taken, and a representative picture is presented for each condition.

EGFR Activates ARF1 to Regulate Growth and Migration
EGFR Activates ARF1 to Regulate Growth and Migration

A

![Graph of ARF1 and ARF6 activation](image1.png)

FIGURE 1. EGF promotes the activation of ARF1 and ARF6. A, MDA-MB-231 cells were treated with EGF for the indicated times. Cells were lysed, and activated ARF1 and ARF6 were captured using GST-GGA3 coupled to glutathione-Sepharose 4B beads in a GST pulldown assay. Representative immunoblots are presented. Input represents 15% of the total protein present in the sample. Quantifications experiments are presented as fold increase over basal and are the mean ± S.E. of five independent experiments. B, activation of ARF1 and ARF6 were assessed as in A in ARF6- and ARF1-depleted MDA-MB-231 cells, respectively. Data (fold over basal) are the mean ± S.E. of three independent experiments.

GTP-bound ARFs. As shown in Fig. 1A, EGF treatment of MDA-MB-231 cells resulted in the rapid and transient activation of endogenous ARF6 and ARF1, where maximal levels of GTP binding were detected after 30 and 60 s, respectively.

To assess the role of endogenous ARF1 in EGFR-dependent cellular responses, we designed siRNAs that specifically targeted the human ARF1 sequence and tested their effects on MDA-MB-231 cells. As illustrated in supplemental Fig. S1A, transfection of either ARF1 siRNA (#1 and #2) reduced the expression of ARF1 without affecting ARF6 levels. Western blot analysis revealed that the minimal concentration of siRNA (ARF1#1) to produce the maximal inhibition of ARF1 expression (90%) was 25 nM (supplemental Fig. S1B), and the optimal time of transfection to achieve this inhibition was 3 days (supplemental Fig. S1C). Efficacy of siRNA transfection for these conditions was over 90%, and cell viability assessed by Trypan blue was similar in scrambled and ARF1 siRNA-transfected cells (data not shown). Because no commercial antibodies are available to examine expression levels of other ARF isoforms, we next quantified mRNA levels of all ARF isoforms using real-time PCR. As illustrated in supplemental Fig. S1D, only ARF1 mRNA levels were reduced in ARF1 siRNA (25 nM, 72h)-transfected cells. To control for siRNA off-target effects, we designed an ARF1 mutant insensitive to ARF1 siRNA #1 (ARF1mut). As illustrated in supplemental Fig. S1E, expression of ARF1mut in cells transfected with ARF1 siRNA resulted in ARF1 expression levels similar to those observed under control conditions. Finally, we confirmed the specificity of the ARF1 antibody against ARF3, because these two isoforms are highly homologous. As illustrated in supplemental Fig. S1F, the anti-ARF1 antibody does not detect overexpressed GFP-tagged ARF3. Additional transfection experiments with ARF6 siRNA reduced expression of this ARF isoform by 80% in MDA-MB-231 cells (supplemental Fig. S1G).

Using the siRNA strategy, we next investigated whether the activation of ARF1 was necessary for the activation of ARF6, and vice versa. It was previously suggested that activated ARF6 recruits ARNO family GEFs for further activation of other ARF isoforms (20). As illustrated in Fig. 1B, depletion of ARF6 did not affect ARF1 activation following EGF stimulation of MDA-MB-231 cells. Similarly, ARF1 depletion did not affect the ability of the EGFR to activate ARF6 suggesting that these two GTP-binding proteins are activated independently following EGF stimulation.

ARF1 and ARF6 Are Both Localized to the Plasma Membrane of MDA-MB-231 Cells—We next examined the cellular distribution of ARF1 and ARF6. As presented in Fig. 2A, ARF1-FLAG is found inside the cytoplasm, but also present on dynamic plasma membrane ruffles, co-localizing with actin. Scanning of the cells, at different confocal planes, confirmed that ARF1-FLAG is present at the Golgi complex where it colocalizes with p115 (supplemental Fig. S2). EGF stimulation led to the formation of membrane protrusions but had no significant effect on the cellular distribution of this ARF isoform, which remained associated with ruffling areas. As expected, overexpressed ARF6-HA associated primarily with the plasma membrane (Fig. 2). Altogether, these data demonstrate that localization of ARF1 to dynamic plasma membrane ruffles is consistent with signaling events emanating from the cell surface.

