The Role of Phosphatidylinositol 3-Kinase in Vascular Endothelial Growth Factor Signaling*

(Received for publication, August 10, 1998, and in revised form, January 11, 1999)

Geeta D. Thakker§, David P. Hajjar¶, William A. Muller§, and Todd K. Rosengart†¶

From the Departments of §Cardiothoracic Surgery and ¶Pathology, Weill Medical College of Cornell University, New York, New York 10021

Vascular endothelial growth factor (VEGF) receptor Flk-1/KDR in endothelial cells is activated during vasculogenesis and angiogenesis upon ligand-receptor interaction. Activated Flk-1/KDR has been shown to recruit Src homology 2 domain-containing signaling molecules that are known to serve as links to the activation of the mitogen-activated protein (MAP) kinase signaling pathway. To define the functional significance of phosphatidylinositol (PI) 3-kinase in VEGF signaling, we have examined its role in human umbilical vein endothelial cell (HUVEC) cycle progression. We show herein that p85, the regulatory subunit of PI-3-kinase, is constitutively associated with Flk-1/KDR. The treatment of HUVECs with VEGF promoted tyrosine autophosphorylation of Flk-1/KDR and also induced phosphorylation of p85. This was followed by an increase in the PI-3-kinase activity, which was sensitive to wortmannin, a potent PI-3-kinase inhibitor. VEGF also induced a striking activation of MAP kinase in a time-dependent manner. Inhibition studies with both a dominant-negative p85 mutant and the PI-3-kinase inhibitor, wortmannin, were employed to show for the first time that VEGF-stimulated PI-3-kinase modulates MAP kinase activation and nuclear events such as transcription from c-fos promoter and entry into the synthesis (S)-phase. Our data demonstrate the importance of PI-3-kinase as a necessary signaling component of VEGF-mediated cell cycle progression.

Vascular endothelial growth factor (VEGF)§ is a secreted glycoprotein specific for endothelial cells (1–3). VEGF is angiogenic in vivo (4, 5) and in vitro (6); its importance in vasculogenesis and angiogenesis has been established through gene deletion studies (7, 8). Flk-1/KDR and Flt-1 are the two receptor tyrosine kinases that regulate the actions of VEGF and are expressed in endothelial cells (9–13), while the related receptor, Flt-4, is found on lymphatic endothelium. The expression pattern of Flt-4 suggests it may play a role during lymphangiogenesis (14).

All of the three VEGF receptors belong to the PDGFR-β family of receptor tyrosine kinases (15). Upon activation, these receptors dimerize and/or oligomerize, following which autophosphorylation and transphosphorylation of their tyrosine residues in the intracellular domain occurs. There are four putative tyrosine phosphorylation sites (Tyr-951, Tyr-996, Tyr-1054, and Tyr-1059) in the KDR intracellular domain (16). These phosphorylated tyrosine molecules act as docking sites for adaptor signaling molecules and non-receptor tyrosine kinases, thereby generating a signal cascade that culminates in a cellular response. The signal transduction pathways involved in mediating the various biological functions of VEGF on endothelial cells such as migration, proliferation, differentiation, or survival remain to be completely defined.

The Ras-MAP (mitogen-activated protein) kinase pathway is a key component in the transduction of signals leading to growth and transformation. It consists of a linear cascade of protein kinases, Raf, MAP kinase kinase, and MAP kinase, which are also called extracellular-regulated kinases (Erks). Erk-1 and Erk-2 are acutely activated upon growth factor stimulation (17).

