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Vascular Endothelial Growth Factor Receptor-2 Activates ADP-ribosylation Factor 1 to Promote Endothelial Nitric-oxide Synthase Activation and Nitric Oxide Release from Endothelial Cells*

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Vascular endothelial growth factor (VEGF) induces angiogenesis and regulates endothelial function via production and release of nitric oxide (NO), an important signaling molecule. The molecular basis leading to NO production involves phosphatidylinositiol-3 kinase (PI3K), Akt, and endothelial nitric-oxide synthase (eNOS) activation. In this study, we have examined whether small GTP-binding proteins of the ADP-ribosylation factor (ARF) family act as molecular switches to regulate signaling cascades activated by VEGF in endothelial cells. Our results show that this growth factor can promote the rapid and transient activation of ARF1. In endothelial cells, this GTPase is present on dynamic plasma membrane ruffles. Inhibition of ARF1 expression, using RNA interference, markedly impaired VEGF-dependent eNOS phosphorylation and NO production by preventing the activation of the PI3K/Akt signaling axis. Furthermore, our data indicate that phosphorylation of Tyr^{801}, on VEGF receptor 2, is essential for activating Src- and ARF1-dependent signaling events leading to NO release from endothelial cells. Lastly, this mediator is known to regulate a broad variety of endothelial cell functions. Depletion of ARF1 markedly inhibits VEGF-dependent increase of vascular permeability as well as capillary tubule formation, a process important for angiogenesis. Taken together, our data indicate that ARF1 is a novel modulator of VEGF-stimulated NO release and signaling in endothelial cells.

The vascular endothelial growth factor (VEGF)[5] is a potent angiogenic factor both in normal and pathological condition (1, 2). The binding of VEGF to its cognate tyrosine kinase receptors, VEGF receptor 1 (VEGFR1, Flt1), 2 (VEGFR-2, Flk-1/KDR) and 3 (VEGFR-3, Flt4) induces dimerization and activation of downstream signaling pathways including, phospholipase C (PLC), the MAPK cascades, Akt, and endothelial nitric-oxide synthase (eNOS) (3, 4). We have shown previously that in endothelial cells, Tyr^{801} present on VEGFR-2, is highly phosphorylated in response to VEGF and is essential for Akt and eNOS activation as well as nitric oxide (NO) release (5). In contrast, phosphorylation of Tyr^{1175} was associated with phosphorylation and activation of PLCγ and Ca^{2+} influx (6). Mutation of this residue in mice (Y1173F) markedly impaired vasculogenesis (7).

Endothelial NOS is an important enzyme in the cardiovascular system responsible for the generation of NO, a key regulator of systemic blood pressure maintenance, vascular remodeling and permeability, angiogenesis, and wound healing (8). eNOS activity has been shown to be regulated by multiple mechanisms including post-translational modifications like multiple site phosphorylation (9), availability of cofactors and substrates, alterations in subcellular localization, and protein-protein interactions with several proteins such as calmodulin (10), caveolin-1 (11), and HSP90 (12). At basal state, eNOS is found enriched in caveolae, and associates to caveolin-1 (13), which maintains it inactive (14). A direct relationship has been observed *in vivo* between the expression of caveolin-1 in endothelial cells and the inhibition of NO release (15). Upon increased intracellular calcium $[Ca^{2+}]_i$ levels, eNOS dissociates from caveolin-1 and HSP90 to form a complex with Ca^{2+}/calmodulin, thereby increasing eNOS activity and NO production (16). Phosphorylation of eNOS was associated with both an increase and a decrease of eNOS activity. For instance, phosphorylation of Ser^{1179} promotes activation of eNOS and increases its sensitivity to Ca^{2+}/calmodulin. In contrast, phosphorylation of Thr^{497} negatively regulates eNOS activity (15). Phosphorylation at Ser^{1179}, can be mediated by protein kinase B/Akt (17, 18). Akt is a Ser/Thr protein kinase involved in many cellular processes including cellular growth and survival (19), angiogenesis, migration, and invasion (20). Akt is activated fol-

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[5] The abbreviations used are: VEGF, vascular endothelial growth factor; NO, nitric oxide; PI3K, phosphatidylinositol-3 kinase; MAPK, mitogen-activated protein kinase; eNOS, endothelial nitric-oxide synthase; ARF, ADP-ribosylation factor; Erk, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyanate; PLC, phospholipase C; EGFR, epidermal growth factor receptor; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; BAEC, bovine aortic endothelial cells; siRNA, small interfering RNA; WT, wild type.

lowing phosphatidylinositol 3,4,5-triphosphate generation, a process involving phosphatidylinositol 3 kinase (PI3K) activation (21). The maximal activation of Akt is reached when Thr^{308} and Ser^{473} are phosphorylated by 3-phosphoinositide-dependent protein kinase 1 and another protein, which remains unidentified (22).

We have shown recently that in invasive breast cancer cells, activation of the epidermal growth factor receptor (EGFR), another tyrosine kinase receptor, promoted the activation of the PI3K/Akt pathway via a mechanism dependent upon the activation of the GTPase ARF1 (23). ARF proteins are a family of six isoforms, and ARF1 and ARF6 are best characterized. Classically, ARF1 has been shown to associate with the Golgi, where it is involved in the formation of Golgi-derived coated vesicles (24, 25). Others and we have reported that this GTPase can also be present at the plasma membrane (23, 26). Similarly, ARF6 is present at the plasma membrane where it appears to be a critical regulator of endocytotic pathways (27, 28) and actin cytoskeleton rearrangement (29). Like all GTPases, ARFs are inactive when bound to GDP and becomes active upon GTP loading. This process is regulated by guanine nucleotide exchange factors, whereas GTP hydrolysis requires GTPase-activating proteins. ARF proteins are activated following the stimulation of seven trans-membrane spanning receptors such as the angiotensin II type 1 receptor as well as tyrosine kinase receptors such as the EGFR (23, 30). Ikeda *et al.* (31) have reported that ARF6 is involved in the regulation of VEGFR signaling and in the control of angiogenesis. In this study, we report that VEGF stimulation of endothelial cells results in rapid and transient activation of ARF1, which acts to regulate activation of PI3K, phosphorylation of Akt and eNOS, as well as NO release. Therefore, depletion of ARF1 results in important physiological consequences such as impaired permeability of endothelial monolayers and the ability of endothelial cells to form capillary tubule, a step necessary for angiogenesis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Bovine aortic endothelial cells (BAEC) were purchased from VEC TECHNOLOGIES (Rensselaer, NY). Dulbecco's minimal essential medium was purchased from Wisent, Inc. (St. Bruno, Quebec, Canada). Fetal bovine serum (Hyclon) was purchased from Fisher (Ottawa, Ontario, Canada). Alexa Fluor 488 polyclonal antibody, Lipofectamine 2000, and fluorescein isothiocyanate (FITC)-labeled dextran were from Invitrogen. The silencer small interfering RNA (siRNA) construction kit was purchased from Ambion (Austin, TX). Recombinant human $VEGF_{A165}$ was obtained from R&D Systems (Minneapolis, MN). Mouse anti-VEGFR-2, p85α (B-9), and Erk1/2 (K-23) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit anti-phospho-Ser^{1179}-eNOS, anti-Akt, anti-phospho-Ser^{473}-Akt, phospho-p44/42 (Erk1/2), anti-PLC, anti-phospho-PLCγ_1 (Tyr^{783}), anti-phospho-p38, p38, pan-actin antibodies, and anti-phospho-Src-Tyr^{416} were purchased from Cell Signaling (Danvers, MA). Mouse anti-eNOS antibody was from BD Transduction Laboratories (Mississauga, Ontario, Canada), ARF1 antibody was from Abcam (Cambridge, MA). Protease inhibitors mixture set IV was from Calbiochem. Src antibodies were from Upstate (Lake Placid, NY). Anti-HA (3F10) was from Roche Applied Science. The *in vitro* angiogenesis assay kit was purchased from Chemicon (Temecula, CA). All other reagents were purchased from Sigma Aldrich.