ARF1 Regulates the EGF-dependent Migration and Proliferation of MDA-MB-231 Cells—Breast cancer cell migration is an important cellular process necessary for metastasis. The role of ARF6 in regulating cellular invasiveness has been previously demonstrated (15, 18). In this study, we therefore focused on ARF1 and next investigated whether this ARF isoform can also act to control cell migration. As expected, EGF stimulation increased the motility and invasive capacity of MDA-MB-231 cells in the wound healing assay as well as in the collagen-coated Boyden chamber assay (Fig. 3). Depletion of ARF1 completely abolished the EGF-dependent effects (Fig. 3, A and B), which was reversed by overexpressing an siRNA-insensitive ARF1 mutant (ARF1mut). Furthermore, overexpression of wild-type ARF1 (1.6-fold, 44% transfection efficiency) increased EGF-promoted migration (Fig. 3C), while overexpression of a dominant negative mutant of ARF1, ARF1T31N (1.5-fold, 63% efficiency), prevented EGF-stimulated cell migration. These data
suggest that modulation of ARF1 activation leads to altered cell migration.

EGFR activation is also associated with enhanced breast cancer cell growth. Accordingly, EGF treatment increased the proliferation rate of MDA-MB-231 cells by 1.6-fold compared with non-stimulated cells assessed after 4 days of culture. Transfection of ARF1 siRNA totally blocked the EGF-mediated response, and co-transfection of ARF1mut increased basal and EGF-stimulated proliferation compared with control conditions (Fig. 4A). As illustrated in Fig. 4B, both basal and EGF-dependent cell proliferation was enhanced in conditions where ARF1 was overexpressed. In contrast, expression of ARF1T31N blocked the EGF-mediated response. Our findings are therefore in agreement with the hypothesis that the small GTPase ARF1 plays an essential role not only in EGF-dependent breast cancer cell migration, but also proliferation.

Depletion of ARF1 Does Not Alter EGFR Expression or Activation—One possible explanation for a reduced migration and growth of breast cancer cells depleted of ARF1 is the possibility that this ARF isoform plays a role in the expression of EGFR and/or its activation by EGF. To test this possibility, we first examined the distribution of endogenous EGFR (identified with EGF coupled to Alexa-Fluor 488) in control and ARF1-depleted (identified by co-transfection with an irrelevant siRNA coupled to Alexa-Fluor 546) cells. Our data indicate that ARF1 knockdown does not affect targeting of the EGFR to the plasma membrane, nor its internalization from the cell surface (Fig. 5A). Alternatively, we next examined the distribution of
EGFR Activates ARF1 to Regulate Growth andMigration

![Diagram](image)

**FIGURE 5. Depletion of ARF1 does not impair the activation of the EGFR by EGF.** A, MDA-MB-231 cells transfected with a scrambled siRNA or ARF1 siRNA (together with an irrelevant siRNA coupled to Alexa-Fluor 546) were incubated with EGF-coupled to Alexa-Fluor 488 for 30 min on ice (0) or for 20 min at 37 °C to assess EGFR distribution. Cells were fixed, and imaging was performed using confocal microscopy. This figure is representative of more than 30 cells observed in 4 independent experiments. B, cells transfected as above were fixed, permeabilized, and labeled using an anti-EGFR, and a secondary antibody coupled to Alexa-Fluor 488. This figure is representative of more than 30 cells observed in 2 independent experiments. Quantification of EGFR immunolabeling at the cell surface expressed as average fluorescence intensity is presented in the right panel. Results are the mean ± S.E. of 10 areas assessed on 5 different cells for each condition. C, cells transfected as in A were stimulated with EGF, fixed, permeabilized, and labeled using a phosphospecific EGFR antibody (Y1148) and a secondary antibody coupled to Alexa-Fluor 488. This figure is representative of more than 30 cells observed in 3 independent experiments. Scale bar, 10 μm. D, MDA-MB-231 cells transfected with either a scrambled or ARF1 siRNA were stimulated with EGF for different times. EGFR levels were examined using an anti-EGFR antibody and phosphorylated EGFR detected using a phospho-specific antibody, P-TYR 1148. This experiment is representative of three others, and input represents 15% of the total protein present in the sample. E, quantification of the data presented in D. EGFR phosphorylation is expressed as fold phosphorylation over basal (non-stimulated). Results are the mean ± S.E. of 4 independent experiments.

