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# Sphingosine Kinase Mediates Vascular Endothelial Growth Factor-Induced Activation of Ras and Mitogen-Activated Protein Kinases

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**Vascular endothelial growth factor (VEGF) signaling is critical to the processes of angiogenesis and tumor growth. Here, evidence is presented for VEGF stimulation of sphingosine kinase (SPK) that affects not only endothelial cell signaling but also tumor cells expressing VEGF receptors. VEGF or phorbol 12-myristate 13-acetate treatment of the T24 bladder tumor cell line resulted in a time- and dose-dependent stimulation of SPK activity. In T24 cells, VEGF treatment reduced cellular sphingosine levels while raising that of sphingosine-1-phosphate. VEGF stimulation of T24 cells caused a slow and sustained accumulation of Ras-GTP and phosphorylated extracellular signal-regulated kinase (phospho-ERK) compared with that after EGF treatment. Small interfering RNA (siRNA) that targets SPK1, but not SPK2, blocks VEGF-induced accumulation of Ras-GTP and phospho-ERK in T24 cells. In contrast to EGF stimulation, VEGF stimulation of ERK1/2 phosphorylation was unaffected by dominant-negative Ras-N17. Raf kinase inhibition blocked both VEGF- and EGF-stimulated accumulation of phospho-ERK1/2. Inhibition of SPK by pharmacological inhibitors, a dominant-negative SPK mutant, or siRNA that targets SPK blocked VEGF, but not EGF, induction of phospho-ERK1/2. We conclude that VEGF induces DNA synthesis in a pathway which sequentially involves protein kinase C (PKC), SPK, Ras, Raf, and ERK1/2. These data highlight a novel mechanism by which SPK mediates signaling from PKC to Ras in a manner independent of Ras-guanine nucleotide exchange factor.**

Activation of Ras proteins is commonly thought to require the activity of guanine nucleotide exchange factors (GEFs) related to the *S. cerevisiae CDC25* gene product (1). However, GEF-independent pathways for Ras activation have been suggested. Downward and coworkers reported that stimulation of the T-cell receptor (TCR) leads to activation of Ras via a mechanism involving protein kinase C (PKC)-dependent downregulation of Ras GTPase-activating protein (Ras-GAP) function. In permeabilized T cells, stimulation of PKC failed to affect the rate at which nucleotides bind to Ras but reduced the rate of GTP hydrolysis on Ras (5). Marais et al. reported that activation of Ras in COS cells by PKC appears to be independent of Ras-GEFs. Stimulation of PKC in COS cells was found to stimulate the formation of a complex of Ras-GTP with the Raf kinase, but activation of Ras was not affected by a dominant-negative Ras mutant (Ras-N17, which sequesters and neutralizes endogenous Ras-GEFs) (17). Taken together, the studies of Downward et al. and Marais et al. suggest that the activation of Ras via PKC occurs via a mechanism that appears not to utilize a Ras-GEF but rather modulates Ras-GAP activity to favor Ras activation. Here, we extend those studies by demonstrating that sphingosine kinase (SPK) links PKC to Ras activation.

In the past decade, sphingosine-1-phosphate (S1P) has received attention because of its role in pathological states such as cancer, angiogenesis, and inflammation. S1P is unusual in that it can function both as an extracellular signaling molecule and as an intracellular second messenger (30). Cell surface receptors for S1P include the EDG family of heterotrimeric G protein-coupled receptors (15, 29). The intracellular targets of sphingosine and S1P as second messengers have remained elusive but affect pathways controlling cell proliferation and cell survival (by opposing the effects of ceramide) (3, 22). A number of growth factors have been shown to activate SPK, which converts sphingosine to S1P, including platelet-derived growth factor (21, 25), nerve growth factor (6, 28), tumor necrosis factor (38, 39), and basic fibroblast growth factor (28). In tumor necrosis factor (38) and platelet-derived growth factor (27) signaling, extracellular signal-regulated kinase (ERK)1/2 activation is at least partially dependent on SPK. Rani et al. (27) also argued that the link between SPK and ERK1/2 is Ras GEF independent because inhibitors of SPK failed to affect signaling events commonly thought to recruit Sos1 (a Ras-GEF) to the plasma membrane.

Vascular endothelial growth factor (VEGF) is one of the most important growth factors involved in angiogenesis. VEGF stimulates endothelial cell growth through PKC and subsequent ERK1/2 activation, although the exact mechanism remains elusive (33, 36). In addition, VEGF receptors have been found in a large number of human tumors, and the mitogenic function of VEGF in these tumors has been suggested (reference 34 and references therein). The recently described role of S1P as a mediator in various growth factor-induced signal transduction pathways and its recently reported role in angiogenesis and tumor biology (14, 16, 35) prompted us to determine whether VEGF signaling might be mediated

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through activation of SPK activity. Our results show that VEGF induces cell proliferation by sequential activation of PKC, SPK, Ras, Raf, and ERK1/2. In contrast to EGF, VEGF activates Ras through a mechanism that modulates Ras-GAP instead of Ras-GEF activity to favor Ras activation. These data highlight a novel mechanism in which SPK mediates signaling from PKC to Ras.

## MATERIALS AND METHODS

**Cell culture.** T24 cells were maintained in RPMI 1640 with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with 5% $CO_2$. In some experiments, a 5 μM concentration of anti-human VEGF (hVEGF) antisense oligonucleotide (5′-TGGCTTGAAGATGTACTCGAT-3′) was added to the medium to inhibit the endogenous VEGF. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection and used before passage 7. The medium for HUVECs was M199 with 25% FBS, 1.25 g of $NaHCO_3$/liter, 10 mM HEPES buffer, 1 mM sodium pyruvate, 60 mg of endothelial cell growth supplement/liter, 5,000 U of heparin/liter, and 1% glutamine penicillin-streptomycin.

**Molecular cloning of murine SPK1.** The murine SPK1 gene was cloned by reverse transcription-PCR with the primers 5′-GACTGAATTCATGGAACCA GAATGCCCTCG-3′ and 5′-GACTGGATCCTTATGGTTCTTCTGGAGGT GG-3′. The PCR products were digested with *Eco*RI/*Bam*HI and cloned into the mammalian expression vector pCMV5/myc1. The SPK1 dominant-negative mutant (DN-SPK1) (pCMV5/myc1-SPK1-G81D) was generated by site-directed mutagenesis with the GeneEditor in vitro site-directed mutagenesis system (Promega). A His-tagged SPK1 bacterial expression vector (pRSET-A-SPK1) was constructed as follows. The pRSET-A vector (Invitrogen) was first digested with *Hin*dIII, filled in with Klenow fragment, and then digested with *Eco*RI. An SPK1 gene fragment was generated by digesting pCMV5/myc1-SPK1 with *Sma*I/*Eco*RI and was ligated to the vector. All constructs were confirmed by DNA sequencing. Plasmids for expression of Flag-tagged SPK1 were provided by Binks Wattenberg (26).

**PKC phosphorylation of recombinant SPK1.** His-tagged recombinant SPK1 was purified from *Escherichia coli* [pRSET-A-SPK1/BL21(DE3)] with standard procedures. In vitro phosphorylation of recombinant SPK1 by PKC was carried out in a 100-μl reaction mixture which contained 20 μl of 5× PKC buffer (100 mM Tris-HCl [pH 7.5], 25 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.5 μM phorbol 12-myristate 13-acetate [PMA]), 10 μl of a His-SPK1/resin complex, 10 μl of 100 μM ATP, 20 ng of PKC (V5261, >90% purity; Promega), and $H_2O$. Whenever PKC inhibitors (Gö6976 and Gö6983) were used, the reaction mixtures were first incubated at 30°C for 20 min before the addition of ATP. After incubation at 30°C for 30 min with rotation, the reaction mixtures were washed three times with buffer (20 mM Tris-HCl [pH 7.5], 0.5 M NaCl, and 50 mM imidazole), and SPK activities were assayed as described below.