Cell Culture and Transfection—BAEC and COS-7 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2.0 mM L-glutamine, and 100 units/ml penicillin/streptomycin. Endothelial cells were used between passages 5 and 8. For VEGF stimulations experiments, cells were starved for 4 h in Dulbecco's modified Eagle's medium supplemented with 2.0 mM L-glutamine and 100 units/ml penicillin/streptomycin. Transfection of siRNAs was performed as described previously (23) using Lipofectamine 2000 according to the manufacturer's instructions. siRNAs targeting human ARF1 and ARF6 (sequence 1) were described previously (23) and synthesized using the Silencer siRNA construction kit from Ambion (Austin, TX). The target sequence for the scrambled siRNA was designed against an irrelevant region in the human genome (5′-AACAGGATAGTCGAGCAGAGT-3′). siRNA-insensitive ARF1 mutant was described previously (23). In this study, measurement of protein expression and experiments were performed 48 and 72 h following ARF6 and ARF1 siRNA transfection, respectively.

Activation of ARF1—BAEC were serum starved for 4 h and stimulated with VEGF (50 ng/ml) at 37 °C for the indicated times. Briefly, cells were lysed in 400 μl of ice-cold lysis buffer E (pH 7.4, 50 mM Tris-HCl, 1% Nonidet P-40, 137 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 20 mM NaF, 1 mM NaPPi, 1 mM Na_3VO_4, and protease inhibitors) as described in Ref. 23. Cell lysates were clarified by centrifugation at 12,000 $\times$ *g* for 10 min and incubated with GST-GGA3 fusion proteins bound to glutathione-Sepharose 4B beads (Amersham Biosciences) for 1 h. Proteins were eluted into 20 μl of SDS sample buffer containing 5% mercaptoethanol by heating to 95 °C for 5 min, resolved on 14% SDS-PAGE, and detected by immunoblot using a specific anti-ARF1 antibody. Secondary antibodies were FITC-conjugated, and proteins were detected using a Typhoon 9410 scanner (Amersham Biosciences). Quantification of the digital images obtained was performed using ImageQuant 5.2 software (Amersham Biosciences).

Western Blot Analysis—BAEC were serum-starved (for 4 h) and treated with VEGF (50 ng/ml) for the indicated times. To prepare lysates, cells were washed twice with phosphate-buffered saline and solubilized with a lysis buffer containing 1% Nonidet P-40, 50 mM Tris-HCl, 125 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% SDS, 0.1% deoxycholic acid, 20 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and protease inhibitor mixture (Calbiochem). Equal amounts of proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature using Tris Buffered Saline/Tween 20 (TBS-T) containing 5% bovine serum albumin or milk and incubated overnight with specific antibodies. Secondary antibodies were FITC-conjugated, and fluorescence was detected using a Typhoon 9410 scanner.

Nitric Oxide Release—BAEC were grown in 6-well dishes. Three days after transfection, cells were serum-starved for 4 h and stimulated with VEGF (50 ng/ml). The amount of NO

released from cells were measured as described in Ref. 5. Briefly, samples of culture medium were taken and processed for the measurement of nitrite (NO_2^-), the stable breakdown product of NO in aqueous solution, by NO-specific chemiluminescence using a NO analyzer (Ionics Instruments).

Confocal Microscopy—BAEC transfected with ARF1-FLAG constructs were serum-starved for 4 h, stimulated with VEGF (50 ng/ml) for 15 min, and fixed using paraformaldehyde (4%) for 15 min at room temperature as described previously (32). Briefly, cells were permeabilized with 0.075% saponin for 10 min and incubated subsequently with a polyclonal anti-FLAG antibody and phalloidin coupled to rhodamine for 1 h, followed by a secondary antibody coupled to Alexa Fluor 488 for 1 h. After labeling, coverslips were mounted using Gel Tol mounting medium. All images were acquired using a 63× oil immersion objective (Carl Zeiss, Oberkochen, Germany), on a Zeiss LSM-510 META laser scanning microscope.

Membrane Protein Recruitment Assay—BAEC transfected with scrambled (control) or ARF1 siRNAs were serum-starved for 4 h and stimulated with VEGF (50 ng/ml) for the indicated times. Cells were then harvested in 300 μl of phosphate-buffered saline containing protease inhibitors, as described previously (23). Cell membranes were disrupted by passing three times through a 27–1/2-gauge needle. Cell lysates were then centrifuged for 10 min at 500 × *g* to discard nucleus and cellular debris, and supernatants were ultracentrifuged at 100,000 × *g* (30 min at 4 °C) to separate cytosolic and membrane fractions. Membrane pellets were then lysed for 10 min in 100 μl of ice-cold Triton X-100, glycerol, HEPES (TGH) buffer containing protease inhibitors. Proteins were eluted into SDS sample buffer containing 5% mercaptoethanol by heating to 95 °C for 5 or 10 min. Proteins were detected by immunoblot analysis using specific antibodies (anti-Akt).

p85α Recruitment to VEGFR-2—BAEC were transfected with scrambled or ARF1 siRNAs, serum-starved for 4 h, and then stimulated with VEGF (50 ng/ml) for the indicated times. Cells were lysed in 100 μl TGH buffer containing protease inhibitors, tumbled at 4 °C for 30 min, and spun at 12,000 × *g* for 10 min. 12.5 μl of the supernatant was taken for total proteins control (total input). Equal concentrations of soluble proteins were incubated with the anti-p85α antibody, and samples were tumbled overnight at 4 °C and then were incubated with protein G-PLUS agarose beads at 4 °C for 2 h. The beads were washed three times with TGH buffer containing protease inhibitors, and proteins were eluted into 20 μl of SDS sample buffer containing 5% mercaptoethanol by heating to 95 °C for 5 min, resolved on 8% SDS-PAGE, and detected by immunoblot using specific anti-VEGFR-2 and anti-p85α antibodies.