Phosphatidylinositol (PI) 3-kinase, a heterodimer of an 85-kDa (p85) adaptor subunit and a 100-kDa (p110) catalytic subunit (18–21), is activated by most growth factors and has been implicated as a critical factor in the control of cell proliferation and cell survival. PI-3-kinase phosphorylates the D-3 position of the inositol ring of phosphoinositides, which in turn act as second messengers. The p85 subunit contains two Src homology 2 (SH2) domains, which bind to tyrosine-phosphorylated receptors after stimulation of cells with growth factors and in this manner recruit p110 into the complex at the cell membrane. The region between the two SH2 domains, the iSH2 region, mediates the association with p110, and this interaction is required for the enzymatic activity of p110 (22). Phosphorylation of the p85 subunit of PI-3-kinase upon VEGF stimulation (23) is suggestive of a potential role for PI-3-kinase in VEGF-mediated signaling. Given this observation, we hypothesized that PI-3-kinase might play a critical role in VEGF signaling, including the Ras-MAP kinase pathway. In this report, we demonstrate for the first time the functional significance of PI-3-kinase in VEGF signaling from Flk-1/KDR leading to MAP kinase activation, followed by transcriptional activation of the c-Fos serum response element that eventually culminates in endothelial cell proliferation.

EXPERIMENTAL PROCEDURES

Cell Culture—Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and were passaged in...
medium M199 (Life Technologies, Inc.) containing 20% fetal bovine serum, 4 mM L-glutamine, penicillin, streptomycin, and 2 ng/ml basic fibroblast growth factor on 80-mm diameter dishes coated with 0.2% gelatin (Sigma). HUVECs were not used after the sixth passage. For experimental purposes, HUVECs were plated on 0.2% gelatin-coated dishes and allowed to form a monolayer. Cultures were treated with unsupplemented medium M199 containing 0.5% fetal bovine serum for 16–18 h. Serum-starved cultures were left untreated or treated with VEGF at the indicated concentrations and time in 5.0 ml of medium M199 at 37 °C and harvested in appropriate lysis buffer at 4 °C.

Materials—Anti-Flik-1, Fli-1, and Erk-2 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-Erk-1/2 polyclonal antibodies were from New England Biolabs, and anti-p85 and anti-phosphotyrosine antibodies (4G10) were from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-hemagglutinin (HA) antibody (12C5A), β-galactosidase and luciferase assay kits, and cell proliferation assay kits containing BrdUrd and anti-BrdUrd antibody were purchased from Boehringer Mannheim. Wortmannin was purchased from Sigma, and PD98059 was bought from Calbiochem. PI 3-kinase substrate, phosphatidylinositol (sodium salt), was bought from Avanti Polar Lipids, Inc. (Alabaster, AL). The ECL reagent was obtained from Amersham Pharmacia Biotech. VEGF 165 was procured from R&D (Minneapolis, MN). Wild type Flik-1 cDNA construct in CMV promoter was provided from Dr. Axel Ullrich (Max-Planck-Institut für Biochemie, München, Germany). Hemagglutinin (HA)-tagged Erk-2 (pCMV-HA-Erk2) in pCMV, SRE-Fos-luciferase (SRE-Fos-Luc), and pCMV-β-galactosidase plasmids were from Dr. Joseph Schlessinger (New York University Medical Center, New York, NY). Dominant-negative (DN) p85 mutant plasmid pSG5 p85ΔSH2-C was from Dr. Julian Doward (Imperial Cancer Research Fund, London, UK), and DN Ras (Ras N17) was from Dr. E. Skolnik (New York University Medical Center, New York, NY).

Treatment with Wortmannin or PD98059—Stock solutions of wortmannin or PD98059 in MeSO were kept at −20 °C. Stocks were diluted in serum-free medium prior to use. Wortmannin, PD98059, or the MeSO carrier control was added to the cells for 15–20 min before the addition of growth factor.

Protein Estimation—Protein was estimated using the BCA protein assay reagent (Pierce) as described by the manufacturer.

Transfections—HUVECs were transiently transfected with 5 μg of SRE-Fos-Luc and 1.0 μg of pCMV-β-galactosidase plasmids using Superfect according to the manufacturer’s instruction (Qiagen). 24 or 48 h later, cultures were serum-starved as indicated earlier and treated as mentioned in the legends. Cells were harvested in appropriate lysis buffer.

For dominant negative studies, HUVECs were transiently transfected with 2.0 μg of HA-Erk-2, 1.0 μg of CMV-β-galactosidase, and increasing concentrations of DN p85 plasmid. The concentration of DNA per transfection was kept constant by supplementing with the vector alone.