**SPK assay.** T24 cells or HUVECs were cultured to confluence in a 60-mm-diameter dish and serum starved overnight. Cells were changed to fresh serum-free medium containing the indicated concentration of PKC inhibitors for 1 h and then stimulated with various amounts of VEGF or PMA. After the indicated time, cells were harvested and SPK activities in cell extracts were assayed as described previously (24). Cellular sphingosine and S1P levels were measured as described previously (7, 23).

**hVEGF immunoassay.** T24 cells were cultured in RPMI 1640-1% FBS and treated with 5 or 10 μM anti-hVEGF antisense oligonucleotide or scramble control oligonucleotide for 40 h. VEGF in the supernatant was measured with a Quantikine hVEGF immunoassay kit according to the manufacturer's protocol (R&D Systems).

**[$^3$H]thymidine incorporation assay.** T24 cells were cultured to 30% confluence in a 24-well dish in RPMI 1640 with 1% FBS. Various concentrations of anti-hVEGF antisense oligonucleotide were added to the medium for 3 days. During the last 4 h, 1 μCi of [$^3$H]thymidine/ml was added to the culture. Cells were then lysed in buffer (20 mM Tris-HCl [pH 7.5], 1% NP-40) and transferred to a nitrocellulose filter and washed with excess 1× phosphate-buffered saline. The incorporation of [$^3$H]thymidine into DNA was counted by a liquid scintillation counter.

**Assay of ERK1/2 activation.** T24 cells or HUVECs were cultured to confluency in a 60-mm-diameter dish and serum starved overnight in the presence of 5 μM anti-hVEGF antisense oligonucleotide. After incubation in fresh serum-free medium with PKC inhibitors (100 nM concentrations of Gö6976 and Gö6983) or SPK inhibitor (10 μM dimethylsphingosine [DMS]) for 30 min, the cells were stimulated with 10 ng of EGF/ml (10 min), 40 ng of VEGF/ml, or the indicated concentrations of PMA (20 min). Cells were lysed in buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM $Na_3VO_4$, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg each of aprotinin and leupeptin/ml). About 20 μg of cell extracts was separated by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE), and the levels of phosphorylated ERK1/2 (phospho-ERK1/2) and total ERK1/2 were detected by Western blotting with specific antibodies (Cell Signaling Technology).

For DN-SPK interference of ERK2 activation, pCMV5/myc1-SPK1, pCMV5/myc-SPK1-G81D, or a control vector (0.5 μg/60-mm-diameter dish) was cotransfected with pHis-ERK2 (1 μg/60-mm-diameter dish) using Lipofectamine Plus reagent (Invitrogen). After serum starvation overnight, cells were stimulated with VEGF, EGF, or PMA and lysed as described above. His-ERK2 was pulled down with ProBond resin (Invitrogen), and the amounts of phospho-ERK2 and total ERK2 were measured as described above.

For siRNA experiments, siRNA for human SPK1 (5′-GGGCAAGGCCTTG CAGCTCdTdT-3′), SPK2 (5′-GCCAGGCCCCGGGGTGGCCdTdT-3′), and the control sequence were synthesized at Dharmacon. Transfection was performed as described in the siRNA user guide (www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html). Two days after transfection, the cells were stimulated with VEGF, EGF, or PMA, and ERK1/2 activation was assayed as described above.

**Ras activation assay.** T24 cells or HUVECs in 60-mm-diameter dishes were serum starved overnight and stimulated with VEGF, PMA, or EGF as indicated. Ras-GTP was pulled down with the Raf-1 Ras binding domain (RBD) and detected by Western blotting with a Ras activation assay kit (Upstate Biotechnology) according to the manufacturer's protocol.

**Raf kinase assay.** T24 cells in 60-mm-diameter dishes were serum starved overnight and stimulated with VEGF or EGF as indicated. Raf-1 kinase was immunoprecipitated from total cell extracts, and the activity was assayed using a Raf-1 immunoprecipitation kinase cascade assay kit (Upstate Biotechnology) according to the manufacturer's protocol.

**Immunofluorescence assay for BrdU incorporation into DNA.** T24 cells were cultured to 50 to 80% confluence on 12-mm-diameter coverslips in a 24-well dish. Transfection was performed with Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. pSGFP plasmid (0.4 μg) and 0.4 μg of pCMV5/myc1-SPK1, pCMV5/myc1-SPK1-G81D, or the control vector were used in each transfection reaction. Four hours after transfection, the cells were changed to fresh starvation medium with a 5 μM concentration of anti-hVEGF antisense oligonucleotide for 20 h. The cells were then stimulated with 50 ng of VEGF/ml for 24 h, and DNA synthesis was assayed with a BrdU (bromodeoxyuridine) Labeling and Detection Kit I (Roche) according to the manufacturer's protocol. Texas Red-conjugated goat anti-mouse immunoglobulin G (10 μg/ml; Molecular Probes) was used as a secondary antibody. Coverslips were mounted with Vectashield mounting medium H1000 (Vector Laboratories) and examined under fluorescence microscopy. DNA synthesis was determined for 100 synthetic green fluorescent protein (SGFP)-positive and 100 SGFP-negative cells under each cotransfection condition and with or without VEGF treatment.

## RESULTS

**VEGF stimulates SPK activity.** We examined VEGF stimulation of SPK in the T24 human bladder carcinoma cell line that expresses both VEGF and its receptor, Flk-1 (data not shown), and primary HUVECs. Cells were starved overnight in serum-free medium prior to the addition of VEGF. As shown in Fig. 1A to C, we found that VEGF stimulates SPK activity in T24 cells (Fig. 1A and B) and HUVECs (Fig. 1B). This stimulation is dependent on the concentration of VEGF and reaches a maximum value (1.7- to 1.9-fold increase) at about 20 min after VEGF addition and decreases slightly thereafter (40 to 60 min).

T24 cells produce VEGF and express the Flk-1 receptor. We therefore tested whether the secreted VEGF might act in an autocrine loop to increase basal levels of SPK activity. T24 cells were grown in suboptimal FBS concentrations (1%) for 3 days in the presence of anti-hVEGF antisense oligonucleotide or control oligonucleotide (19). We found that the antisense oli-