Permeability Assay—48 h after transfection, BAEC were plated, at a density of 200,000 cells per well, onto collagen-coated Transwell units (with a 6.5-mm diameter and 3.0-μm pore size polycarbonate filter, Corning Costar) and cultured for 3 days, until the formation of monolayer. Cells were serum-starved for 1 h in Dulbecco's modified Eagle's medium containing 1% bovine serum albumin. Cells were then stimulated with VEGF (40 ng/ml, upper chambers) in presence of 1 mg/ml FITC-labeled dextran. Permeability through endothelial cell monolayers was measured by collecting sample and measuring the fluorescence at 520 nm (excitation at 492 nm) using a spectra Max M2 spectrophotometer (Molecular Devices).

Angiogenesis Assay—In vitro angiogenesis was assessed in three-dimensional fibrin matrices using an angiogenesis assay kit (Chemicon), according to the manufacturer's instructions. Diluted ECMatrix™ solution containing growth factors was transferred to each well of a 96-well tissue culture plate and incubated at 37 °C for 1 h to allow the matrix solution to solidify. BAEC were harvested and seeded at a density of 15,000 cells per well onto the surface of the polymerized ECMatrix™, followed by incubation at 37 °C for 8 h. Tube formation was observed with an inverted light microscope connected to a digital camera (LEICA DMRIB) at magnifications between 10 and 40×.

Statistical Analysis—Statistical analysis was performed using a one-way or two-way analysis of variance followed by a Bonferroni's multiple comparison test using GraphPad Prism (version 4.0a; San Diego, CA).

RESULTS

VEGF Stimulates ARF1 Activation in BAEC—We first examined the ability of VEGF to stimulate the activation of ARF1 in endothelial cells. BAEC were harvested at different times post-VEGF treatment and analyzed for endogenous ARF1-GTP levels using the GST-GGA3 pulldown assay. As illustrated in Fig. 1*A*, VEGF stimulation promoted the rapid and transient activation of ARF1, where maximal levels were observed after 2 min of stimulation. We next examined the distribution of this GTPase in endothelial cells. BAEC were transiently transfected with ARF1-FLAG. Immunolabeling experiments revealed that this ARF isoform was present in the cytosol and co-localized with actin (phalloidin coupled to rhodamine) at dynamic plasma membrane ruffles (Fig. 1*B*). Scanning of the cells at different confocal planes confirmed that, as expected, ARF1-FLAG was also present at the Golgi (data not shown) as reported previously (23). VEGF stimulation led to reorganization of the actin cytoskeleton and formation of lamellipodia. To explore the functional significance of ARF1 activation following VEGF treatment, we examined signaling to the PI3K and MAPK pathways.

Depletion of ARF1 Inhibits VEGF-induced Akt Activation but Not MAPK—To define the role of ARF1 on VEGF-induced PI3K activation, we used the RNA interference approach to knock down expression of the GTPase. Fig. 2*A* shows that transfection of BAEC with our well characterized siRNA designed against ARF1 effectively blocked the expression of this ARF isoform (75%). First, control and ARF1-depleted cells were incubated with VEGF for up to 30 min, and Akt phosphorylation was assessed by Western blot analysis using an antibody that selectively detected Ser^{473} phosphorylated Akt. As illustrated in Fig. 2, *B* and *C*, VEGF stimulation promoted transient Akt phosphorylation, where maximal levels were detected 5 min after stimulation. Depletion of ARF1 totally abolished this VEGF-induced response. To control for siRNA off-target effects, we overexpressed an ARF1 construct insensitive to the ARF1 siRNA in ARF1-depleted cells. In these conditions, VEGF stimulation resulted in the phosphorylation of Akt, similar to what we observed in control conditions (Fig. 2*C*). We next examined activation of the MAPK pathway. VEGF treatment