For co-immunoprecipitation of p85 with Flik-1, HUVECs were transiently transfected with 10 μg of Flik-1 plasmid.

For studies of cell cycle progression with DN p85, cells were transiently transfected with 2 μg of CMV-β-gal and increasing concentrations of either DN p85 or DN Ras plasmids.

Immunoprecipitations and Immunoblotting—HUVECs were lysed in cell extraction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na3VO4, 1% Nonidet P-40, and protease inhibitors). Equal amount of lysate was resolved in 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, the blots were immunoblotted with anti-phospho-specific Erk-1/2 antibodies that recognize phosphorylation of Thr202 and Tyr204. For inhibition studies, serum-starved cells were preincubated with the indicated concentrations of wortmannin or PD98059 for 15 min prior to VEGF treatment.

For assays of transfected HA-Erk-2, affinity-purified anti-HA monoclonal antibody was used for immunoprecipitations (IP). The amount of cell lysate used in the IP was normalized for β-galactosidase activity levels to account for transfection efficiencies. Erk-2 immune complex assays were initiated by adding 30 μl of kinase assay buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 5 μM ATP, and 400 μM adenosine) containing 0.2 mg/ml phosphatidylinositol and 10 μCi of [γ-32P]ATP (4000 Ci/mmol; Amersham Pharmacia Biotech) and 0.5 μg of antibody and incubated for 15 min at room temperature. Kinase complexes were resolved in an 8% SDS-PAGE. Gels were treated with KOH for 2 h at 60 °C to remove serine and threonine phosphorylation. Gels were fixed, dried, and autoradiographed.

In Vitro PI 3-Kinase Assay—PI 3-kinase activity was assayed essentially as described by Krook et al. (24). Serum-starved HUVECs were transfected with pEGF for 5 min. Anti-phosphotyrosine antibody was used for immunoprecipitation at a concentration of 4 μg/ml of lysate. PI 3-kinase assay was carried out in kinase assay buffer (30 mM HEPES, pH 7.4, 30 mM MgCl2, 50 μM ATP, and 400 μM adenosine) containing 0.2 mg/ml phosphatidylinositol and 10 μCi of [γ-32P]ATP for 15 min at 25 °C. Reactions were terminated by the addition of 100 μl of 1 M HCl. The reaction products were extracted with 200 μl of CHCl3/methanol (1:1). The chloroform phase was collected, separated by thin layer chromatography, autoradiographed, and quantified using a phosphorimager.

MAP (Erk1/2) Kinase Assay—Cells were washed twice with ice-cold phosphate-buffered saline and extracted in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na3VO4, 1% Nonidet P-40, and protease inhibitors). Equal amount of lysate was resolved in 10% SDS-PAGE. Following transfer to nitrocellulose membrane, the blots were immunoblotted with anti-phosphospecific Erk-1/2 antibodies that recognize phosphorylation of Thr202 and Tyr204. For inhibition studies, serum-starved cells were preincubated with the indicated concentrations of wortmannin or PD98059 for 15 min prior to VEGF treatment.

For assays of transfected HA-Erk-2, affinity-purified anti-HA monoclonal antibody was used for immunoprecipitations (IP). The amount of cell lysate used in the IP was normalized for β-galactosidase activity levels to account for transfection efficiencies. Erk-2 immune complex assays were initiated by adding 30 μl of kinase assay buffer (50 mM Tris, pH 7.5, 10 mM MgCl2) containing 5 μCi of [γ-32P]ATP (>4000 Ci/mmol) and 2.5 μg of bovine brain-derived myelin basic protein (MBP). The kinase complex was resolved in 14% SDS-PAGE and analyzed for autoradiography. MBP runs as an 18-kDa protein.