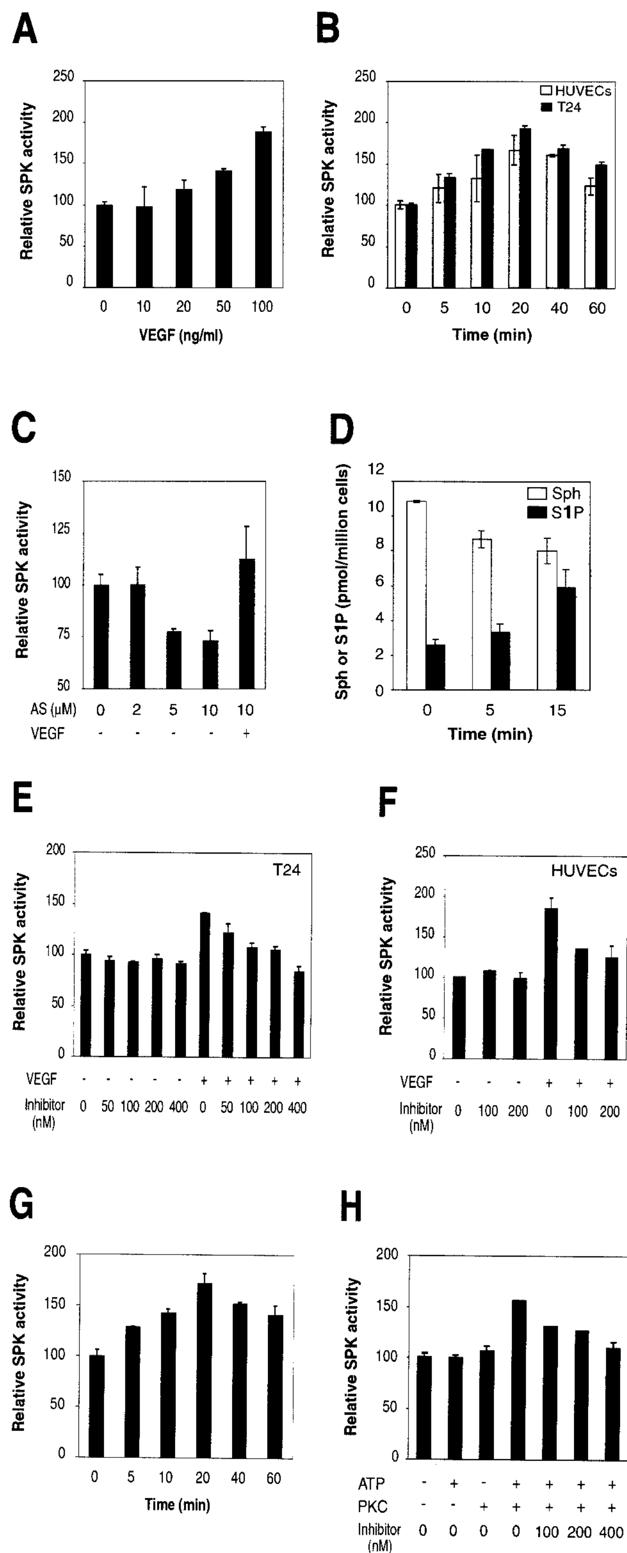


FIG. 1. Stimulation of SPK activity by VEGF, PMA, and PKC. (A) Concentration-dependent VEGF stimulation of SPK activity in T24 cells. Cells were stimulated with the indicated amount of VEGF for 20 min, and the SPK activity in cell extracts was assayed by measuring [$^{32}$P]ATP incorporation into S1P (relative activity of 100 = 56 pmol/min/mg of protein). Error bars indicate standard deviation. (B) Time course of VEGF stimulation of SPK activity in T24 cells (100 ng of VEGF/ml; closed bars) and HUVECs (20 ng of VEGF/ml; open bars). SPK activity was determined as described for panel A. (C) SPK activity in T24 cells grown in RPMI 1640-1% FBS in the presence of the indicated concentration (0 to 10 μM) of anti-VEGF antisense (AS) oligonucleotide. Where indicated, cells were treated with VEGF (100 ng/ml) for 20 min prior to harvesting. SPK activity was determined as described for panel A. (D) Analysis of total cellular sphingosine (Sph) and S1P levels in VEGF-stimulated T24 cells (100 ng of VEGF/ml for the indicated times). Sph and S1P levels were determined by previously described methods (7, 23). (E) VEGF stimulation (100 ng/ml, 15 min) of SPK activity in T24 cells preincubated with the indicated concentrations of the PKC inhibitors (Gö6976 and Gö6983). SPK activity was determined as described for panel A. (F) VEGF stimulation (20 ng/ml, 15 min) of SPK activity in HUVECs preincubated with the indicated concentrations of the PKC inhibitors. SPK activity was determined as described for panel A. (G) PMA stimulation (20 nM for the indicated times) of SPK activity in T24 cells. (H) In vitro stimulation of SPK activity by PKC. His-tagged recombinant SPK1 protein was first incubated with PKC (V5261; Promega) in vitro with or without the indicated concentrations of the PKC inhibitors (Gö6976 and Gö6983), and then the activity of the resin-bound His-tagged SPK1 was determined as described for panel A.

TABLE 1. Inhibition of VEGF secretion in T24 cells after treatment with antisense oligonucleotide that targets VEGF[a]

| Oligonucleotide | VEGF secretion (%)[b] by T24 cells after treatment with oligonucleotide at concn | | |
|---|---|---|---|
| | 0 μM | 5 μM | 10 μM |
| Scramble | 100.0 | 104.2 | 99.0 |
| AS-VEGF | 100.0 | 36.4 | 19.1 |

[a] T24 cells (triplicate wells) were treated with antisense oligonucleotide that targets VEGF (AS-VEGF) or scrambled oligonucleotide for 40 h. VEGF in the supernatant was measured with a Quantikine hVEGF immunoassay kit according to the manufacturer's protocol (R&D Systems).

[b] The values are normalized to those for untreated cells (100% is approximately 2 ng/ml).

gonucleotide, but not the control oligonucleotide, reduced the amount of VEGF in the medium by 80% (Table 1) and reduced [$^3$H]thymidine incorporation by 80% (after the addition of a 5 μM concentration of antisense oligonucleotide, [$^3$H]thymidine incorporation in T24 cells dropped to 32.5% of that observed in untreated cells, and the addition of a 10 μM concentration lowered [$^3$H]thymidine incorporation to 17.1% of that in untreated cells). Thus, we examined SPK activity in anti-hVEGF antisense oligonucleotide-treated T24 (3 days with 1% FBS) and found that the antisense oligonucleotide lowered the basal level of SPK activity and that this effect could be reversed by exogenous VEGF (Fig. 1C). We conclude that the endogenous VEGF produced in T24 cells causes a partial stimulation of SPK activity and that the antisense oligonucleotide can inhibit this stimulation. In subsequent experiments where T24 cells were starved, anti-hVEGF antisense oligonucleotide was included in the starvation conditions to block the mitogenic VEGF autocrine loop.

We measured the total cellular sphingosine and S1P levels in T24 cells before and after VEGF stimulation. T24 cells were serum starved overnight (with anti-hVEGF antisense oligonucleotide) and then stimulated with VEGF. The level of sphingosine was higher than that of S1P in quiescent T24 cells. VEGF stimulation increased the S1P level (about 2.3-fold) and slightly decreased the sphingosine level such that after 15 min

approximately equal amounts of S1P and sphingosine were found in total cell extracts (Fig. 1D). Together, these data demonstrate that VEGF stimulation of T24 cells leads to activation of SPK.

**PKC mediates VEGF stimulation of SPK.** We next examined the signaling events leading to VEGF stimulation of SPK. Since phospholipase C gamma (PLC-γ) and PKC are well-known downstream effectors of VEGF signaling (31, 33), we tested the effect of inhibitors of PKC on the VEGF stimulation of SPK activity in HUVECs and T24 cells. We used a combination of Gö6976 and Gö6983, which are known to specifically inhibit the α, β, γ, δ, ζ, θ, and μ isoforms of PKC (10, 18). These inhibitors result in near complete inhibition of VEGF stimulation of SPK activity in both T24 cells (Fig. 1E) and HUVECs (Fig. 1F).

We also examined SPK activity in T24 cells following treatment with PMA, a well-known activator of PKC (Fig. 1G). In these experiments, PMA treatment stimulated SPK activity, which reached a maximum level (1.7-fold increase) after 20 min and decreased slightly thereafter (40 to 60 min). Together, these data suggest that PKC mediates VEGF stimulation of SPK activity in vivo. Using a recombinant, His-tagged murine SPK1 expressed in *E. coli*, we tested whether direct phosphorylation by PKC could stimulate SPK activity in vitro (Fig. 1H). The data show that PKC treatment in the presence of ATP stimulates SPK activity by approximately 1.6-fold. Inclusion of [$\gamma$-$^{32}$P]ATP in the reaction mixture followed by SDS-PAGE and autoradiography showed that SPK1 was indeed phosphorylated (data not shown). Furthermore, we found that PKC-induced stimulation of SPK was blocked by the PKC-specific inhibitors (Gö6976 and Gö6983) (Fig. 1H), whereas the unstimulated SPK activity was not affected by these inhibitors (data not shown). These in vivo and in vitro results suggest that VEGF stimulation of SPK in cells results from the direct phosphorylation of SPK by PKC.

**DN-SPK mutant inhibits VEGF stimulation of DNA synthesis.** VEGF stimulation of endothelial cell proliferation is reported to be PKC dependent. We showed that VEGF stimulation leads to PKC-dependent SPK activation. We next examined whether SPK is involved in the mitogenic function of VEGF in T24 cells. A dominant-negative mutation (G82D) was recently identified in the putative ATP-binding region of human SPK1 (26). We created a homologous mutation (G81D) in a myc-tagged murine SPK1 by site-directed mutagenesis. Using a vector expressing this mutant protein, we tested whether DN-SPK1 could interfere with VEGF stimulation of DNA synthesis in T24 cells. In these experiments, we used the SGFP reporter construct to identify transfected cells and BrdU incorporation (red fluorescence) to assess DNA synthesis. The results demonstrate that the DN-SPK1 mutant inhibited VEGF stimulation of DNA synthesis by about 50%, whereas wild-type SPK1 (Wt-SPK1) and the empty vector did not (Table 2).

