Recruitment and Activation of Phospholipase Cγ1 by Vascular Endothelial Growth Factor Receptor-2 Are Required for Tubulogenesis and Differentiation of Endothelial Cells

Rosana D. Meyer, Catharina Latz, and Nader Rahimi‡

From the Departments of Ophthalmology and Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

Abstract

Vascular endothelial growth factor-mediated angiogenic signal transduction relay is achieved by coordinated induction of endothelial cell proliferation, migration, and differentiation. These complex cellular processes are most likely controlled by activation of both cooperative and antagonistic signals by vascular endothelial growth factor receptors (VEGFRs). Here, we investigated the contribution of tyrosine-phosphorylated residues of VEGFR-2/fetal liver kinase-1 to endothelial cell proliferation and differentiation and activation of signaling proteins. Mutation of tyrosine 1006 of VEGFR-2 to phenylalanine severely impaired the ability of this receptor to stimulate endothelial cell differentiation and tubulogenesis. Paradoxically, the mutant receptor stimulated endothelial cell proliferation far better than the wild-type receptor. Further analysis showed that tyrosine 1006 is responsible for phospholipase Cγ1 (PLCγ1) activation and intracellular calcium release in endothelial cells. Activation of PLCγ1 was selectively mediated by tyrosine 1006. Mutation of tyrosines 799, 820, 949, 994, 1080, 1173, and 1221 had no measurable effect on the ability of VEGFR-2 to stimulate PLCγ1 activation. Association of VEGFR-2 with PLCγ1 was mainly established between tyrosine 1006 and the C-terminal SH2 domain of PLCγ1 in vitro and in vivo. Taken together, the results indicate that phosphorylation of tyrosine 1006 is essential for VEGFR-2-mediated PLCγ1 activation, calcium flux, and cell differentiation. More importantly, VEGFR-2-mediated endothelial cell proliferation is inversely correlated with the ability of VEGFR-2 to associate with and activate PLCγ1.

Vascular endothelial growth factor (VEGF) exerts its cellular responses by binding to one of its receptors, VEGFR-2/fetal liver kinase-1, and stimulating its autophosphorylation. VEGFR-2 belongs to a subfamily of receptor tyrosine kinases (RTKs) whose activation plays an essential role in a large number of biological processes such as embryonic development, wound healing, and pathological angiogenesis (1,2). Although many cellular events involved in angiogenesis, including endothelial cell proliferation, migration, and differentiation, have been extensively characterized, the signal transduction pathways downstream of VEGFR-2, which might mediate these events, are largely limited. Elucidating the precise molecular...
mechanisms of signal transduction relays involved in angiogenesis is required for design of better anti-angiogenic strategies.

Recent studies on VEGFR-2 signal transduction relay have shown that many well characterized signaling proteins such as phospholipase Cγ1 (PLCγ1) and phosphatidylinositol 3-kinase (PI3K) are activated following stimulation of endothelial cells with VEGF (3,4). However, the role of individual tyrosine residues of VEGFR-2 that might contribute to association and activation of PLCγ1 is not clear and is subject to a great deal of inconsistency within the literature. For example, Cunningham et al. (5) suggested that tyrosines 801 and 1175 of human VEGFR-2 (corresponding to tyrosines 799 and 1173 of mouse VEGFR-2, respectively) are binding sites for PLCγ1. A study by Takahashi et al. (3) showed that tyrosine 1175 is a PLCγ1-binding site in VEGFR-2 and that phosphorylation of tyrosine 1175 is required for association and activation of PLCγ1 by VEGFR-2. In contrast, Wu et al. (6) suggested that tyrosine 952 (but not tyrosines 801 and 1175) is the PLCγ1-binding site in VEGFR-2. How can these discrepancies within the literature be explained? One possibility is that VEGF-mediated VEGFR-2 autophosphorylation and the ability of VEGFR-2 to recruit signaling proteins are influenced by other endothelial cell-surface receptors such as VEGFR-1, neuropilin-1, and neuropilin-2, which are normally expressed by endothelial cells. Because VEGF binds to all of these receptors, it is highly possible that activation of these receptors and their signal transduction relays are influenced by the presence of these receptors likely due to establishment of receptor homo- and heterodimerization in endothelial cells (7-10). A second possibility is that activation of VEGFR-2 and stimulation of its associated signaling proteins are affected by endothelial cadherins and integrins, adding an additional level of complexity to VEGFR-2-induced signal transduction relays in endothelial cells (11,12). Finally, it is also possible that individual autophosphorylation sites in VEGFR-2 are not stringently required for the recruitment and association of PLCγ1. If this is true, VEGFR-2 autophosphorylation sites may be compensatory in their ability to associate with PLCγ1.