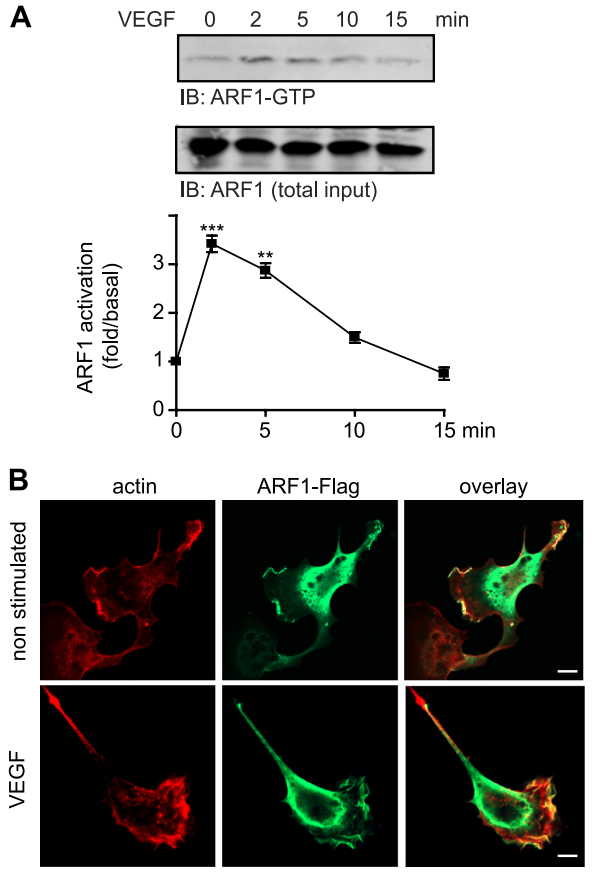

FIGURE 1. **Stimulation of the VEGF receptor promotes activation of ARF1.** *A*, serum-starved BAEC were stimulated with 50 ng/ml VEGF for the indicated time and GTP-bound ARF1 captured using a pulldown assay (GST-GGA3) as described under "Experimental Procedures." Activated and total endogenous ARF1 were detected by Western blot analysis using an ARF1-specific monoclonal antibody. *Inputs* represent 5% of the total protein present in the sample. Quantifications are presented as fold over basal and are the mean ± S.E. of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ compared with non-stimulated conditions. *B*, serum-starved BAEC transfected with ARF1-FLAG were left untreated or stimulated with VEGF (50 ng/ml) for 15 min. Cells were then fixed and incubated with a FLAG antibody (polyclonal), a secondary rabbit antibody coupled to Alexa Fluor 488, and phalloidin coupled to rhodamine. Confocal planes best depicting membrane ruffles were acquired using a LSM 510 META confocal microscope from Zeiss. This figure is representative of four independent experiments, where >30 cells were examined. *Scale bar*, 10 μm. *IB*, immunoblotting.

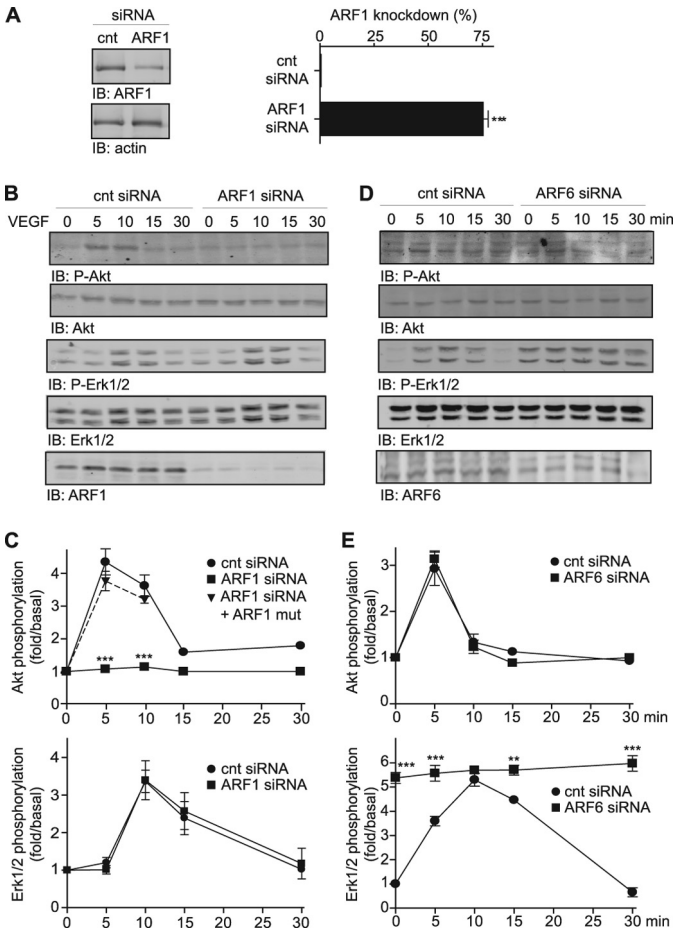

FIGURE 2. **Depletion of ARF1 impairs VEGFR signaling to the PI3K pathway.** *A*, control or ARF1 siRNA were transfected into BAEC for 72 h. Knockdown of ARF1 was confirmed by Western blotting. Quantification of the inhibition of ARF1 expression is expressed as the percent of ARF1 knockdown. Data are the mean ± S.E. of four independent experiments. ***, $p < 0.001$ compared with the control siRNA transfection. *B–E*, BAEC transfected with either a scrambled (control; *cnt*), an ARF1 (*B* and *C*), or ARF6 (*D* and *E*) siRNA were serum-starved and treated with 50 ng/ml VEGF for the indicated time. Cells lysates were run, and levels of phosphorylated Akt and Erk1/2 were detected using phospho-specific antibodies. Total Akt and Erk1/2 were also assessed. *C* and *E*, quantifications are presented as fold over basal and are the mean ± S.E. of four independent experiments. **, $p < 0.01$; ***, $p < 0.001$ when values are compared with the control siRNA-transfected condition. *IB*, immunoblotting.

promoted phosphorylation of Erk1/2, where maximal levels were observed after 10 min of stimulation. Transfection of ARF1 siRNA had no effect on VEGF-induced Erk1/2 phosphorylation (Fig. 2, *B* and *C*). These experiments demonstrate that ARF1 selectively regulates activation of the PI3K/Akt pathway in endothelial cells.

To determine whether regulation of the Akt pathway is specific to ARF1 proteins, we examined the effect of depleting ARF6, the other ARF isoform present on the plasma membrane. As illustrated in Fig. 2, *D* and *E*, depletion of ARF6 had no effect on Akt phosphorylation. However, depletion of this ARF isoform enhanced the activation of Erk1/2 at basal level similarly to what we had observed in other endothelial cell types (32).

PI3K Recruitment to VEGFR-2 and Akt Membrane Translocation Is Dependent upon ARF1—Upon stimulation, VEGFR-2 activates PI3K by a mechanism involving the recruitment of the regulatory subunit of this enzyme (5). We therefore monitored the interaction between the receptor and p85α. As illustrated in Fig. 3*A*, VEGF stimulation of endothelial cells resulted in the formation of a complex including the endogenously expressed receptor and regulatory subunit of PI3K. This response was, however, abolished when ARF1 expression was inhibited. Activation of PI3K leads to the generation of phosphatidylinositol 3,4,5-triphosphate and translocation of Akt to the plasma membrane. To better understand the molecular mechanisms regulated by ARF1, we also examined the recruitment of Akt to isolated membranes. Here, control and ARF1-depleted cells were stimulated with VEGF for various times. As shown in Fig. 3*B*, VEGF stimulation promoted the recruitment of Akt to membranes, a process markedly impaired by depletion of ARF1. These data suggest that ARF1 acts as a molecular switch to control the recruitment of PI3K to the receptor thereby regulating relocalization of Akt, a step required for its phosphorylation and signal transduction. Because eNOS is one of the