TABLE 2. Inhibition of VEGF-stimulated DNA synthesis in T24 cells by a DN-SPK mutant (SPK1-G81D)[a]

| Agent | % of BrdU-positive cells among SGFP-positive cells | | % of BrdU-positive cells among SGFP-negative cells | |
|---|---|---|---|---|
| | Without VEGF | With VEGF | Without VEGF | With VEGF |
| Empty vector | 17 | 82 | 19 | 82 |
| Wt-SPK | 21 | 80 | 23 | 74 |
| SPK-G81D | 16 | 44 | 27 | 77 |

[a] T24 cells were cotransfected with pSGFP and pCMV5/myc-1, pCMV5/myc-1-mSPK1(Wt), or pCMV5/myc-1-mSPK1-G81D. VEGF stimulation of DNA synthesis was determined by assaying BrdU incorporation by immunofluorescence microscopy. The percentages of cells exhibiting BrdU incorporation (DNA synthesis) in transfected cells (SGFP positive) and untransfected cells (SGFP negative) were determined with 100 cells under each condition. Relative to cells transfected with the vector control or the plasmid harboring Wt-SPK, the cells transfected with SPK1-G81D exhibited approximately 50% inhibition of VEGF-induced DNA synthesis ($P < 0.006$). Relative to SGFP-negative cells in the same dish, SGFP-positive cells cotransfected with DN-SPK exhibited a 42% inhibition of VEGF-induced DNA synthesis ($P < 0.004$).

**SPK mediates VEGF stimulation of ERK1/2.** ERK1/2 activation is essential for VEGF stimulation of endothelial cell growth, and this pathway has been reported to be PKC dependent (31, 36, 40). We determined whether SPK activation is involved in this pathway by inactivating SPK with a pharmacological inhibitor, a DN-SPK mutant, or siRNA that downregulates SPK expression. First, we tested whether VEGF-stimulated ERK1/2 activation (phosphorylation) in T24 cells could be inhibited by DMS, a known inhibitor of SPK (8). The data show that VEGF stimulation of ERK1/2 phosphorylation is completely inhibited by DMS as well as the PKC inhibitors (Gö6976 and Gö6983) (Fig. 2A, top panel). Furthermore, we found that stimulation of ERK1/2 phosphorylation by PMA (Fig. 2A, middle panel) was also inhibited by DMS, whereas EGF-stimulated ERK1/2 phosphorylation (which is not PKC or SPK dependent [27]) was unaffected by DMS or the PKC inhibitors (Fig. 2A, bottom panel). We found the time courses of activation of ERK1/2 by VEGF and PMA to be remarkably similar, showing a slow and prolonged activation (Fig. 2B). In contrast, EGF results in a rapid and transient activation of ERK1/2 (Fig. 2B). We examined the ability of VEGF to induce the phosphorylation of a His-tagged ERK2 in T24 cells by transfection of a plasmid expressing His-ERK2 together with a vector control or plasmids expressing Wt-SPK1 or DN-SPK1. Western blotting for total His-ERK2 demonstrated that equivalent amounts of ERK2 were present under each condition (data not shown). VEGF induced the phosphorylation of His-ERK2 in cells transfected with a control vector or Wt-SPK1 but not in cells transfected with the DN-SPK1 (Fig. 2C, top panel). The inhibition of His-ERK2 phosphorylation by the DN-SPK1 could be completely reversed by overexpression of Wt-SPK (Fig. 2C, top panel). The DN-SPK1 had no effect on EGF-induced phosphorylation of His-ERK2. We found in some experiments that transfection of T24 cells with Wt-SPK1 enhanced the ability of VEGF to activate ERK2 (Fig. 2C, bottom panel). We consistently found that the DN-SPK1 mutant blocked VEGF activation of ERK2 and that overexpression of Wt-SPK1 can reverse the effects of the DN-SPK1 (Fig. 2C). Lastly, we used siRNA to downregulate the levels of SPK1 and SPK2 and determined whether this affected VEGF or EGF activation of ERK1/2. In T24 cells treated with siRNAs that target SPK1 and SPK2, EGF activation of ERK1/2 was unaffected (Fig. 2D). In contrast, siRNA that targets SPK1 completely blocked VEGF activation of ERK1/2, while siRNA that targets SPK2 produced no effect (Fig. 2D). While immunological reagents that can detect endogenous SPK1 are not