In this study, we have addressed the enigma concerning the recruitment and activation of PLCγ1 by VEGFR-2 by using a unique system of a VEGFR-2 chimera and constructing a panel of VEGFR-2 tyrosine mutants, including tyrosines 799, 820, 949, 994, 1006, 1080, and 1173. In this system, VEGFR-2 is selectively activated by CSF-1 without any contributions from other VEGFRs such as VEGFR-1 and neuropilins. Here, we report the following results. 1) Tyrosine 1006 (but not tyrosines 799, 949, 994, 820, 1080, 1173, and 1221) of VEGFR-2 is responsible for association with and activation of PLCγ1. 2) Association of PLCγ1 with VEGFR-2 is established primarily by the C-terminal SH2 domain of PLCγ1. 3) PLCγ1 activation is required for endothelial cell tubulogenesis and differentiation, but not for VEGFR-2-induced endothelial cell proliferation.

MATERIALS AND METHODS

Reagents and Antibodies

Human recombinant CSF-1 was purchased from R&D Systems. Mouse anti-phosphotyrosine (PY-20) and anti-PLCγ antibodies and anti-mouse and anti-rabbit secondary antibodies were purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-MAPK and anti-phospho-MAPK antibodies were purchased from New England Biolabs Inc. (Beverly, MA). Rabbit anti-phospho-PLCγ antibody was purchased from BIOSOURCE (Camarillo, CA). Rabbit anti-VEGFR-2 antibody was made against amino acids corresponding to the kinase insert or carboxyl terminus of VEGFR-2 (9). U73122 was purchased from Calbiochem. Mouse anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).
**Cell Lines**

Porcine aortic endothelial (PAE) cells expressing CKR and tyrosine mutant receptors were established by a retroviral system as described previously (4,9). Briefly, cDNAs encoding CKR and tyrosine mutant receptors were cloned into retroviral vector pLNCX^2^ and transfected into 293-GPG cells. Viral supernatants were collected for 7 days, concentrated by centrifugation, and used as previously described (9).

**Site-directed Mutagenesis**

The VEGFR-2 chimera CKR was used as a template to construct the mutations. CKR was subcloned into the pGEMT cloning vector, and site-directed mutagenesis was carried out using a PCR-based site-directed mutagenesis method (4,9,30). The resultant mutations were verified by sequencing and were subsequently cloned into the pLNCX^2^ vector at _Not_I and _Sal_I sites.

**Immunoprecipitation and Western Blotting**

PAE cells expressing CKR and tyrosine mutant CKRs were grown under semiconfluent culture conditions in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and supplemented with glutamate, penicillin, and streptomycin and serum-starved overnight in DMEM. Cells were either left resting or stimulated with 40 ng/ml CSF-1 for 10 min at 37 °C. Cells were washed twice with buffer containing 25 m[^i] M HEPES (pH 7.4), 150 m[^i] M NaCl, and 2 m[^i] M Na_3^VO_4 and lysed in lysis buffer (10 m[^i] M Tris-HCl, 10% glycerol (pH 7.4), 5 m[^i] M EDTA, 50 m[^i] M NaCl, 50 m[^i] M NaF, 1% Triton X-100, 1 m[^i] M phenylmethylsulfonyl fluoride, 2 m[^i] M Na_3^VO_4, and 20 μg/ml aprotinin). Proteins were immunoprecipitated using appropriate antibodies. Immunocomplexes were bound to protein A-Sepharose and washed three times with 1.0 ml of lysis buffer. Immunoprecipitates were resolved on an SDS-polyacryl-amide gel, and the proteins were transferred to Immobilon membranes. For Western blot analysis, the membranes were incubated for 60 min in blocking solution containing 10 m[^i] M Tris-HCl (pH 7.5), 150 m[^i] M NaCl, 10 mg/ml bovine serum albumin, and 0.05% Tween 20. The membranes were then incubated with primary antibodies diluted in blocking solution for another 60 min, washed three times with Western rinse, incubated with horseradish peroxidase-conjugated secondary antibodies, washed, and developed with ECL (Amersham Biosciences). Finally, the membranes were stripped by incubation in stripping buffer containing 6.25 m[^i] M Tris-HCl (pH 6.8), 2% SDS, and 100 m[^i] M β-mercaptoethanol at 50 °C for 30 min; washed with Western rinse; and reprobed with the antibody of interest.