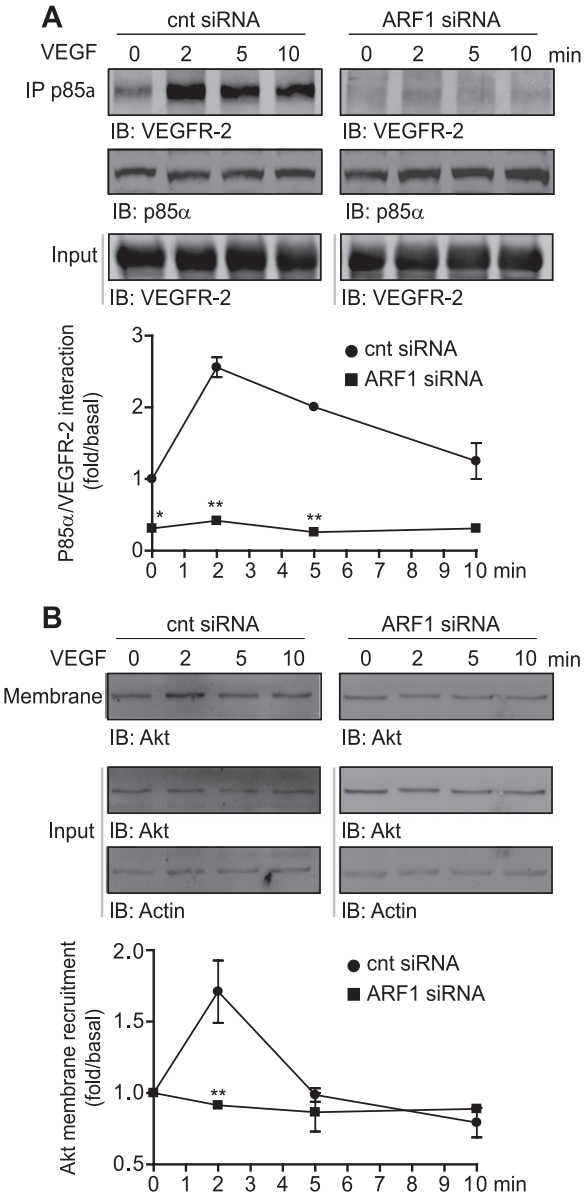

FIGURE 3. **Expression of ARF1 is essential for VEGF-dependent recruitment of p85α to the VEGFR-2 and plasma membrane translocation of Akt.** *A*, BAEC were transfected with a scramble (control; *cnt*) or ARF1 siRNA, serum-starved, and stimulated with VEGF for the indicated times. P85α was immunoprecipitated (*IP*) using the anti-p85α antibody and interacting VEGFR-2 was detected by Western blot analysis. *Inputs* represent 12.5% of the total protein present in the sample. *B*, BAEC were transfected as in *A* and then deprived of serum and stimulated with VEGF at indicated times. Cell fractionation was performed, and membrane-associated Akt was detected by Western blotting. *Inputs* represent 5% of the total protein present in the sample. Quantifications are presented as fold over basal and are the mean ± S.E. of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ when values are compared with the control siRNA-transfected condition. *IB*, immunoblotting.

downstream effectors of Akt in endothelial cells, we next studied activation of this enzyme.

ARF1 Regulates eNOS Activation and NO Release—As illustrated in Fig. 4*A*, VEGF stimulation resulted in the transient phosphorylation of eNOS on Ser^{1179}, where maximal levels were detected after 5 min of agonist stimulation. In ARF1-depleted cells, VEGF failed to phosphorylate eNOS on this residue. This effect was specific to this ARF isoform because depletion of ARF6 had no effect on eNOS activation following VEGF stimulation. Furthermore, expression of an ARF1 siRNA-insensitive construct, when expressed at levels similar to the endogenous protein, allowed recovery of eNOS phosphorylation (Fig. 4*B*).

We next examined whether NO release in the cell media was affected by knockdown of ARF1 expression. As depicted in Fig. 4*C*, VEGF stimulation of BAEC promoted the generation of NO. Depletion of ARF1 completely abolished the ability of the cells to produce this important intracellular messenger following VEGF stimulation, further supporting a role for this ARF isoform in controlling activation of the PI3K/Akt/eNOS/NO axis.

VEGFR-2 Tyr^{801} Is Required for the Activation of ARF1—VEGF-stimulated NO release from endothelial cells requires the autophosphorylation of tyrosine residues on VEGFR-2. We reported previously that Tyr^{801} present on the intracellular domain of VEGFR-2 is essential for the activation of the PI3K/Akt pathway, phosphorylation of eNOS at Ser^{1179}, and NO release (5). Considering our previous findings, we sought to examine whether phosphorylation of this residue was required for the activation of ARF1. To delineate the molecular mechanisms by which VEGFR-2 stimulation activated ARF1, we conducted experiments in COS-7 cells transfected with wild type (WT) or mutated Y801F, Y1175F, or Y1214F VEGFR-2, as these cells express ARF1 but not VEGFR-2. As illustrated in Fig. 5*A*, stimulation of VEGFR-2-transfected cells resulted in the rapid and transient activation of ARF1, whereas VEGFR-2 Y801F expressing cells failed to respond to VEGF. VEGF-stimulated VEGFR-2 Y1175F- and Y1214F-expressing cells did not significantly impair the activation of ARF1 when compared with overexpressed WT VEGFR-2.

We next examined whether in endothelial cells, phosphorylation of Tyr^{801} following VEGF treatment also was a key event to mediate ARF1 activation. BAEC were transfected with WT or mutant VEGFR-2. Similar to what we observed in COS-7 cells, mutation of Tyr^{801}, in VEGFR-2, markedly impaired the ability of the receptor to activate ARF1 but also phosphorylate eNOS (Fig. 5, *A* and *B*). Expression of VEGFR-2 Y1175F or Y1214F led to the activation of the GTPase but not PLCγ and p38, respectively (Fig. 5, *B* and *C*). To gain further insights on the molecular mechanisms by which VEGF promotes ARF1 activation, we examined Src activity. As illustrated in Fig. 5*D*, the phosphorylation of this soluble tyrosine kinase as well as the activation of ARF1 were inhibited when cells were pretreated with 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) (10 μM for 30 min), a Src family kinase inhibitor. To address specifically the role of Src, BAEC were transiently transfected with an empty vector or a dominant-negative form of Src (SrcK298M-HA). Expression of this Src mutant markedly impaired VEGF-dependent ARF1 activation (Fig. 5*E*). Taken together, these data demonstrate that Tyr^{801} of VEGFR-2, previously identified as the key residue to mediate VEGF dependent activation of eNOS, also is essential for ARF1 activation. Furthermore, GTP-binding on ARF1 requires the activation of Src.