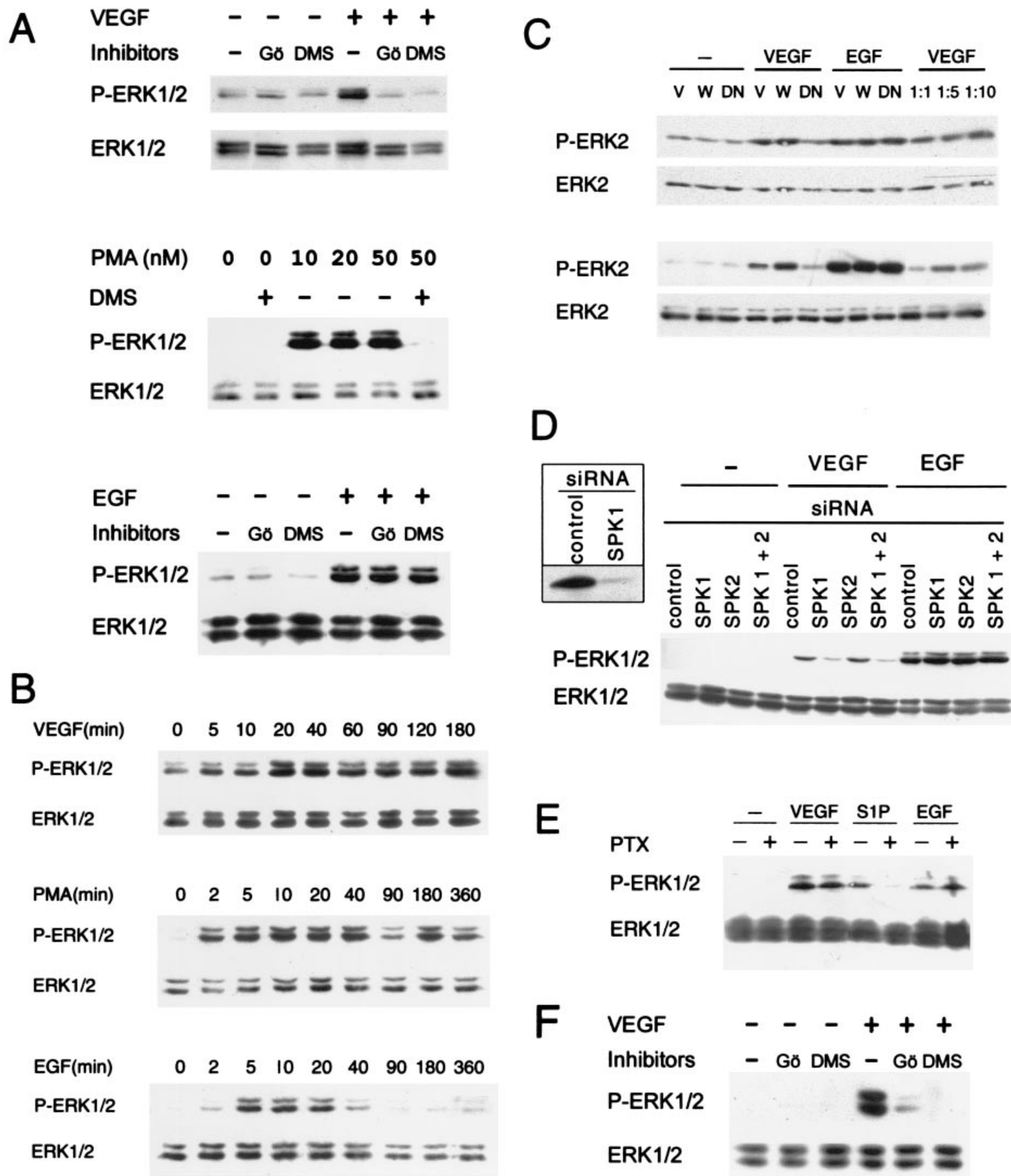


FIG. 2. Analysis of ERK1/2 activation in cells treated with VEGF, PMA, or EGF. (A) ERK1/2 activation in T24 cells stimulated with VEGF (40 ng/ml for 15 min; top panel), PMA (10 to 50 nM for 20 min; middle panel), or EGF (10 ng/ml for 10 min; bottom panel). Where indicated, cells were pretreated for 30 min with the PKC inhibitors (Gö) or the SPK inhibitor, DMS. ERK1/2 activation (phosphorylation) in total cell extracts was analyzed by immunoblotting with anti-phospho-ERK1/2 antibody, and the amount of total ERK1/2 protein was determined by reprobing the same blot with anti-ERK1/2 antibody. (B) Time course of ERK1/2 activation. T24 cells were treated with VEGF, PMA, or EGF as described above, and phospho-ERK1/2 levels were determined at the indicated times. (C) The DN-SPK mutant blocks VEGF- but not EGF-induced ERK2 activation in T24 cells. T24 cells were cotransfected with pHis-ERK2 and pCMV5/myc1-SPK1 (W), pCMV5/myc1-SPK1-G81D (DN), or a control vector (V). Where indicated, the ratio of DN-SPK1 to Wt-SPK1 plasmids was varied. At 24 h after transfection, cells were stimulated with VEGF or EGF as described for panel A. His-ERK2 was precipitated from cell extracts by affinity for nickel-agarose beads and subjected to immunoblotting as described for panel A. Data show the results from two independent experiments. (D) siRNA for human SPK1 interferes with VEGF, but not EGF, stimulation of ERK1/2. T24 cells were stimulated with VEGF or EGF or left unstimulated as described above for panel A. P-ERK1/2 and total ERK1/2 levels were determined as described for panel A. The inset shows a Western blot to detect Flag-tagged SPK1 overexpressed in T24 cells after treatment with siRNA that targets SPK1 or control siRNA. (E) PTX blocks S1P, but not VEGF or EGF, stimulation of ERK1/2. T24 cells were pretreated with 100 nM PTX for 30 min and then stimulated with VEGF, EGF, or S1P (100 nM, 10 min), and ERK1/2 activation was assayed as described for panel A. (F) VEGF stimulation (10 ng/ml, 10 min) of ERK1/2 in HUVECs is blocked by PKC and SPK inhibitors. Where indicated, PKC or SPK inhibitors were used to treat cells prior to stimulation as described for panel A. Levels of P-ERK1/2 and total ERK1/2 were determined as described for panel A.

readily available, we found that Flag-tagged human SPK1 levels (driven by an expression vector introduced into T24 cells) are reduced by 95% following treatment with siRNA that targets SPK1 (Fig. 2D, inset). Together, these results demonstrate that the blocking of SPK activity by pharmacological or genetic means results in the loss of VEGF-to-ERK1/2 signaling.

Because S1P can act as an extracellular ligand for EDG receptors and lead to the activation of ERK1/2, we tested whether VEGF might be acting through EDG receptors. EDG receptor signaling is blocked by pretreatment of cells with pertussis toxin (PTX). We stimulated T24 cells with VEGF, S1P, or EGF in the presence or absence of PTX. Each stimulus led to increased ERK1/2 phosphorylation. While PTX completely blocked S1P activation of ERK1/2, it was without effect on VEGF and EGF signaling (Fig. 2E).

Like T24 cells, we found that HUVECs that are stimulated with VEGF display elevated levels of activated ERK1/2 that is sensitive to PKC and SPK inhibitors (Fig. 2F). Also, three tumor cell lines which are known to express VEGF receptors (T-47D breast carcinoma, DU-145 prostate carcinoma, and SK-N-SH neuroblastoma) showed a VEGF-induced phosphorylation of ERK1/2 that is sensitive to PKC and SPK inhibitors (data not shown). The inability of DMS, DN-SPK1, and siRNA that targets SPK1 to affect EGF-to-ERK1/2 signaling under conditions where they block VEGF signaling indicates that DMS, DN-SPK1, and siRNA-SPK1 are not generalized or nonspecific inhibitors of signaling that links tyrosine kinase receptors to ERK1/2 activation. These data strongly suggest that VEGF stimulation of the ERK1/2 pathway involves SPK.

**SPK mediates VEGF stimulation of Ras in a Ras-GEF-independent manner.** PKC-dependent receptor-mediated events have been reported to lead to the activation of ERK1/2 via Ras-dependent or Ras-independent signaling events (13, 17). Marais et al. reported that PKC-dependent activation of ERKs involves Ras-GTP accumulation that is insensitive to dominant-negative Ras and concluded that Ras activation occurs via a mechanism distinct from that of the EGF receptor (17). To determine whether the VEGF/PKC-induced activation of ERK1/2 involves Ras signaling, we tested whether VEGF treatment resulted in the accumulation of Ras-GTP. Using a glutathione *S*-transferase fusion of the Ras-GTP-binding domain of Raf-1, we precipitated Ras-GTP from T24 cell extracts following treatment of the cells with different stimuli in the presence or absence of PKC and SPK inhibitors. VEGF treatment stimulated an increase in Ras-GTP within 10 min that was sustained over 3 h (Fig. 3A, bottom panel), and at 10 min, PKC and SPK inhibitors (Fig. 3A, top panel) blocked this stimulation. Furthermore, we found that PKC activation by PMA stimulated an increase in Ras-GTP accumulation at 10 min and that PKC and SPK inhibitors (Fig. 3A, top panel) blocked this stimulation. Like T24 cells, HUVECs stimulated with VEGF show an increase in the levels of Ras-GTP that is sensitive to PKC and SPK inhibitors (Fig. 3A, middle panel).

To rule out the possibility that the effects of the PKC and SPK inhibitors on Ras-GTP accumulation were due to nonspecific pleiotropic effects, we studied their effects on EGF stimulation of Ras, which occurs independently of PKC and SPK. We found that EGF stimulation of Ras-GTP accumulation was rapid and transient (Fig. 3A, bottom panel) and insensitive to PKC and SPK inhibitors (Fig. 3A, top panel). Pharmacological inhibitors pose a potential problem in that the specificity of the inhibitors cannot be conclusively determined. siRNA that targets mRNAs in a sequence-dependent manner allows the silencing of a signaling protein in a highly specific mechanism. We used siRNA designed to target SPK1 or SPK2 to determine whether the elimination of these affects VEGF-induced Ras-GTP accumulation. As shown in Fig. 3B, incubation of cells with siRNA that targets SPK1, but not SPK2, resulted in near complete blocking of VEGF-induced Ras-GTP accumulation. EGF-stimulated accumulation of Ras-GTP was unaffected by the targeting of either SPK1 or SPK2. Together, these data demonstrate that VEGF stimulates the accumulation of Ras-GTP by a PKC- and SPK-dependent mechanism.

Ras activation is generally thought to occur through stimulation of Ras-GEFs (1). However, Ras activation by a GEF-independent mechanism involving GAP inhibition has also been suggested, but a molecular mechanism for this has not been proposed (5). Ras-GEFs can be inhibited by a Ras dominant-negative mutant (Ras-N17) through formation of an inactive complex with Ras-GEFs. We determined whether Ras-N17 expression in T24 cells could affect phosphorylation of transiently expressed His-ERK2. We found that Ras-N17 had no effect on VEGF-induced His-ERK2 phosphorylation (Fig. 3C). Consistent with the observations of Marais et al., we found that PMA stimulation of His-ERK2 phosphorylation was unaffected by Ras-N17 (Fig. 3C). We also examined the effects of Ras-N17 in T24 cells on EGF stimulation of His-ERK2 (which is known to require Ras-GEFs). As expected, we found that EGF-stimulated His-ERK2 phosphorylation was blocked by Ras-N17 (Fig. 3C). Together, the results presented here demonstrate that SPK links PKC to the activation of Ras via a Ras-N17-insensitive mechanism.