**Cell Proliferation**

The proliferation assay was performed as described (4,9). Briefly, cells were plated at 2 × 10[^5]/ml in 24-well plates containing DMEM supplemented with 10% fetal bovine serum and incubated at 37 °C for 12 h. Cells were then washed once with phosphate-buffered saline and serum-starved overnight in DMEM, and various concentrations of CSF-1 were added. During the last 4 h of incubation, cells were pulsed with[^3]H]thymidine (0.2 μCi/ml) and harvested. Results for each group were collected from four samples. Each experiment was repeated three times, and essentially the same results were obtained. The data are presented as -fold increase over the control.

**Calcium Flux Assay**

PAE cells expressing either CKR or tyrosine mutant CKRs were grown on 25-mm round glass coverslips and serum-starved for 12–18 h. Cells were incubated in HEPES-buffered saline solution (137 m[^i] M NaCl, 5 m[^i] M KCl, 4 m[^i] M MgCl_2, 3 m[^i] M CaCl_2·2H_2O, 25 m[^i] M glucose, and 10 m[^i] M HEPES) with 4 μM Fluo-3/AM supplemented with 0.02% pluronic acid in Me_2SO for 30 min at 37 °C. After rinsing two times in HEPES-buffered saline solution, the live cells were placed in an open chamber (Molecular Probes, Inc., Eugene, OR) with 500 μl of HEPES.

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solution and positioned on the stage of a Zeiss LSM 510 Axiovert confocal laser scanning microscope equipped with an argon laser. For each experiment, cells were scanned for at least 5–10 s before the addition of CSF-1 to establish a base-line fluorescence reading. All readings were made while continuously scanning the cells every 789 ms (33).

**Measurement of Inositol Phosphate Production**

The production of inositol phosphates was measured using 60-mm dishes of PAE cells expressing CKR and tyrosine mutant CKRs labeled for 48 h in DMEM supplemented with 0.1% bovine serum albumin and 1 μCi/ml myo-[H]inositol. The cultures were washed and incubated for 15 min with DMEM containing 0.1% bovine serum albumin, 15 mM HEPES (pH 7.5), and 20 mM LiCl. The medium was then aspirated, and fresh medium with or without 40 ng/ml CSF-1 was added and incubated at 37 °C for 20 min. The culture plates were placed on ice, and the medium was removed before the addition of 1.5 ml of ice-cold methanol/HCl (100:1). The quenched samples were collected, and the plates were rinsed with an additional 1.5 ml of methanol/HCl (100:1). To each sample were added 1.5 ml of HO and 3 ml of CHCl₃, and the tubes were mixed by vortexing and left on ice for 30 min. The water-soluble phase was collected, diluted with 2 volumes of water and 1 ml of AG 1-X8 formate resin (Bio-Rad), and incubated for 2 h. The samples were extensively washed with water and then with a solution containing 5 mM disodium tetraborate and 60 mM sodium formate. Finally, the total inositol phosphates were eluted from the resin with a solution of 0.1 M formic acid and 1.0 M ammonium formate and subjected to scintillation counting. The CHCl₃-phase samples containing the phospholipids were dried, redissolved in methanol, and subjected to scintillation counting.

**In Vitro Angiogenesis/Tubulogenesis Assay**

Endothelial cell spheroids were generated as previously described (31). A defined number of cells were suspended in DMEM containing 1% fetal bovine serum and 0.24% (w/v) carboxymethylcellulose (4000 centipoise) in non-adherent round-bottom 96-well plates under standard cell culture conditions. After 24 h, all cells formed one single spheroid per well (750 cells/spheroid). Spheroids were cultured for 2 days before using them in the *in vitro* angiogenesis assay in the following manner. Spheroids containing 750 cells were embedded in collagen gels. Eight volumes of collagen were mixed with 1 volume of 10× HEPES-buffered saline solution containing 10% 10× DMEM with phenol red. The pH was adjusted to 7.4 with 0.1 M NaOH. Spheroids were centrifuged and suspended in 9 ml of DMEM containing 0.96% carboxymethylcellulose. Collagen and spheroids were mixed and transferred to prewarmed 24-well plates, and the gels were allowed to polymerize in the incubator. After 30 min, 100 μl of DMEM containing various concentrations of CSF-1 were added on top of the gel. Sprouting and tubulogenesis were observed after 2 days under an inverted phase-contrast microscope (Nikon), and pictures were taken using the SPOT camera system.