Moreover, analysis of EGFR levels present at the plasma membrane, using MetaMorph software, confirmed that the intensity of the fluorescence was similar in control and ARF1-depleted cells. Finally, the ability of EGF to promote activation of its endogenously expressed receptors as assessed by receptor tyrosine phosphorylation appears similar in control and ARF1 siRNA-transfected MDA-MB-231 cells (Fig. 5, C–E). These data strongly suggest that EGFRs are functional in ARF1-depleted cells. Because growth factors such as the EGFR use the mitogen-activated protein kinase (MAPK) and PI3K pathways to transmit signal from their receptors, we next examined the role of ARF1 in regulating the activation of these two signaling cascades.

**Depletion of ARF1 Significantly Impairs the Ability of the EGFR to Signal through the PI3K but Not the MAPK Pathway—**EGF promoted migration and proliferation of MDA-MB-231 cells can be significantly reduced by pretreatment with biochemical inhibitors of either the MAPK or the PI3K pathway, the PD98059 and LY294002 compounds, respectively (Fig. 6, A and B). Fig. 6 (C and D) demonstrate that these inhibitors are indeed effective in blocking the EGFindependent phosphorylation of Erk1/2 as well as Akt, respectively, in cells transfected with a scrambled siRNA. To test the involvement of ARF1 in the regulation of these two signaling pathways, we measured EGFR-induced phosphorylation of Erk1/2 and Akt in ARF1-depleted cells. ARF1 knockdown did not affect the ability of EGF to activate the MAPK pathway (Fig. 6, E and G). However, activation of the PI3K pathway following EGF stimulation was completely abolished under these conditions (Fig. 6, F and H). To further support the role of ARF1 in regulating the PI3K/Akt pathway, we examined the effect of overexpressing the ARF1 dominant negative mutant. As illustrated in supplemental Fig. S3A, transfection of ARFI T31N blocked the EGFindependent phosphorylation of Akt, whereas it had no effect on receptors using a specific EGFR antibody. As expected, endogenous EGFRs were found in dynamic plasma membrane ruffles and, to a lesser extent, on cytoplasmic structures (Fig. 5B).
**EGFR Activates ARF1 to Regulate Growth and Migration**

Erk1/2 activation. In addition, our data show that depletion of ARF6 had no effect on Akt phosphorylation while it markedly reduced the ability of the EGFR to promote the activation of Erk1/2 (supplemental Fig. S3B).

These data therefore support the idea that ARF1, at the plasma membrane, specifically regulates the activation of the PI3K pathway, whereas ARF6 is an important player in the activation of the MAPK pathway. Hence, we next investigated the role of ARF1 in regulating PI3K activation.

**Impaired Recruitment of PI3K in ARF1-depleted Cells—**
Upon RTK stimulation, class 1A PI3K are classically recruited to the activated receptor at the plasma membrane to convert phosphatidylinositol 1,4,5-bisphosphate into phosphatidylinositol 1,4,5-trisphosphate, leading to the recruitment of Akt allowing its phosphorylation (27, 28). Here, we first used a biochemical approach to examine the plasma membrane translocation of p110, the catalytic subunit of PI3K as well as Akt. As illustrated in Fig. 7A, EGF stimulation led to the enrichment of p110α and Akt in membrane fractions. Depletion of ARF1 completely abolished the agonist-dependent recruitment of the catalytic subunit of PI3K and Akt to the plasma membrane, which was reversed by expression of the ARF1 siRNA-insensitive construct (ARF1mut).