Ras activation of ERK1/2 requires the recruitment and activation of Raf kinase. Therefore, we predicted that VEGF stimulation of ERK1/2 would require Raf kinase function. To test this, we treated T24 cells with VEGF or EGF in the presence or absence of a Raf kinase inhibitor (catalog no. 553008; CalBiochem, Inc.). We found that both VEGF- and EGF-stimulated ERK1/2 phosphorylations were blocked by the Raf kinase inhibitor (Fig. 3D). This is consistent with the work of Takahashi et al. with sinusoidal endothelial cells where VEGF stimulates Raf kinase activity (31). Similarly, we found that VEGF stimulation of T24 cells leads to activation of Raf kinase activity and that SPK and PKC inhibitors block Raf activation (Fig. 3E). These data implicate a VEGF-induced pathway leading to activation of ERK1/2 that involves PKC- and SPK-dependent induction of an active Ras-GTP/Raf complex. The observation that Ras-N17 failed to block VEGF activation of ERK1/2 reported here and by others (31) suggests that activation of Ras by VEGF occurs via a mechanism independent of Ras-specific GEFs. We note that the activation of Ras by VEGF is consistently slower than that induced by EGF (Fig. 3A), which is consistent with a GEF-independent mechanism. Also, we note that the intrinsic exchange rate of Ras in permeabilized cells is significantly higher than that observed in vitro. [$\alpha$-$^{32}$P]GTP was found to saturate Ras binding in 5 min or less in permeabilized T24 cells (data not shown), and the GTP was rapidly converted to GDP, consistent with the results of previous studies with T cells (5).

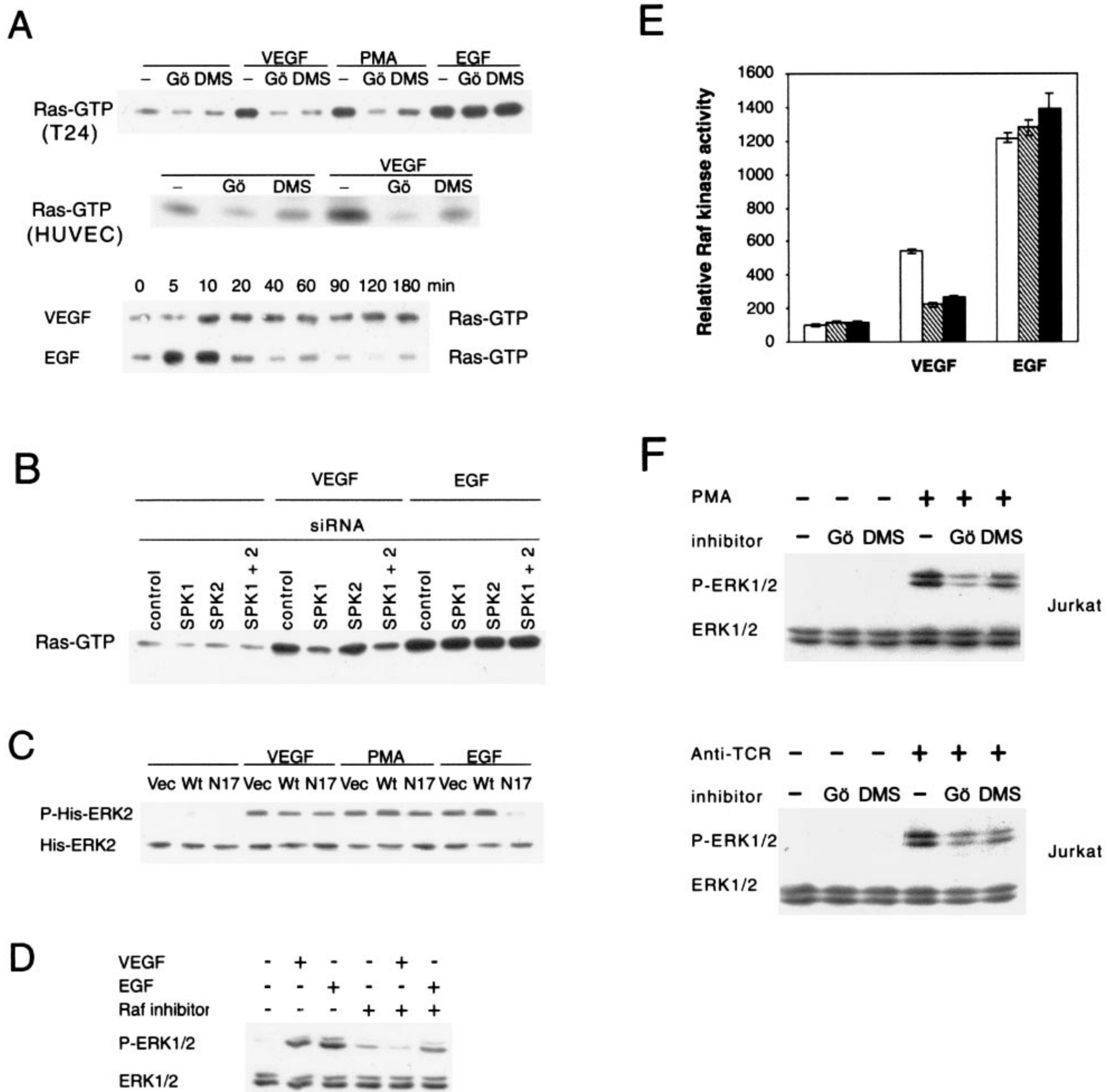


FIG. 3. VEGF stimulation of Ras via a Ras-GEF-independent mechanism. (A) Ras-GTP accumulation in T24 cells following stimulation. Ras-GTP levels in cell extracts were measured by pulldown assays using immobilized GST–Raf-1–RBD. The results of Western blot analysis using a panspecific anti-Ras antibody is shown. In the top panel, where indicated, cells were pretreated for 30 min with the PKC inhibitors (Gö) or the SPK inhibitor (DMS) and then stimulated with VEGF (40 ng/ml, 20 min), PMA (10 nM, 20 min), or EGF (10 ng/ml, 10 min). The middle panel shows Ras-GTP levels in HUVECs before and after VEGF (20 ng/ml) treatment and with or without preincubation with PKC inhibitors (Gö) or the SPK inhibitor (DMS). The bottom panel shows the time course of Ras activation in T24 cells treated with VEGF (40 ng/ml) or EGF (10 ng/ml). (B) The levels of Ras-GTP in T24 cells stimulated with VEGF or EGF after preincubation (2 days) with siRNA that targets SPK1 or SPK2 or control siRNA as indicated. Ras-GTP levels were determined as described above. (C) Effect of the dominant-negative Ras mutant (Ras-N17) on ERK2 activation by VEGF, PMA, and EGF. T24 cells were cotransfected with pHis-ERK2 and either pZIP (Vec), pZIP-Ras-Wt (Wt), or pZIP-Ras-N17 (N17). Phosphorylated His-ERK2 (P-His-ERK2) and total His-ERK2 were determined by immunoblotting following precipitation of His-ERK2 with nickel-agarose from cell extracts. (D) Effect of Raf-1 kinase inhibitor on ERK1/2 activation by VEGF and EGF. T24 cells were pretreated for 30 min with 100 nM Raf-1 kinase inhibitor (catalog no. 553008; CalBiochem, Inc.) and then stimulated with VEGF (40 ng/ml, 15 min) or EGF (10 ng/ml, 10 min). Phospho-ERK1/2 (P-ERK1/2) and total ERK 1/2 levels were determined as described in the legend to Fig. 2. (E) Raf-1 activation in T24 cells stimulated with VEGF or EGF. T24 cells were pretreated with SPK inhibitor (hatched bars) or PKC inhibitors (solid bars) or left untreated (open bars) and then stimulated with VEGF (50 ng/ml, 15 min) or EGF (10 ng/ml, 5 min). Raf-1 was immunoprecipitated from cell extracts, and the activity was measured with an Raf-1 immunoprecipitation kinase cascade assay kit. (F) ERK1/2 activation in Jurkat T cells stimulated with PMA (100 nM for 10 min; top panel) or anti-CD3 antibody (Anti-TCR) (OKT-3, 10 μg/ml; bottom panel). Where indicated, cells were pretreated for 30 min with the PKC inhibitors (Gö) or the SPK inhibitor (DMS). Phospho-ERK1/2 (P-ERK1/2) and total ERK 1/2 levels were determined as described in the legend to Fig. 2.