**RESULTS**

**Role of Individual Tyrosine Residues of VEGFR-2 in the Activation of PLCγ1**

VEGF binds to multiple endothelial cell-surface receptors, including VEGFR-1, VEGFR-2, neuropilin-1, and neuropilin-2 (7-10,13), by generating potentially homologous and heterologous signaling networks. Because these receptors are very often expressed in endothelial cells, it is difficult to address the activation of specific signaling molecules by the individual receptors. We have recently constructed a VEGFR-2 chimera containing the extracellular domain of human CSF-1 receptor/c-fms fused with the transmembrane and cytoplasmic domains of murine VEGFR-2 (9). This model allowed us to dissect the function of VEGFR-2 in endothelial cells by selectively stimulating the receptor with CSF-1 (4,29). Throughout this work, the VEGFR-2 chimera is called CKR. In this study, we used PAE cells
expressing CKR and aimed to address the role of individual tyrosine residues in the recruitment and activation of PLCγ1.

Initially, we evaluated the kinetics of tyrosine phosphorylation of PLCγ1 by CKR following CSF-1 stimulation. For this purpose, cells were stimulated with CSF-1, and the kinetics of phosphorylation of PLCγ1 was evaluated by subjecting total cell lysates to Western blot analysis using anti-phospho-PLCγ1 antibody that specifically recognizes the active form of PLCγ1. CKR-mediated phosphorylation of PLCγ1 peaked after 10 min of stimulation, was significantly reduced after 30 min, and was not detectable after 45 min of stimulation (Fig. 1A). To test the role of individual tyrosine sites in VEGFR-2-mediated activation of PLCγ1 in PAE cells, we individually replaced tyrosines 799, 820, 949, 994, 1006, 1173, and 1212 with phenylalanine. Fig. 1C shows the schematic location of these tyrosine sites in VEGFR-2. We initially tested the ability of F799/CKR and F1173/CKR to stimulate PLCγ1 activation. These tyrosine sites are conserved in both human and mouse VEGFR-2, and the corresponding tyrosines in human VEGFR-2 are at positions 801 and 1175, respectively. These tyrosine sites have previously been suggested to bind PLCγ1 (3). As demonstrated in Fig. 1D, individual mutation of tyrosines 799 and 1173 in mouse VEGFR-2 had no significant effect on their ability to activate PLCγ1. The data suggest that tyrosines 799 and 1173 are not responsible for PLCγ1 activation and furthermore argue that other tyrosine sites in VEGFR-2 might mediate its activation. To test the contribution of other tyrosine sites in VEGFR-2 to the activation of PLCγ1, additional sites were mutated and expressed in PAE cells. To this end, PAE cells expressing tyrosine mutant CKRs, including tyrosines 820, 949, 994, 1006, and 1221, were stimulated with CSF-1 and analyzed for activation of PLCγ1. As demonstrated in Fig. 1F, all of the tyrosine mutant receptors, viz., F820/CKR, F949/CKR, F994/CKR, and F1221/CKR, were able to stimulate PLCγ1 activation. In contrast, F1006/CKR failed to stimulate robust activation of PLCγ1 compared with the wild-type receptor and other tyrosine mutant CKRs (Fig. 1F). The data also suggest that, in addition to tyrosines 799 and 1173, the presence of tyrosines 820, 949, 994, and 1221 is not required for PLCγ1 activation by VEGFR-2.

Role of Tyrosines 1006 and 1080 of VEGFR-2 in PLCγ1 and MAPK Activation

Tyrosines 1006 and 1080 are located in the kinase domain of VEGFR-2, adjacent to the N and C termini of the activation loop, respectively (Fig. 2A). To test the putative contribution of tyrosines 1006 and 1080 to PLCγ1 activation, we generated a double tyrosine mutant receptor in which both tyrosines 1006 and 1080 were replaced with phenylalanine. The resultant receptor was similarly expressed in PAE cells and tested for its ability to activate PLCγ1. As Fig. 2B shows, ligand-stimulated cells expressing the double mutant receptor (F1006/F1080/CKR) maintained their ability to induce residual activation of PLCγ1. The ability of the double mutant receptor to activate PLCγ1 was not completely diminished and indeed was similar to that of F1006/CKR (Fig. 2B), suggesting that tyrosine 1080 is not involved in the activation of PLCγ1 by VEGFR-2. In addition, the single tyrosine 1080 mutant was also fully capable of activating PLCγ1, similar to the wild-type receptor (data not shown).