Although the regulatory subunit of PI3K exhibits no enzymatic activity, it is crucial for regulating the activation of class 1A PI3K by RTKs. Upon EGF stimulation, p85α has been shown to be recruited to the activated receptor and become phosphorylated. As illustrated in Fig. 7B, EGF stimulation led to the formation of a complex, including the EGFR and p85α. Depletion of ARF1 markedly impaired the interaction between the activated receptor and the regulatory subunit of PI3K. We also monitored tyrosine phosphorylation of the p85α regulatory subunit. As shown in Fig. 7C, EGF stimulation led to the phosphorylation of p85α as shown by a phospho-specific p85α (Y508) antibody and a total phospho-tyrosine (Y99) antibody. Depletion of ARF1 abrogated the EGF-dependent phosphorylation of p85α. Collectively, our data suggest that ARF1 is a key protein regulating the PI3K pathway in breast cancer cells.

**Migration and Proliferation of MDA-MB-435 and SKBR3 Cells Are Also Regulated by ARF1—**
To determine whether the mechanism we uncovered is general, we examined the role of ARF1 in regulating migration and growth of other breast cancer cell lines. We chose MDA-MB-435 cells, which are similar to MDA-MB-231, as well as SKBR3 cells, which express high levels of HER2 (29). All cell lines expressed high level of EGFR when compared with a control immortalized, non-cancerous human mammary epithelial cell line, MCF10a (Fig. 8A). MDA-MB-435 cells expressed low levels of ARF1, similar to those of MCF10a, whereas MDA-MB-231 cells contained levels of ARF1 higher than MDA-MB-435. SKBR3 cells expressed much

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**FIGURE 6. ARF1 regulates the activation of the PI3K pathway following EGF stimulation.** A, MDA-MB-231 cells were treated with vehicle, PD98059, an inhibitor of the MAPK pathway or LY294002, an inhibitor of the PI3K pathway (15 μM, 30 min). Migration of non-stimulated and EGF-stimulated cells was assessed after 6 h using the Boyden chamber assay. Results are the mean ± S.E. of four independent experiments and expressed as -fold increase in migration over basal (non-stimulated, control conditions). B, as in A, cells were treated with vehicle, PD98059, or LY294002, and proliferation was performed after 4 days. Data are expressed as -fold over basal and are the mean ± S.E. of four independent experiments. C and E, cells transfected with a scrambled (C) or ARF1 siRNA were treated with vehicle or PD98059, stimulated or not with EGF (0 or 2 min), and activation of Erk1/2 was determined by Western blotting. Membranes were blotted with phospho-specific anti-Akt and anti-Akt antibodies. Immunoblots are representative of four independent experiments. Input represents 15% of the total lysate. G, quantification of the data presented in C and E (Erk1/2 activation). Results are presented as the mean ± S.E. of four independent experiments. H, quantification of the data presented in D and F (Akt activation). Results are presented as the mean ± S.E. of four independent experiments. **, p < 0.01; ***, p < 0.001; ***, p < 0.001 are values compared with the control unstimulated condition.
EGFR Activates ARF1 to Regulate Growth and Migration

higher levels of ARF1. In all cancer cell lines, EGF stimulation resulted in the activation of ARF1 (data not shown).

EGF stimulation led to a 3.7- and 1.7-fold increased of MDA-MB-435 and SKBR3 cell migration, respectively. Depletion of ARF1 markedly reduced the EGF-mediated response (Fig. 8B). Interestingly, overexpression of wild-type ARF1 had no effect on the extent of the EGF-dependent response in MDA-MB-435 cells, whereas it markedly increased the migration of SKBR3 cells (supplemental Fig. S4A). As previously observed, expres-

sion of the ARF1 dominant negative mutant inhibited the EGF-dependent response in the two cell lines studied (supplemental Fig. S4A).