ARF1 Is Essential for VEGF-induced BAEC Permeability and Capillary Tubule Formation—BAEC form a tight barrier when grown to confluence, and VEGF-dependent NO

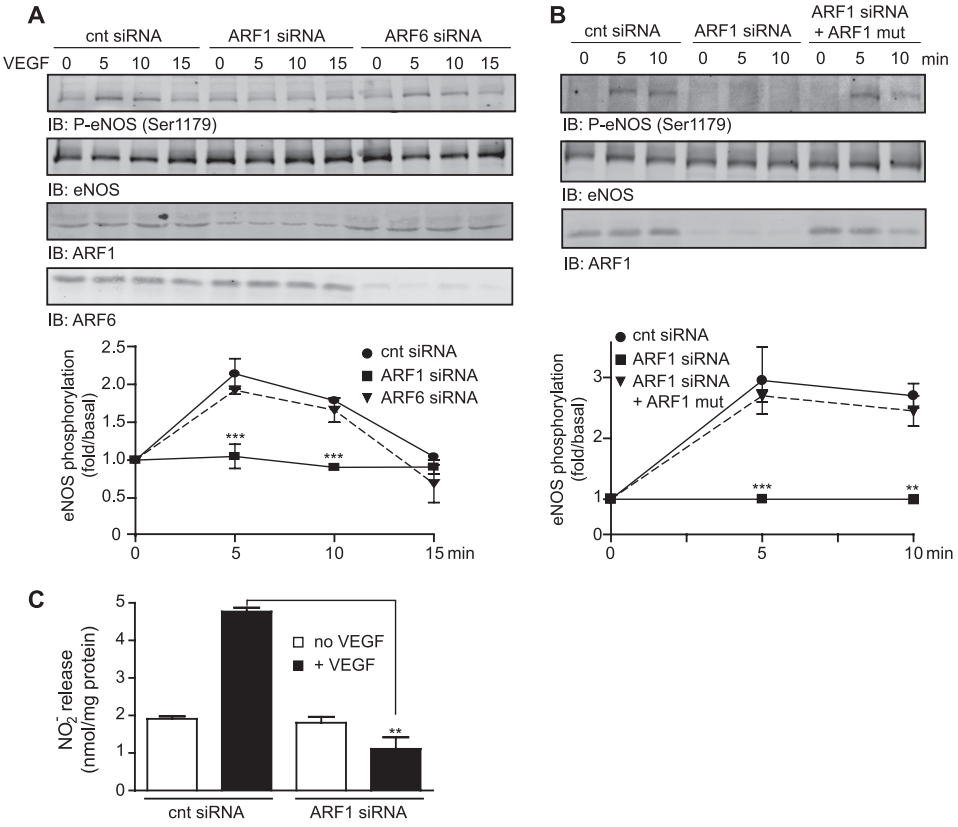

FIGURE 4. **ARF1 modulates the activation of eNOS and the production of NO following VEGF stimulation in BAEC.** *A*, BAEC transfected with a control, ARF1, or ARF6 siRNA were serum-starved and stimulated with VEGF (50 ng/ml) for the indicated times. Phosphorylation of eNOS was examined by Western blotting using a phospho-specific antibody (Ser^{1179}). Levels of eNOS, ARF1, and ARF6 present in each sample also were assessed. Quantifications are presented as fold over basal and are the mean ± S.E. of four independent experiments. ***, $p < 0.001$ when values are compared with the control siRNA-transfected condition. *B*, BAEC transfected with control siRNA, ARF1 siRNA, or ARF1 siRNA together with siRNA-insensitive ARF1 mutant (*ARF1 mut*) were serum-starved and stimulated with VEGF for the indicated times. Phosphorylation of eNOS, was determined by immunoblotting (*IB*). Levels of eNOS and ARF1 present in each sample also were assessed. Quantifications are presented as fold over basal and are the mean ± S.E. of three experiments. **, $p < 0.01$ and ***, $p < 0.001$ when values are compared with the control siRNA-transfected condition. *C*, BAEC transfected with a scrambled (control; *cnt*) or an ARF1 siRNA were serum-starved and stimulated with VEGF for 30 min. Samples of culture medium were taken and subjected to nitrite quantification as described under "Experimental Procedures." Data are the mean ± S.E. of five independent experiments performed in duplicate. **, $p < 0.01$ when values are compared with the control siRNA-transfected condition.

release increases permeability of the monolayer (33). In our experiments, this process was measured by quantifying the movement of fluorescently labeled dextran across confluent endothelial cell cultures. VEGF stimulation of cells transfected with a scrambled (control) siRNA resulted in increased permeability (Fig. 6*A*). Depletion of ARF1 markedly inhibited this cellular response. These results further support a role for ARF1 in NO release and associated biological responses.

Because endothelial cell permeability and activation of eNOS are essential events regulating angiogenesis, we next examined whether ARF1 was required for capillary tube formation. BAEC transfected with either a scrambled or ARF1 siRNA were seeded onto a fibrin matrix, and tube formation was assessed after 8 h. As illustrated in Fig. 6*B*, control cells were able to form capillary-like structures. In contrast, ARF1-depleted cells failed to form tubes. Relative tube length was reduced by 54% when expression of the GTPase was knocked down (Fig. 6*B*).

DISCUSSION

In endothelial cells, binding of VEGF to its receptor results in the autophosphorylation of several tyrosine residues and activation of multiple signaling pathways. Here, we show that VEGF stimulation promotes the rapid and transient activation of ARF1, a member of the ARF family of proteins. Our findings demonstrate that in endothelial cells, ARF1 GTPase coordinates VEGF-induced eNOS activation and NO release through the activation of the PI3K/Akt pathway. We present evidence that VEGFR-2 phosphorylation on tyrosine 801 leads to the rapid activation of ARF1 and downstream signaling cascades.

VEGF was shown previously to promote ARF6 activation, another member of the ARF family of proteins (31, 34). This GTPase can regulate Rac activity, thereby controlling endothelial cell migration and angiogenesis (31, 34). Others and we have recently shown that ARF1, known for its role in coordinating the recruitment of coat protein complexes on endomembranes to form transport vesicles, can be present on dynamic plasma membrane ruffles (23, 26) and control growth factor receptor signaling (23). Using the RNA interference approach, we demonstrate that the presence of this GTPase is necessary for VEGF-dependent activation of the PI3K pathway. In contrast, we show that activation of ARF6 regulates signaling to the Erk pathway. Similar to what we have observed in other endothelial cell types such as EaHY926 and human umbilical vein endothelial cells, depletion of ARF6 enhances basal Erk phosphorylation levels by altering focal adhesion complexes, namely focal adhesion kinase and Src activity (32). The molecular mechanisms by which ARF1 regulates PI3K/Akt activation in endothelial cells appears to be similar to what we have reported in breast cancer cells (23). Depletion of ARF1 resulted in the inability of the catalytic subunit of the PI3K to generate phosphatidylinositol 3,4,5-triphosphate and activate Akt. We have shown that ARF1 in breast cancer cells can interact directly with p85α providing a mean by which this molecular switch might regulate the localization and therefore the function of this enzyme (23).