To test the role of tyrosines 1006 and 1080 in the ligand-dependent autophosphorylation VEGFR-2, we also analyzed their ligand-dependent tyrosine phosphorylation. As Fig. 2D demonstrates, mutation of tyrosines 1006 and 1080 had no significant effect on the tyrosine phosphorylation of the receptor, suggesting that these tyrosine residues are not involved in the regulation of ligand-dependent receptor autophosphorylation. To test whether tyrosine 1006 plays a role in VEGFR-2-dependent activation of MAPK and also to test whether activation of PLCγ1 modulates MAPK activation in this system, we analyzed the ability of this receptor to stimulate MAPK activation. As Fig. 2F shows, both single mutant (F1006/CKR) and double mutant (F1006/F1080/CKR) receptors were fully able to activate MAPK. The data strongly
suggest that activation PLCγ1 is not required for VEGFR-2-mediated phosphorylation of MAPK.

**The SH2 Domains of PLCγ1 Cooperatively Associate with Ligand-stimulated VEGFR-2**

Association of PLCγ1 with RTKs is established by its N- and C-SH2 domains (14). However, the N- and C-SH2 domain requirement of PLCγ1 for its activation appears to be distinctive among RTKs whose stimulation leads to PLCγ1 activation (14,15,31). To test which SH2 domain of PLCγ1 is involved in association with VEGFR-2, we made recombinant GST fusion proteins consisting of the N-SH2, C-SH2, or C- and N-SH2 domains of PLCγ1 and tested their ability to associate with ligand-stimulated CKR in vitro. As shown in Fig. 3B, no significant association between CKR and GST-N-SH2 was observed. Only a modest association of CKR with the C-SH2 domain was detected. In contrast, when both the N- and C-SH2 domains were fused to GST, a strong association between CKR and the GST fusion proteins containing the N- and C-SH2 domains was detected. These results suggest that the presence of the N-SH2 domain of PLCγ1 alone is not sufficient to mediate the association of PLCγ1 with VEGFR-2. In contrast, the C-SH2 domain is, in part, able to associate with VEGFR-2 without the N-SH2 domain. However, the presence of the N-SH2 domain greatly facilitated the ability of the C-SH2 domain to interact with VEGFR-2 (Fig. 3B), suggesting that the C- and N-SH2 domains of PLCγ1 cooperatively associate with VEGFR-2.

To test the contribution of tyrosines 1006 and 1173 to complex formation between CKR and the SH2 domains of PLCγ1, PAE cells expressing either F1006/CKR or F1173/CKR were stimulated with CSF-1, and cell lysates were incubated with GST-C-SH2 and GST-C+N-SH2 recombinant proteins. As shown in Fig. 3C, the ability of CKR to associate with the SH2 domains of PLCγ1 was significantly reduced when tyrosine 1006 was mutated. In contrast, mutation of tyrosine 1173 did not alter the association of CKR and the SH2 domains of PLCγ1. Altogether, the data suggest the following. (i) The N-SH2 domain of PLCγ1 alone is not sufficient to bind VEGFR-2. (ii) The C-SH2 domain of PLCγ1 binds to VEGFR-2 weakly, but its association with VEGFR-2 is greatly enhanced by the presence of the N-SH2 domain. This suggests that the C- and N-SH2 domains of PLCγ1 are engaged in association with VEGFR-2 in a cooperative manner. (iii) The presence of tyrosine 1006 (but not tyrosine 1173) is required for association of PLCγ1 with VEGFR-2.

**The Presence of Tyrosine 1006 Is Required for VEGFR-2-mediated Inositol 1,4,5-Triphosphate (IP3) Generation and Intracellular Calcium Release**

The immediate consequence of PLCγ1 activation by RTKs is accumulation of IP3 and diacylglycerol. IP3 accumulation stimulates the release of calcium from intracellular stores (14,15). To test the requirement for tyrosines 1173 and 1006 of VEGFR-2 in IP3 production and calcium release, we tested the ability of F1173/CKR and F1006/CKR to stimulate IP3 production and intracellular calcium release in PAE cells. As depicted in Fig. 4A, stimulation of both CKR and F1173/CKR resulted in robust production of IP3. In contrast, F1006/CKR failed to stimulate a significant amount of IP3 production (Fig. 4A). In addition, we also evaluated the ability of CKR and tyrosine mutant CKRs to stimulate the intracellular calcium release in PAE cells after stimulation with CSF-1. Fig. 4B shows that stimulation of PAE cells expressing either CKR or F1173/CKR with CSF-1 caused rapid intracellular calcium release as measured using Fluo-3/AM as a probe. Unlike CKR and F1173/CKR, F1006/CKR completely failed to stimulate intracellular calcium release (Fig. 4B). Collectively, these results suggest that the presence of tyrosine 1006 (but not tyrosine 1173) of VEGFR-2 is required for IP3 production and intracellular calcium release.
Mutation of Tyrosine 1006 of VEGFR-2 Enhances Its Ability to Stimulate Proliferation of PAE Cells