Knockdown of ARF1 expression blocked the EGF-stimulated proliferation of both MDA-MB-435 and SKBR3 cells. In the case of the latter, ARF1 knockdown also reduced basal growth (Fig. 8C). Overexpression of ARF1 increased proliferation of both cell lines, whereas expression of the ARF1 dominant negative (ARFITN) was inhibitory (supple-

mental Fig. S4B). As observed in MDA-MB-231 cells, depletion of ARF1 in SKBR3 cells differentially affected the PI3K and MAPK path-

ways: it totally abolished the ability of the EGFR to induce phosphorylation of Akt (supplemental Fig. 4C) but did not impair activation of Erk1/2 (supplemental Fig. S4D). Taken together, these studies sug-

gest that ARF1 plays a general role in the migratory and proliferative abilities of breast cancer cell lines through the regulation of the PI3K pathway.

**DISCUSSION**

In this study, we describe a critical role for the small GTPase ARF1 in EGF-dependent breast cancer cell migration and proliferation. Specifically, we show that this ARF isoform is present at the plasma membrane and regulates activation of the PI3K pathway. Although ARF1 has classically been associated with the Golgi and the secretory pathway (13), our data together with recent reports (20) clearly illustrate that it can also be found at the cell periphery and regulate signaling from the plasma membrane. It was proposed that the ability of ARF1 to cycle off the Golgi to the cytosol upon GTP hydrolysis makes it avail-

able for recruitment onto other membrane (20). In addition, Li and colleagues (30) have shown that insulin stimulation promotes the transient recruitment of ARF1-GFP to the plasma membrane of HeLa cells. Here, we demonstrate that in MDA-MB-231 cells, this ARF isoform partially localized to dynamic plasma membrane ruffles in non-stimulated conditions, and it was totally lost upon GTP hydrolysis due to ARF1 interaction with the Golgi apparatus, suggesting a role for ARF1 in the regulation of membrane dynamics.

**FIGURE 7. Role of ARF1 and the recruitment of PI3K.** A, MDA-MB-231 cells transfected with a scrambled, ARF1 siRNA, or ARF1 siRNA and ARF1 siRNA insensitive construct (ARF1 mut) were stimulated with EGF for the indicated times. Membrane fractions were prepared, and amounts of associated p110α and Akt were assessed by Western blotting. Input represents 15% of the sample. Data are the mean ± S.E. of four to eight experiments. B, cells transfected with a scrambled or ARF1 siRNA were stimulated or not with EGF (0, 1, or 2 min). Endog-

enously expressed EGFRs were immunoprecipitated using the anti-EGFR antibody, and associated p85α was examined by Western blotting using a p85α antibody. Input represent 15% of the total cell lysate. Data are the mean ± S.E. of four experiments. C, MDA-MB-231 cells were stimulated with EGF (0 or 2 min), and phosphorylation of p85α (Y208) was determined by Western blotting using a phospho-specific p85α or an anti-phospho-

tyrosine antibody (Y99). Input represent 15% of the total cell lysate. Data are the mean ± S.E. of five experi-

ments. ***, p < 0.001 are values compared with the control unstimulated condition.
EGFR Activates ARF1 to Regulate Growth and Migration

To study the role of ARF1 in cell migration and proliferation, we have used two different strategies: the reduction of cellular expression levels by RNA interference, and overexpression of wild-type or mutant ARF1 proteins. Altogether, these complementary approaches yielded consistent results demonstrating that ARF1 is an important regulator of both the migration and proliferation of invasive breast cancer cells. Because this ARF isomor is a central regulator of the secretory pathway, we first investigated whether EGFR expression and signaling might be altered in ARF1-depleted cells, thereby resulting in decreased migration and proliferation. Using a different cell line, Volpi-cell-Daley and colleagues previously reported that no single ARF was essential for any step of membrane trafficking (31). Our data revealed similar findings. Depletion of ARF1 did not impair EGFR expression, targeting to the cell surface, activation by EGF, or signaling through the MAPK pathway. In addition, expression of an ARF1 mutant insensitive to ARF1 siRNA effectively restored EGF-dependent cell migration and proliferation, thereby excluding off-target effects of the ARF1 siRNA. Therefore, the inhibition of migration and growth observed following ARF1 depletion are unlikely to be due to impaired EGFR function, but specific for EGFR-dependent signaling events.