One important physiological responses associated with VEGF stimulation is the secretion of NO, and generation of this signaling molecule has been associated with the activation of PLCγ (35) and PI3K/Akt (17, 18). *In vivo*, NO modulates VEGF-

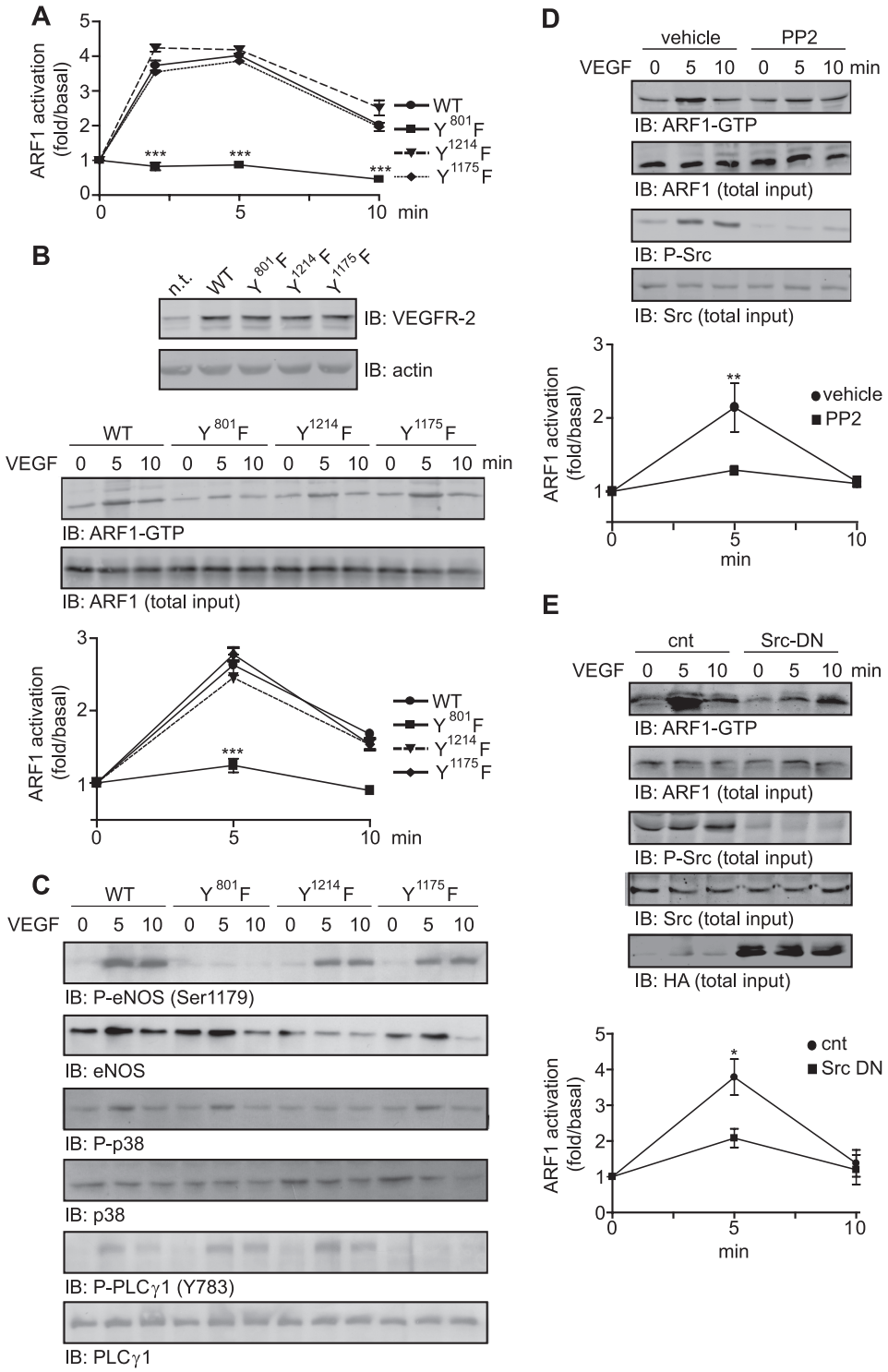

FIGURE 5. **Tyrosine 801 on the VEGFR-2 is a mediator of ARF1 activation.** *A*, COS-7 cells were transfected with expression vectors coding for the WT or mutant VEGFR-2, as indicated. 48 h following transfection, serum-starved cells were stimulated with VEGF for the indicated times, and activated ARF1 was captured using GST-GGA3 as described under "Experimental Procedures." Levels of GTP-bound ARF1 were assessed by Western blotting and quantifications are the mean ± S.E. of three independent experiments. *Inputs* represent 5% of the sample. ***, $p < 0.001$ when values are compared with the paired control (overexpressed WT VEGFR-2) condition. *B*, BAEC were transfected as in *A*. Expression levels of endogenous and overexpressed VEGFR-2 are depicted. This blot is representative of three other experiments. Cells were stimulated with VEGF for the indicated times, and activated ARF1 was detected by Western blot analysis. Quantifications are the mean ± S.E. of four independent experiments. ***, $p < 0.001$ when values are compared with the paired control (overexpressed WT VEGFR-2) condition. *C*, BAEC-transfected cells were stimulated with VEGF as in *A* and *B*, and activation of eNOS, p83, and PLCγ was determined by immunoblotting (*IB*) cell lysates with the corresponding phospho-specific antibodies. Total levels of proteins were also assessed. *D*, BAEC were pretreated with vehicle or PP2 (10 μM) for 30 min. Cells were then stimulated with VEGF for the indicated times, and activated ARF1 was detected by Western blot analysis. Quantifications are the mean ± S.E. of four independent experiments. **, $p < 0.001$ when values are compared with the paired control condition. *E*, control and SrcK298M-HA expressing cells were stimulated with VEGF for the indicated times, and activation of ARF1 was assessed as in *B* and *D*. Quantifications are the mean ± S.E. of four independent experiments. *, $p < 0.001$ when values are compared with the paired control condition. *DN*, dominant-negative; *cnt*, control. *n.t.*, non-transfected.