One of the functions of VEGFR-2 in endothelial cells is the induction of endothelial cell proliferation (9). To test the potential role of tyrosine 1006 of VEGFR-2 in endothelial cell proliferation, we subjected cells expressing CKR, F1173/CKR, F949/CKR, and F1006/CKR to a proliferation assay. As shown in Fig. 5, stimulation of PAE cells expressing CKR, F949/CKR, and F1173/CKR with CSF-1 induced proliferation of PAE cells in a CSF-1-dependent manner. As previously reported, mutation of tyrosine 1173 partially abolishes VEGFR-2-mediated proliferation of PAE cells (4). In contrast to wild-type CKR, F949/CKR, and F1173/CKR, stimulation of F1006/CKR-expressing cells resulted in augmented proliferation of PAE cells. Interestingly, an increase in the concentration of CSF-1 resulted in even greater cell proliferation. This observation was in contrast to what we observed with wild-type VEGFR-2 or CKR. At subsaturated concentrations of ligand (1–5 ng/ml CSF-1 and 10–20 ng/ml VEGF), stimulation of CKR and VEGFR-2 induced cell proliferation. However, at saturated concentrations of ligand (10–40 ng/ml CSF-1 and 20–100 ng/ml VEGF), VEGFR-2 stimulation caused cell differentiation (Ref. 4 and this work). Collectively, these results suggest that phosphorylation of tyrosine 1006 and its involvement in the recruitment of PLCγ1 to VEGFR-2 may negatively regulate the ability of VEGFR-2 to stimulate endothelial cell proliferation.

Tyrosine 1006 of VEGFR-2 Is Required for Differentiation and in Vitro Angiogenesis of PAE Cells

Angiogenesis is a complex cellular process that involves endothelial cell proliferation, migration, and differentiation. Activation of VEGFR-2 is known to provoke these cellular events in endothelial cells. To test the requirement for tyrosines 1006 and 1173 in VEGFR-2-mediated cell differentiation, PAE cells expressing wild-type CKR, F1006/CKR, and F1173/CKR were subjected to differentiation and in vitro angiogenesis assays. As shown in Fig. 6A, stimulation of both wild-type CKR and F1173/CKR stimulated morphological changes in PAE cells. In contrast, stimulation of F1006/CKR resulted in no apparent morphological change in PAE cells, suggesting that tyrosine 1006 of VEGFR-2 is involved in mediating endothelial cell differentiation. To further test the contribution of tyrosine 1006 to VEGFR-2-mediated cell differentiation, we assessed the ability of F1006/CKR to stimulate tubulogenesis of PAE cells. As shown in Fig. 6B, stimulation of both wild-type CKR and F1173/CKR induced tubulogenesis; however F1006/CKR failed to stimulate tubulogenesis (Fig. 6B). To test the role of PLCγ1 in endothelial cell tubulogenesis, we also attempted to inhibit PLCγ1 by a pharmacological approach using U73122, a selective PLCγ1 inhibitor. As shown in Fig. 7, CKR-induced tubulogenesis was inhibited by pretreatment of cells with U73122. Collectively, these results suggest that the presence of tyrosine 1006 of VEGFR-2 is essential for its ability to stimulate endothelial cell differentiation and tubulogenesis. Furthermore, activation of PLCγ1 by VEGFR-2 is required for these cellular responses in endothelial cells.

DISCUSSION

In recent years, it has been suggested that PLCγ1 activation is associated with VEGFR-2-mediated endothelial cell proliferation and MAPK activation (3), and there are several studies...
that have also investigated the VEGFR-2 tyrosine residues that are important in PLCγ1 activation. However, the mechanism by which PLCγ1 is activated by VEGFR-2 and the biological importance of PLCγ1 in endothelial cells remain elusive and controversial (3-6). We found that tyrosine 1006 of VEGFR-2 is a critical residue required for VEGFR-2-mediated PLCγ1 activation, IP3 production, and intracellular calcium release in PAE cells. There are several possible explanations for the discrepancies between our present results and the results of previous studies. Many of the previous studies used wild-type systems, including endothelial cell lines, most of which endogenously express VEGFR-1, VEGFR-2, neuropilin-1, and neuropilin-2. VEGF binds to all of these receptors. Thus, it is highly possible that activation of these receptors and their signal transduction relays are influenced by the presence of these receptors likely due to establishment of receptor homo- and heterodimerization in endothelial cells (8,9,12). The chimeric model of VEGFR-2 employed in our study is highly selective, and contribution of other VEGFRs to PLCγ1 activation is unlikely. Another possibility is that murine VEGFR-2, in which the chimeric receptor CKR was created, unexpectedly differs from human VEGFR-2.