SRE-Fos-Luc Assay—HUVECs transiently transfected with SRE-Fos-Luc and CMV-β-galactosidase plasmids were treated with wortmannin at the concentration mentioned in the figure legend and stimulated with VEGF. Cultures were harvested 6 h later in reporter lysis buffer, and analyzed for luciferase and β-galactosidase activities.

Cell Proliferation Assay—HUVECs were plated on gelatin-coated coverslips in 12-well plates at a density of 5 × 104 cells, allowed to adhere for 12 h, and serum-starved for 24 h. After 12 h of incubation in serum-free medium M199 supplemented with 10 ng/ml VEGF containing 10 μg 5-bromodeoxyuridine (BrdUrd), cells were fixed and stained with anti-BrdUrd monoclonal antibody and alkaline phosphatase-conjugated anti-mouse immunoglobulin.

For DN studies, cells transiently transfected with CMV-β-gal and DN p85 or DN Ras were serum-starved for 36 h and then incubated in medium M199 containing 10 μM BrdUrd and 10 ng/ml VEGF for 10 h. Cells were fixed and stained with X-gal, followed by anti-BrdUrd as described above. The percentage of X-gal-positive cells that had incorporated BrdUrd was evaluated using a light microscope.

Statistical Analysis—Statistical analysis was carried out by Student’s t test. Results are expressed as mean ± standard deviation of the mean.

RESULTS

p85 Constitutively Associates with Flik-1/KDR and Undergoes Tyrosine Phosphorylation in VEGF-treated Endothelial Cells—In order to investigate the mitogenic response to VEGF in a physiologically relevant model, we chose to study the regulation in HUVECs rather than cell lines. Unfortunately, available antibodies did not immunoprecipitate KDR from HUVECs, so we transfected these cells with a cDNA encoding Flik-1, the murine homologue of KDR. Transfection efficiencies in HUVECs varied from 20% to 40%, and transfection of HUVECs did not change their gross morphology. We demonstrate the co-immunoprecipitation of Flik-1/KDR with p85 from VEGF unstimulated and stimulated human endothelial cells following immunoprecipitation with anti-p85 antibodies (Fig. 1A). In
PI 3-Kinase in VEGF Signaling

contrast to these findings, the anti-Flk-1 antibodies did not immunoprecipitate p85 (Fig. 1B). We conclude from these results that p85 can directly and constitutively associate with Flk-1/KDR. p85 has been shown to associate with Flt-1 in a yeast two-hybrid system (25). We therefore looked for Flt-1 association with p85 and Flk-1/KDR in the above immunoprecipitates by immunoblotting with Flt-1 specific antibodies. Under our experimental conditions, we could not detect Flt-1 in either p85 or Flk-1 immunoprecipitates (data not shown).

We next confirmed previous observations that KDR, which has intrinsic kinase activity, was tyrosine-phosphorylated as a result of VEGF treatment (Fig. 2A). We found that, in addition to KDR, two other proteins migrating with approximate molecular masses of 46 and 70 kDa were also tyrosine-phosphorylated. Concomitant phosphorylation of the p85 subunit of PI 3-kinase in response to VEGF stimulation was demonstrated by in vitro kinase assay following immunoprecipitation with a specific anti-p85 antibody (Fig. 2B).

VEGF Promotes PI 3-Kinase Activation—We sought to examine whether p85 phosphorylation in response to VEGF treatment was able to regulate the catalytic counterpart of the PI 3-kinase enzyme, p110. We found VEGF treatment resulted in about 80 ± 25% increase in PI 3-kinase activity when examined by an in vitro kinase assay using phosphatidylinositol as a substrate (Fig. 3, A and B). This increase in PI 3-kinase activity could be blocked when endothelial cells were preincubated with wortmannin (10 and 100 nM), a fungal metabolite that is a potent inhibitor of PI 3-kinase (p < 0.01 versus VEGF-stimulated). Wortmannin also significantly inhibited the unstimulated background PI 3-kinase at 100 nM concentration (p < 0.001 versus control).