Elevated levels of Ras-GTP are found in neurofibromin 1 (NF1)-deficient Schwann cells (11) and hematopoietic progenitor cells (41), indicating that loss of a Ras-GAP function is sufficient for Ras-GTP accumulation. In addition, Ras activation in TCR signaling has been suggested by Downward and coworkers to occur by a mechanism involving inhibition of GAP function as opposed to activation of Ras-GEFs (5). In that study, PKC was found to be upstream of GAP regulation; however, activation of PKC did not result in an increase in GAP phosphorylation, suggesting that an unknown molecule links PKC to GAP signaling. Our observation that SPK is downstream of PKC activation but upstream of Ras activation prompted us to examine whether SPK might link TCR-mediated activation of ERK1/2. Indeed, we found that the SPK inhibitor DMS blocks ERK1/2 activation mediated by PMA (Fig. 3F, top panel) and TCR cross-linking (Fig. 3F, bottom panel) in Jurkat T-cells. These results suggest that SPK mediates ERK1/2 signaling downstream of both TCRs and PKC.

## DISCUSSION

The results presented here demonstrate that VEGF activates SPK in T24 cells and HUVECs. The kinetics of activation is slow, peaking at 20 min. Several lines of evidence suggest that PKC mediates the effects of VEGF on SPK activation. First, VEGF activation of the VEGF receptor, Flk-1, has been reported to recruit PLC-$\gamma$ and lead to the subsequent activation of PKC (31, 36, 37, 40). Second, PKC has been implicated in the activation of SPK in some, but not all, receptor-mediated events leading to SPK activation (2–4, 12). Third, we found that inhibitors of PKC can effectively block SPK activation. Fourth, we found that purified PKC can phosphorylate and activate recombinant SPK1 in an ATP-dependent manner. Several lines of evidence also suggest that SPK can mediate the downstream effects of PKC. First, PMA is able to activate SPK in T24 cells. Second, we observed phosphorylation and activation of recombinant SPK by purified PKC. Third, the SPK inhibitor, DMS, can block PMA-dependent activation of ERK1/2. We note that the concentration of DMS used here has been reported not to affect PKC activity in cells (8). Taken together, these results support our conclusion that VEGF stimulation of SPK is mediated by PKC.

We note that in vitro phosphorylation of SPK by PKC results in the incorporation of [$^{32}$P]phosphate into SPK resolved by SDS-PAGE when [$\gamma$-$^{32}$P]ATP is included in the kinase reaction (data not shown). The incorporation of radioactivity could be inhibited by the inclusion of PKC inhibitors in the kinase reaction. In $^{32}$P-labeled cells overexpressing SPK1, SPK1 was found to be a phosphoprotein in quiescent T24 cells, and VEGF treatment did not affect its phosphorylation (data not shown). This suggests that SPK has multiple phosphorylation sites (some of which are not PKC-mediated), and subsequent phosphorylation by PKC is masked by the phosphorylation sites already present in resting cells. We are currently seeking to determine the site(s) in SPK1 phosphorylated by PKC.

VEGF is reported to activate the ERK1/2 signaling pathway in a PKC-dependent manner in a variety of cells. Not surprisingly, we found that VEGF stimulation of ERKs in T24 cells was blocked by PKC inhibitors. We extend the results of previous studies by showing that SPK is also involved in VEGF/PKC-dependent activation of ERKs. We used three independent methods to assess the role of SPK in VEGF activation of ERKs. We found that the SPK inhibitor, DMS, effectively blocks VEGF activation of ERK1/2. In vitro, DMS is reported to inhibit PKC. However, at the concentration used in our study, DMS had no effect on cellular PKC activity or translocation to membranes in PC12 or Swiss 3T3 cells (8). We also blocked cellular SPK activity by expression of a DN-SPK mutant. DN-SPK completely blocked VEGF activation of ERK2. The effects of DN-SPK could be reversed by overexpression of Wt-SPK. Consistent with the finding of Olivera and coworkers, we found that cells overexpressing Wt-SPK1 exhibit an increase in SPK activity of more than 100-fold (22). Because the Wt-SPK1 and DN-SPK1 plasmids are identical except for a single point mutation, it is likely that DN-SPK is also expressed at very high levels relative to the endogenous SPK. Together, these results suggest that a large excess of DN-SPK relative to the endogenous SPK can block the activity of the endogenous SPK. The ability of a 1-to-1 ratio of plasmids expressing Wt-SPK and the DN-SPK to allow VEGF-to-ERK signaling indicates that when the amount of the DN-SPK is similar to that of Wt-SPK, the dominant-negative effects are silenced. Lastly, we used siRNA to target SPK in T24 cells and examined VEGF activation of ERK1/2. Treatment of T24 cells with siRNA that targets SPK1, but not SPK2, resulted in a 50% blockage of VEGF activation of ERK1/2. The combination of siRNAs that target both SPK1 and SPK2 was no more effective than siRNA that targets SPK1 alone. Neither siRNA had any effect on EGF activation of ERK1/2. Using polyclonal antisera for SPK1, we were unable to detect endogenous SPK1 but could readily detect an overexpressed Flag-tagged SPK1. Therefore, to assess the efficiency of the siRNA that targets SPK1, we treated cells overexpressing Wt-SPK1 with the siRNA. We found that the siRNA reduced the levels of SPK1 by more than 95%. Because overexpression of SPK1 results in SPK activities that are $>$100-fold greater than endogenous levels, the siRNAs are likely to effectively reduce endogenous SPK1 levels. We conclude that SPK1 links VEGF to ERK1/2 signaling.

We found that VEGF stimulation of T24 cells leads to a slow and sustained activation of Ras (Fig. 3A). This is similar to the slow and sustained activation of Ras in PMA-treated COS cells reported by Marais and coworkers (17). We also found that VEGF treatment of T24 cells leads to a slow activation of SPK (Fig. 1B) and ERK1/2 (Fig. 2B). The similar kinetics of activation of SPK, Ras, and ERK and the similarity of PMA and VEGF activation of Ras are consistent with a linear pathway involving these signaling molecules. Furthermore, that Ras activation by VEGF is downstream of PKC and SPK was indicated by our observation that PKC and SPK inhibitors (both pharmacological inhibitors and siRNA) block Ras-GTP accumulation in response to VEGF.

The ability of VEGF to activate Ras has been investigated by many laboratories. Several groups of researchers have reported that VEGF does not lead to Ras activation. What might explain these discrepancies? First, there are discrepancies in the time periods for which groups monitored the accumulation of Ras-GTP. Activation of Ras signaling is often rapid and transient. Ras activation in response to many stimuli, such as EGF, occurs within minutes and begins to decline rapidly to basal levels after 10 min. As a consequence of the slow kinetics

of Ras activation (Fig. 3A) induced by VEGF in some cells, such as T24 cells, monitoring for Ras activation at an early time point may cause researchers to miss Ras activation. Another possible reason for the conflicting claims of the involvement of Ras in VEGF signaling is that some studies have relied on the use of dominant-negative Ras-N17 in concluding that Ras was not involved in VEGF signaling events. However, Ras-N17 does not directly block Ras function but rather the GEFs that can activate Ras. Thus, as noted by Marais et al., the use of Ras-N17 cannot determine whether Ras is excluded from a signaling pathway. Importantly, the Ras-GEF-independent mechanism of Ras activation studied by Marais and coworkers was a PKC-mediated pathway, like that which we propose for VEGF signaling. In determining whether Ras is involved in a signaling event, the RBD of Raf is likely to be more reliable than Ras-N17. This domain binds directly and specifically to Ras-GTP and therefore blocks Ras from interacting with endogenous substrates. In this regard, Doanes et al. reported that overexpression of the RBD of Raf blocked the ability of VEGF to activate ERKs in HUVECs (4a). Thus, the results presented here, together with previous observations, indicate that in some cell types, such as T24 and HUVECs, VEGF activates Ras and that this leads to the activation of ERKs. Another possible explanation for the discrepancy over whether Ras is or is not involved in VEGF signaling is the heterogeneity of the VEGF signaling pathway in different cells. For example, T24 cells express Flk-1 but not Flt-1 or Tie-1 (data not shown), while various endothelial cells express various amounts of the multiple VEGF receptors.