Our results also demonstrate that the N-SH2 domain of PLCγ1 alone is not able to associate significantly with CKR. Only a modest association of CKR with the C-SH2 domain was detected. In contrast, when the both N- and C-SH2 domains were fused to GST, a strong association between CKR and the GST fusion proteins containing the N- and C-SH2 domains was detected. These results suggest that the presence of the N-SH2 domain of PLCγ1 alone is not sufficient to mediate association of PLCγ1 with VEGFR-2. In contrast, the C-SH2 domain is, in part, able to associate with VEGFR-2 without the N-SH2 domain. However, the presence of the N-SH2 domain greatly facilitated the ability of the C-SH2 domain to interact with VEGFR-2, suggesting that the C- and N-SH2 domains of PLCγ1 cooperatively associate with VEGFR-2. Also our results demonstrate that the presence of tyrosine 1006 (but not tyrosine 1173) is required for association of PLCγ1 with VEGFR-2.

Stimulation of many RTKs, including the platelet-derived growth factor receptor, fibroblast growth factor receptor, hepatocyte growth factor receptor, and VEGFR-2, has been shown to activate PLCγ1 (3,14-19). Nevertheless, the significance of PLCγ1 in the RTK-initiated cellular responses such as cell proliferation and differentiation still is not clear. In some cases such as the platelet-derived growth factor receptor, activation of PLCγ1 is suggested to be involved in cell proliferation (20). Unlike these observations, many recent elegant studies on the biological importance of PLCγ1 demonstrated a negative role for PLCγ1 in cell proliferation induced by RTKs. For instance, fibroblast cells obtained from PLCγ1 knockout mice are normal in their ability to proliferate in response to epidermal growth factor and other growth stimulation (21,32). Overexpression of PLCγ1 in fibroblast cells also appears to have no effect on platelet-derived growth factor- and basic fibroblast growth factor-dependent cell proliferation (22). A point mutation of the fibroblast growth factor receptor that abolishes PLCγ1 association with the receptor also shows no negative effect on the ability of the fibroblast growth factor receptor to stimulate cell proliferation (23,24). Moreover, the transforming potentials of the epidermal growth factor receptor and nerve growth factor receptor/Trk have been shown to inversely correlate with their ability to associate with PLCγ1 (25). Altogether, these studies suggest that activation of PLCγ1 is dispensable or compensatory for cell proliferation.

How can mutation of tyrosine 1006 of VEGFR-2 and the lack of PLCγ1 activation by this receptor enhance the mitogenic effect of VEGFR-2 in endothelial cells? One possibility is that the recruitment of PLCγ1 by VEGFR-2 through tyrosine 1006 promotes endothelial cell tubulogenesis and differentiation, thus preventing cells from proliferation. This possibility is supported by the observation that mutation of tyrosine 1006 to phenylalanine not only did not decrease the ability of the VEGFR-2 chimera CKR to stimulate cell proliferation, but rather
caused the receptor to stimulate cell proliferation even better than wild-type CKR. This is consistent with the elimination of the ability of CKR to stimulate cell differentiation.

We have recently shown that activation of CKR stimulates endothelial cell proliferation in a PI3K-dependent manner and that tyrosines 799 and 1173 of VEGFR-2 are required for both PI3K activation and cell proliferation (4). The opposing effects of PLCγ1 and PI3K on cellular functions reported in the literature are consistent with our proposed model of VEGF-2-mediated cell proliferation and differentiation. The role of PI3K in RTK-mediated mitogenic responses has been documented extensively (26). In contrast, activation of PLCγ1 has been described as a negative feedback regulator of cell proliferation induced by RTKs (25,27). Moreover, recent studies suggest that PLCγ1 activation is directly linked to cell differentiation (28,29), suggesting that PLCγ1 activation may even encounter cell proliferation by promoting cell differentiation.