Wortmannin and DN PI 3-Kinase Inhibit VEGF-stimulated MAP Kinase Activation—MAP kinase plays a central role in controlling signals for growth from most growth factor receptor tyrosine kinases. We looked at the kinetics of MAP kinase activation in VEGF-stimulated endothelial cells and found a time-dependent response, which was maximum at 10 min (data not shown).

To assess the role of PI 3-kinase in MAP kinase activation by VEGF, cells were preincubated with different concentrations of wortmannin prior to VEGF stimulation. Interestingly, wortmannin in a dose-dependent manner inhibited VEGF-induced Erk1/2 activation (Fig. 4A), indicating that PI 3-kinase is involved in MAP kinase activation by VEGF. About 70% inhibition of MAP kinase activation was observed with 100 nM wortmannin (concentration relevant to PI 3-kinase inhibition). The MAP kinase inhibitor PD98059, was used a positive control (Fig. 4A). The blot was stripped and reprobed with Erk-2 antibodies to confirm equal loading of protein (Fig. 4B).

To confirm that the PI 3-kinase pathway was responsible for activating MAP kinase in response to VEGF, cells were transfected with HA epitope-tagged Erk-2 and increasing concentrations of a DN form of p85 (p85ΔSH2-C). This DN p85 lacks the amino acids 475–523 in the SH2 domain and therefore does not bind p110, the catalytic subunit of PI 3-kinase (26). Co-expression of p85 with HA-tagged Erk-2 was necessary to facilitate the evaluation of dominant negative effects in only the transfected cells. As expected, stimulation with VEGF caused an activation of the tagged Erk-2. Importantly, overexpression of a DN p85 mutant inhibited VEGF-induced HA-Erk-2 activation in a dose-dependent manner. Expression of HA-Erk-2 and DN p85 at a 1:5 ratio suppressed Erk-2 activation almost 70% (Fig. 5).

VEGF Promotes PI 3-Kinase Activity—To examine the biological effects of VEGF-activated PI 3-kinase signaling events further downstream, experiments were carried out to determine BrdUrd incorporation as a measure of VEGF-induced growth. Following VEGF treatment, 65 ± 4.6% of the serum starved HUVECs entered into the S-phase as detected by staining with anti-BrdUrd antibody. Wortmannin blocked VEGF-induced BrdUrd incorporation by approximately 40% at 10 nM and about 50% at 100 nM concentration (Fig. 7A). Nonspecific effects of wortmannin were eliminated by studies of cell cycle progression with DN p85. Interestingly, DN p85 inhibited in a dose-dependent manner entry of the cells into S-phase (Fig. 7B). DN Ras was used as positive control. Both DN p85 and DN Ras failed to block cells entering into the

![Figure 1](image1.png)

**Fig. 1.** Association of p85 with Flk-1/KDR in HUVECs. Serum-starved HUVECs were either left untreated (−) or treated (+) with 10 ng/ml VEGF for 5 min; extracts were immunoprecipitated with either anti-p85 (A) or anti-Flk-1 (B) polyclonal antibodies, followed by immunoblotting with antibodies to Flk-1 and p85. Immunoprecipitation of Flk-1 with p85 is demonstrated.

![Figure 2](image2.png)

**Fig. 2.** Tyrosine phosphorylation of Flk-1/KDR and p85 in HUVECs in response to VEGF stimulation. Serum-starved HUVECs were either left unstimulated (−) or stimulated (+) with VEGF (10 ng/ml for 5 min) and lysed in modified RIPA buffer. ~800 µg of total protein was immunoprecipitated with Flk-1 polyclonal antibodies (A) or with p85 polyclonal antibodies (B). Immunocomplex kinase assays were performed using [γ-32P]ATP, and complexes were resolved in an 8% SDS-PAGE. Tyrosine phosphorylation of KDR and p85 is indicated.
S-phase of the cell cycle in media containing 20% serum (data not shown).