The T24 bladder tumor cell line was used in the identification of the first human oncogene, H-*ras* (32). The oncogenic Ras protein was found to contain a point mutation which results in a dramatic impairment of the GTPase activity of Ras. The VEGF-induced accumulation of Ras-GTP that we observed was likely due to wild-type Ras protein. By using pan-Ras antibodies that are specific for the three major Ras proteins (H-, K-, and N-Ras), we determined the species of Ras that accounted for the VEGF-induced increase in Ras-GTP (Fig. 3A). We detected N-Ras-GTP only after VEGF stimulation of T24 cells; however, we detected some H-Ras-GTP in resting cells and the level of H-Ras-GTP increased more than threefold after VEGF treatment (data not shown). We found little K-Ras-GTP present in T24 cells. Thus, the accumulation of Ras-GTP shown in Fig. 3A was predominantly due to the wild-type *ras* alleles (one H-*ras* and two N-*ras*).

We note that SPK inhibition results in blockage of Ras-GTP accumulation in VEGF- and PMA-stimulated tumor cell lines (Fig. 3A and B). Importantly, DMS and siRNA that targets SPK1/2 failed to affect EGF activation of Ras, thus arguing against a pleiotropic effect. These observations suggest that one of the downstream targets of S1P leads to Ras activation. Because VEGF induction of ERK1/2 phosphorylation is insensitive to Ras-N17 (suggesting that Ras-GEFs related to the yeast CDC25 [1] are not involved), we suggest that Ras-GAPs (p120 and NF1) are possible direct targets of these sphingolipids during VEGF stimulation of tumor and endothelial cells. Indeed, it has recently been found that sphingosine can activate Ras-GAP activity in vitro and that S1P can block the ability of sphingosine to activate Ras-GAPs (X. Shu, unpublished observations). We propose that membrane-associated sphingosine in cells attenuates the basal Ras activity by stimulating Ras-GAP activity. VEGF stimulation of SPK results in the conversion of sphingosine to S1P such that S1P levels are sufficient to displace sphingosine from GAPs. Overall, this would decrease GAP activity and increase the level of activated Ras-GTP without involving Ras-GEFs. In resting T24 cells, sphingosine levels were found to be four- to fivefold higher than that of S1P. We found that total cellular S1P increases after VEGF stimulation, while the level of sphingosine decreases such that after 15 min approximately equal amounts of S1P and sphingosine were found (Fig. 1D). While it is not possible to measure the relative levels of S1P and sphingosine in the immediate vicinity of VEGF receptors or Ras-GAPs, these data suggest that in some subcellular locations the amount of S1P exceeds that of sphingosine. We are currently exploring the molecular mechanism by which the SPK substrate and product can influence GTP hydrolysis on Ras.

Recently, several Ras-GEFs that have diacylglycerol (DAG)-binding motifs have been described, and some of these are reported to respond to PMA and DAG. Might VEGF signaling, which can activate PLC (to produce DAG), use such DAG-responsive Ras-GEFs? We report here that VEGF activation of Ras is blocked by siRNA that targets SPK1. Thus, if DAG-responsive Ras-GEFs are involved, then they would require SPK1 function for Ras-GEF activity. Also, we and others have found that VEGF activation of ERK1/2 is insensitive to Ras-N17. Consequently, if a DAG-responsive Ras-GEF is involved, then this must be an atypical Ras-GEF that is unaffected by a dominant-negative Ras mutant. We note that all Ras-GEFs (from organisms ranging from yeasts to humans) for which the effects of Ras-N17 have been tested have been found to be inhibited by Ras-N17. Lastly, we found that PKC inhibitors block VEGF-induced activation of Ras; consequently, if a DAG-responsive Ras-GEF is involved, then it must be sensitive to PKC inhibitors or dependent on SPK1 activity. We currently favor the model in which Ras-GEFs are not involved in the VEGF signaling described here.

S1P can act as an intracellular second messenger as well as an extracellular ligand for EDG G protein-coupled receptors. S1P activation of EDG receptors is reported to activate Ras and ERK signaling. Can we exclude the possibility that S1P produced by VEGF stimulation of cellular SPK is secreted and activates EDG receptors leading to Ras and ERK activation, rather than functioning as an intracellular second messenger? Several lines of evidence strongly favor the view that S1P functions as an intracellular second messenger in VEGF signaling. First, Endo and coworkers reported that treatment of HUVECs with S1P leads to activation of ERK that is completely reversed by pretreatment of the cells with PTX (9). In a parallel experiment, PTX was without effect on VEGF-induced activation of ERK, indicating that the S1P/EDG receptor and VEGF activate ERK by distinct mechanisms (9). We found similar results with T24 cells where VEGF, EGF, and S1P each lead to ERK activation but only S1P signaling is blocked by pretreatment of cells with PTX (Fig. 2E). Furthermore, S1P/EDG receptor activation of ERKs is blocked by Ras-N17, while VEGF activation of ERKs in T24 cells is insensitive to Ras-N17 (20). The kinetics of ERK activation for VEGF and S1P/EDG are also dramatically different. Whereas VEGF stimulation of ERKs is slow and sustained, S1P/EDG

activation of ERKs peaks near 1 min of stimulation. Lastly, Olivera and coworkers reported that no detectable increase of S1P in the medium was found in cells which overexpress SPK1 (500-fold excess) (22). Thus, it appears that VEGF signaling and that of extracellular S1P are distinct, suggesting that the intracellular S1P generated by VEGF leads to activation of downstream signaling without engaging extracellular EDG receptors.

Is the VEGF signaling pathway described here unique to T24 cells or does it operate in other cell types or tumor cells expressing VEGF receptors? A number of observations suggest that the pathway we described is operational in a significant number of cell types. Like the T24 cell line, many tumor cell lines express the Flk-1 receptor. It will be important to determine whether the VEGF signaling described for T24 cells is widely utilized by other human tumor cells. We discuss here our analysis of three additional human tumor cell lines that were reported to express VEGF receptors. For each of these cell lines, VEGF activation of ERKs could be blocked by SPK and PKC inhibitors. A review of the literature indicates that more than 50% of human tumor cell lines may overexpress one or more VEGF receptor, and the presence of an autocrine loop has been suggested in many cases. Our analysis of 19 bladder tumor cell lines found that 13 of them express one or more VEGF receptor (data not shown). Also, 70% of pancreatic carcinomas are found to coexpress VEGF and VEGF receptors, and a VEGF-mitogenic autocrine loop was found in pancreatic cell lines (even when an activated allele of *ras* is present) (34). Given that hypoxic conditions in tumors as well as activated oncogenes, such as *ras*, can stimulate VEGF expression and secretion, an autocrine function for VEGF in human tumors may be very important.

Many types of endothelial cells have been found to respond to VEGF by activation of PKC, Ras, Raf, and ERKs. In many cases, activation of ERKs was not blocked by Ras-N17 or was only partially blocked. We also show that VEGF does activate SPK in HUVECs and that this activation is blocked by PKC inhibitors. Furthermore, VEGF stimulation of ERK1/2 in HUVECs is blocked by the SPK inhibitor, DMS. Thus, all of the VEGF signaling events in HUVECs that we have studied to date show similarity with those in T24 cells. A link between PKC and Ras activation has been observed in other systems, such as PMA stimulation of COS cells and TCR stimulation of Jurkat T cells. Interestingly, both of these systems lead to the activation of Ras that is insensitive to Ras-N17. Furthermore, for both of these systems, PKC was proposed to activate Ras via a Ras-GEF-independent mechanism. For TCR stimulation of ERK1/2 in Jurkat cells, we found that DMS blocks ERK activation, suggesting that SPK mediates this signaling. It is interesting to speculate that in a variety of signaling networks, PKC-dependent activation of Ras may employ SPK, as we suggest here for T24 cells.


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