To confirm our data, we next examined the impact of ARF1 overexpression using a method that yielded elevated transfection efficiency. Under these conditions, EGF-dependent cell migration and both basal and stimulated growth were enhanced. In contrast, transfection of a dominant negative mutant, ARF1T31N, causing ARF1 to exist primarily in a GDP-bound state, blocked the EGF-dependent effects. Expression of this mutant has previously been shown to result in the redistribution of β-COP from the Golgi membranes to the cytosol and the collapse of the Golgi into the endoplasmic reticulum (32). In our experiments and conditions, activation of the MAPK pathway in ARF1T31N-transfected cells remained intact suggesting that EGF-dependent signaling events are not altered by expression of this ARF1 mutant.

The nature of the ARF-GEF responsible for ARF activation upon EGF treatment remains unknown. Although overexpression of ARNO has been previously reported to increase basal migratory phenotypes (33), further experiments are required to identify the endogenous ARF-GEF(s) responsible for ARF1 activation upon EGFR stimulation. In our experiments, ARF6 is not required to promote the EGF-dependent activation of ARF1 or the activation of the PI3K pathway. Furthermore, treatment of MDA-MB-231 cells with a PI3K inhibitor (LY 294002) has no effect on ARF1 activation (data not shown) suggesting that the endogenous GEF promoting GTP loading on ARF1 does not require phosphatidylinositol 1,4,5-trisphosphate production. Our data suggest that the two GTPases are activated independently following EGFR stimulation to regulate distinct signaling pathways acting in concert to enhance breast cancer cell migration and proliferation.

It is well known that class IA PI3Ks are translocated to activated RTKs to regulate cell migration and growth downstream of RTKs (8). In this study, we demonstrate that depletion of ARF1 markedly inhibits activation of the PI3K pathway, as assessed by the phosphorylation of Akt, the plasma membrane recruitment of p110α, the interaction of p85α with the EGFR, and the tyrosine phosphorylation of p85α. Conversely, overexpression of ARF1 potentiated EGF-dependent effects. Whether in EGF-stimulated MDA-MB-231 cells, p85α interacts directly or via an adaptor protein remains to be determined. In either case, our data suggest that ARF1 might act as a switch to regulate this key event.

In our experiments, depletion of ARF1 greatly impaired the activation of the PI3K pathway without affecting the activation of the MAPK pathway. It was previously reported that ARF6, highly overexpressed in invasive breast cancer cells (15), can regulate tumor cell invasion through the activation of the MEK-Erk pathway in a human melanoma cell line (LOX) (18). Our data indicate that ARF6 controls Erk activation in MDA-MB-231 cells as well. Taken together, these findings suggest that
ARF1 and ARF6 may both contribute to cancer cell progression and invasiveness by controlling the PI3K and MAPK pathways, respectively.

We previously observed that, in HEK 293 cells, depletion of ARF6 led to spontaneous membrane ruffling and cell migration (25). Here, we show that depletion of ARF1 or overexpression of a dominant negative mutant does not affect basal cell migration but significantly reduces EGF-dependent effects. In HEK 293 cells, basal migration and angiotensin II-promoted migration are not affected by the depletion of ARF1. These contrasting findings highlight the complexity of the signaling pathways activated by stimulation of G-protein-coupled and tyrosine kinase receptors in different cellular contexts. We have also observed that EGF-induced proliferation of MDA-MB-231 cells is greatly impaired by the depletion of ARF1. The exact mechanism by which this phenomenon occurs needs to be further investigated. Altan-Bonnet et al. (34) have suggested that ARF1 plays an important role in the orchestration of mitotic Golgi breakdown, chromosome segregation, and cytokinesis. In our experiments, depletion of ARF1 or expression of the ARF1T31N mutant did not significantly impair basal cell growth, after 4 days, but consistently blocked EGF-stimulated effects.

The fact that ARF1 depletion had similar effects on several different breast cancer cell lines suggests that the mechanisms that we have uncovered may be widely used. Taken together, our findings reveal an unsuspected role for ARF1 and indicate that this small GTPase may be a potential therapeutic target for the treatment of invasive breast cancers. It is of special interest to note the recent identification of an inhibitor that specifically prevents ARF1 activation (35). Further studies using such compounds will provide insights into the precise role of ARF1 during tumorigenesis.

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