**DISCUSSION**

To further our knowledge of the signal transduction pathways from VEGF receptor, we have analyzed the functional importance of PI 3-kinase in VEGF signaling from Flk-1/KDR. It has been shown that p85, the adaptor subunit of PI 3-kinase, is capable of associating with the VEGF receptor (23, 25). The functional significance of PI 3-kinase activation in VEGF signaling has, however, not been defined clearly. In our study, we show that phosphorylation of p85 in response to VEGF regulates its catalytic counterpart, i.e., p110, and furthermore demonstrate that PI 3-kinase activation contributes to MAP kinase activation, transcription of c-Fos SRE, and cell cycle progression in human endothelial cells.

Several reports have shown that activated Flk-1/KDR receptors will associate with signaling intermediates such as phospholipase C-γ, GTPase-activating protein, Nck-Grb2, and Shc-Grb2 (23, 28–30). These interactions result in tyrosine phosphorylation of some of the potential substrates. The Ras-MAP kinase pathway has been reported to signal for VEGF-induced growth, as has the PKC-MAP kinase pathway (30, 31).

Our results show for the first time that PI 3-kinase not only gets phosphorylated upon VEGF stimulation but also contributes to cell cycle progression following MAP kinase activation.

One of the early events observed upon interaction of VEGF with its receptor Flk-1/KDR is activation of the receptor’s intrinsic tyrosine kinase activity. In agreement with previous studies (28, 30), Flk-1/KDR was strongly phosphorylated in response to VEGF in our study. Two other proteins phosphorylated and recruited to the activated receptor had approximate molecular masses of 46/47 and 67/70 kDa. The 46/47-kDa...
protein (p46/47) phosphorylated in our study could either be p46Shc or p47Nck, both of which are known to bind to Flk-1/KDR (30) as well as to other receptor tyrosine kinases like PDGFR and epidermal growth factor receptor (32–34). Although we did not characterize the identity of these proteins, we subscribed to the idea that these were SH2 domain-containing tyrosine-phosphorylated proteins, which potentially couple to the activation of Ras-MAP kinase pathway (35).

We also show that Flk-1/KDR constitutively associates with p85 as this association was independent of VEGF stimulation. Flk-1/KDR has a candidate motif YYXM in its intracellular region which is a potential site for p85 to bind. It should be noted here that p85 contains two SH2 domain and a SH3 domain, the SH2 domain of p85 is responsible for interaction with phosphorylated tyrosine in the context of above motif. However, mutational analysis of tyrosine residues in Flk-1/KDR would be required to verify this notion. Flt-1, the other receptor for VEGF, has also been shown to associate with p85 in a two-hybrid system, the binding site for which has been identified as a YVNA motif (25). However, Flt-1 does not apparently signal for proliferation, which has been underscored by knockout studies wherein only the tyrosine kinase domain of Flt-1 has been deleted (36). Flt-1 tyrosine kinase homozygous mice (Flt-1TK−/−) develop normal blood vessels and survive, which suggests that Flt-1 tyrosine kinase domain does not signal for migration, proliferation, and differentiation, essential features of endothelial cells during vasculogenesis. Although activation of the kinase counterpart of PI 3-kinase may or may not require p85 to be phosphorylated (37), we found that VEGF treatment promoted p85 phosphorylation that in turn increased PI 3-kinase activity.

In accordance with published literature (30, 38, 39), we have also observed MAP kinase activation in endothelial cells upon VEGF stimulation. We demonstrate for the first time in our study a link between PI 3-kinase and the downstream activation of MAP kinase in VEGF stimulated cells by 1) significantly inhibiting MAP kinase activation with wortmannin, a potent PI 3-kinase inhibitor; and 2) blocking MAP kinase activation with a dominant negative p85 mutant. These observations suggest that the lipid products generated by the PI (3) kinase

![Graph](image1.png)

**Fig. 6.** Inhibition of VEGF-induced c-Fos SRE-dependent transcription by wortmannin. HUVECs were transiently transfected with 5 μg of SRE-c-Fos-Luc plasmid and 1 μg of pCMV-β-gal. 24 h after transfection, cells were serum-starved for 16 h, pretreated with indicated concentrations of wortmannin, and stimulated with VEGF for 6 h. Cells extracts were assayed for luciferase and β-galactosidase activity. The data presented are the amount of SRE-Luc activity divided by the β-galactosidase activity present in the cell extracts. Data shown are the mean ± standard deviation of three samples. This is a representative experiment performed independently three times. *, standard deviation was less than 1.