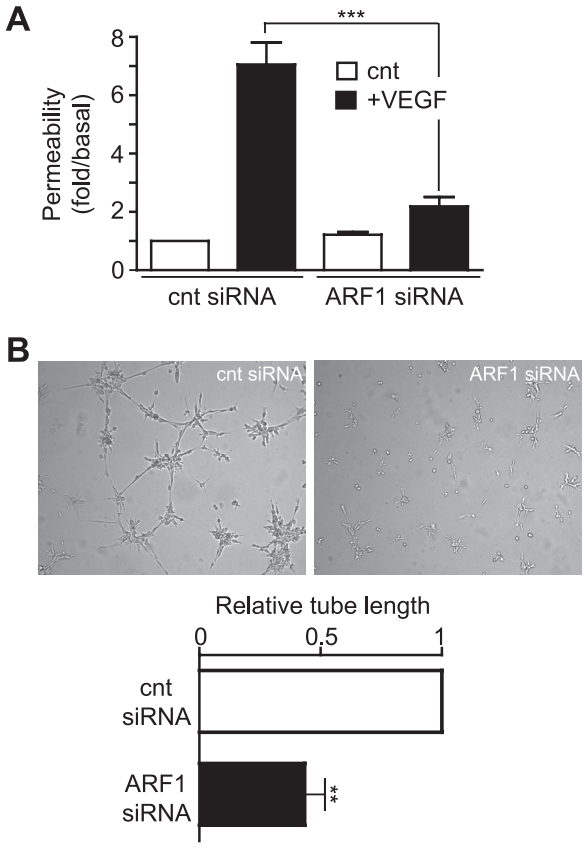

FIGURE 6. **ARF1 is essential for endothelial monolayer permeability and angiogenesis.** *A*, permeability to FITC-dextran was determined in confluent BAEC monolayers transfected with a scrambled or an ARF1 siRNA. Cells were stimulated or not with VEGF for 30 min. Data represent permeability to FITC-dextran expressed as the mean fold increase over basal compared with scrambled unstimulated cells. *B*, the ability of control (*cnt*) and ARF1-depleted BAEC to form capillary-like structures on Matrigel was assessed as described under "Experimental Procedures." *Images*, are representative of three independent experiments. Quantification of relative tube length formed by BAEC for each condition was performed. Data are expressed as mean ± S.E. of three independent experiments. **, $p < 0.001$ when compared with the control condition.

induced angiogenesis and vascular permeability (36). Others and we have previously reported that phosphorylation of tyrosine 801, on the VEGFR-2, is involved in PI3K activation (5, 37). Akt, the major downstream effector of this signaling pathway, is the best documented kinase regulating the activation of eNOS. Phosphorylation of this enzyme at serine 1179/1177 leads to activation (15) and can be catalyzed by at least five kinases (38): Akt (17, 39, 40), protein kinase A (41), protein kinase G (42), AMP-activated protein kinase (43), and CaM kinase II (44). Inhibition of Akt by the pharmacological agents wortmannin or LY-294002, inhibits the activation of eNOS and the release of NO by VEGF in endothelial cells (45, 46). In this study, we have shown that mutation of tyrosine 801 on the VEGFR-2 completely abolishes the ability of the receptor to activate ARF1, further supporting the importance of this residue in activating the PI3K/Akt/eNOS/NO signaling axis. Furthermore, we present data supporting a role for Src. We and others have previously reported that this non-receptor tyrosine kinase was an important regulator of VEGF-dependent NO release as well as for modulation of permeability of endothelial cells (35, 47, 48). The mechanisms by which Src modulates VEGFR-2 signaling remain somewhat elusive. However, many evidences point toward a role upstream of PI3K/Akt activation (49–51). This is consistent with our present findings that delineate Src kinase activity as being necessary for ARF1 activation by VEGFR-2 and for Akt-dependent eNOS phosphorylation.

Activation of ARF6 by VEGFR-2 was recently reported to require ARNO (ARF-nucleotide-binding site opener), an ARF guanine nucleotide exchange factor (34). Alternatively, activation of this ARF isoform by the EGFR required a direct interaction between the receptor and GEP100/BRAG2 in breast cancer cells (52). Tyrosine 801-phosphorylated VEGFR-2 may promote the recruitment of an ARF guanine nucleotide exchange factor to the plasma membrane, by a mechanism dependent on the activation of Src, which would act to specifically promote GTP-binding on ARF1. In our experiments, mutation of tyrosine 1175 on VEGFR-2 was effective in blocking phosphorylation of PLCγ but did not have any effect on ARF1 activation suggesting that phosphorylation of tyrosine 801 only, might regulate the ARF1/Akt/eNOS/NO signaling axis. Phosphorylation of both tyrosine 801 and 1214 on VEGFR-2 was shown to be important for the phosphorylation of Gab1, a scaffolding adaptor protein, and activation of Akt, Erk1/2, and Src (53, 54). In our experiments, mutation of tyrosine 801 significantly impaired ARF1 and eNOS activation. Taken together, these data suggest that although phosphorylation of other tyrosine residues might regulate activation of the PI3K pathway or NO release, tyrosine 801 phosphorylation is specifically required for ARF1-dependent signaling.

It is generally accepted that release of NO, from endothelial cells, leads to important physiological consequences. First, VEGF stimulation enhanced permeability of the endothelial monolayer. This response was previously shown to involve eNOS activation and NO release (36). As expected, depletion of ARF1 markedly reduced the ability of VEGF to increase permeability of cells grown to confluence without affecting basal levels. In addition, cells transfected with the ARF1 siRNA were found to be impaired in their ability to form capillary tubules, an *in vitro* angiogenesis assay. Our assay was performed on a three-dimensional matrix containing a variety of growth factors acting complementary to promote this complex cellular response. Our observation that ARF1 depletion inhibits capillary tube formation suggests that this GTPase may regulate the function of other factors important for this response. In a previous study, we reported that depletion of ARF6 also resulted in diminished relative tube length (32), further highlighting the role of GTPases in this complex cellular response.

In summary, our findings demonstrate the importance of ARF1 in mediating VEGFR-2-dependent activation of the PI3K/Akt pathway leading to eNOS activation and NO release. Together with our previous work, these data suggest that ARF1 might be a new pharmacological target to treat diseases associated with impaired NO generation and characterized by pathological angiogenesis and vascular leak.

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