![Graph](image2.png)

**Fig. 7.** Effects of wortmannin and dominant-negative p85 on VEGF-induced cell cycle progression. A. HUVECs were plated onto wells coated with 0.2% gelatin, allowed to adhere for 12 h, and then serum-starved overnight. Cells were either left untreated (-) or treated with wortmannin at the indicated concentration for 20 min, and then stimulated with 10 ng/ml VEGF in the presence of 10 μM BrdUrd in serum-free medium M199. Cells were fixed 12 h later and stained for X-gal followed by anti-BrdUrd. Values represent mean ± standard deviation of four samples. *, p < 0.02 versus VEGF-stimulated; @, p < 0.003 versus VEGF-stimulated.

![Graph](image3.png)

**Fig. 7.** Effects of wortmannin and dominant-negative p85 on VEGF-induced cell cycle progression. B. HUVECs were transiently transfected with 2 μg of CMV-β-gal plasmid; 5 and 10 μg of DN p85 plasmid (p85ΔSH2-C) or 3 and 6 μg of DN Ras (N17 Ras). 12 h after transfection, cells were serum-starved for 36 h and stimulated with 10 ng/ml VEGF in M199 containing 10 μM BrdUrd. Cells were fixed 10 h later and stained for X-gal followed by anti-BrdUrd. Values represent mean ± standard error of four samples. *, p < 0.02 versus VEGF-stimulated; @, p < 0.003 versus VEGF-stimulated.
following VEGF stimulation may bind to a signaling protein which could activate MAP kinase. Candidate proteins could be pleckstrin homology domain-containing proteins that require PI 3-kinase products to be fully activated (35, 40).

Finally, we demonstrate for the first time the critical role of PI 3-kinase activation in generating a maximal mitogenic response to VEGF. These observations are consistent with previous evidence supporting a role for PI 3-kinase in PDGF-induced and granulocyte-macrophage colony-stimulating factor-induced mitogenic signaling in smooth muscle cells and macrophages, respectively (41, 42). PI 3-kinase activation has been linked to a number of biologically diverse processes such as cell survival, membrane trafficking, and insulin-stimulated glucose transport (43–45). Very recently, the constitutively active forms of PI 3-kinase have been ingeniously used to identify and study responses specifically induced by PI 3-kinase, an approach that has shown that PI 3-kinase activation alone is sufficient to promote entry into S phase of the cell cycle (46). The fact that our inhibition studies with either DN p85 or wortmannin did not abolish VEGF-induced MAP kinase activation and mitogenesis suggests the presence of additional PI 3-kinase-independent pathways in VEGF-induced growth promoting effects, such as the PKC-MAP kinase (31) and the Ras-MAP kinase pathway (30). In summary, results of our study identify PI 3-kinase as an important mediator of VEGF-induced MAP kinase activation and subsequent endothelial cell proliferation.

Acknowledgments—We thank Dr. Axel Ullrich, Dr. Joseph Schlessinger, Dr. Julian Downdward, and Dr. E. Skolnik for kindly providing us the cDNA clones used in this study and Dr. Enrique Meiri for useful comments on the manuscript.

REFERENCES

1. Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1306–1309
2. Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. (1989) Science 246, 1309–1312
3. Senger, D., Ferrara, N., and Davis-Smyth, T. (1995) J. Biol. Chem. 270, 6729–6733
4. Grooth, H.-J., Giedlin, M. A., and Williams, L. T. (1998) Mol. Cell. Biol. 18, 2551–2577
5. Kumar, V. A., and Schlessinger, J. (1995) Mol. Cell. Biol. 15, 979–977
6. Tyn, J., Keat, C., and Schlessinger, J. (1992) Mol. Cell. Biol. 12, 115–126