Thus, based on our previous (4) and current data, we propose that VEGFR-2-mediated signal transduction relay (in particular, the decision whether endothelial cells proliferate or differentiate) depends on reciprocal activation of PI3K and PLCγ1 by VEGFR-2. Consistent with this idea, tyrosine 799 and 1173 mutations of CKR enhance its ability to stimulate cell differentiation, whereas these mutations abolish the mitogenic ability of CKR (4). Interestingly, F1006/CKR displayed an inverse phenotype in its ability to stimulate cell differentiation versus cell proliferation. Coordinated endothelial cell proliferation and differentiation are essential prerequisites for angiogenesis. Our study demonstrates that VEGFR-2 initiates these opposing cellular events by recruiting and activating PLCγ1. Activation of PLCγ1 is inversely correlated with the ability of VEGFR-2 to stimulate cell proliferation. Activation of PLCγ1 by VEGFR-2 promotes tubulogenesis and differentiation. Furthermore, studies are required to determine the underlying mechanisms involved in PLCγ1-mediated endothelial cell differentiation.

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REFERENCES
1. Risau W. Nature 1997;386:671–674. [PubMed: 9109485]
2. Folkman J, D’Amore PA. Cell 1996;87:1153–1155. [PubMed: 8980221]
3. Takahashi T, Yamaguchi S, Chida K, Shibuya M. EMBO J 2001;20:2768–2778. [PubMed: 11387210]
4. Dayanir V, Meyer RD, Lashkari K, Rahimi N. J. Biol. Chem 2001;276:17686–17692. [PubMed: 11278468]
5. Cunningham SA, Arrate MP, Brock TA, Waxham MN. Biochem. Biophys. Res. Commun 1997;240:635–639. [PubMed: 9398617]
6. Wu L-W, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, Wang D, Warren RS, Donner DB. J. Biol. Chem 2000;275:5096–5103. [PubMed: 10671553]
7. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Cell 1998;92:735–745. [PubMed: 9529250]
8. Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M. J. Cell. Biochem 2002;85:357–368. [PubMed: 11948691]
9. Rahimi N, Dayanir V, Lashkari K. J. Biol. Chem 2000;275:16986–16992. [PubMed: 10747927]
10. Conway EM, Collen D, Carmeliet P. Cardiovasc. Res 2002;49:507–521. [PubMed: 11166264]
11. Rahimi N, Kazlauskas A. Mol. Biol. Cell 1999;10:3401–3407. [PubMed: 10512875]
12. Soldi R, Mitola S, Strasry M, Defilippi P, Tarone G, Bussolino F. EMBO J 1999;18:882–892. [PubMed: 10022831]
13. Whitaker GB, Limberg BJ, Rosenbaum JS. J. Biol. Chem 2001;276:25520–25531. [PubMed: 11333271]
14. Rhee SG. Annu. Rev. Biochem 2001;70:281–312. [PubMed: 11395409]
15. Chattopadhyay A, Vecchi M, Ji Q, Mernaugh R, Carpenter G J. Biol. Chem 1999;274:26091–26097. [PubMed: 10473558]
16. Carpenter G, Ji Q. Exp. Cell Res 1999;253:15–24. [PubMed: 10579907]
17. Cross MJ, Hodgkin MN, Roberts S, Landgren E, Wakelam MJ, Claesson-Welsh L. J. Cell Sci 2000;113:643–651. [PubMed: 10652257]
18. Poulin B, Sekiya F, Rhee SG J. Biol. Chem 2000;275:6411–6416. [PubMed: 10692443]
19. Derman MP, Chen JY, Spokes KC, Songyang Z, Cantley LG. J. Biol. Chem 1996;271:4251–4255. [PubMed: 8626770]
20. Valius M, Kazlauskas A. Cell 1993;73:321–334. [PubMed: 7682895]
21. Ji QS, Ermini S, Baulida J, Sun FL, Carpenter G. Mol. Biol. Cell 1998;9:749–757. [PubMed: 9529375]
22. Mammili B, Zilberstein A, Franks C, Felder S, Kremer S, Ullrich A, Rhee SG, Skorecki K, Schlessinger J. Science 1990;248:607–610. [PubMed: 233512]
23. Mohammadi M, Dionne CA, Li W, Li N, Spivak T, Honegger AM, Jaye M, Schlessinger J. Nature 1992;358:681–684. [PubMed: 1379698]
24. Peters KG, Marie J, Wilson E, Escobedo J, Del Rosario M, Mirda D, Williams LT. Nature 1992;358:678–681. [PubMed: 1379697]
25. Obermeier A, Tinhofer I, Grunicke HH, Ullrich A. EMBO J 1996;15:73–82. [PubMed: 8598208]
26. Cantley LC. Science 2002;296:1655–1657. [PubMed: 12040186]
27. Chen P, Xie H, Wells A. Mol. Biol. Cell 1996;7:871–881. [PubMed: 8816994]
28. Xie Z, Bikle DD. J. Biol. Chem 1999;274:20421–20424. [PubMed: 1040667]
29. Bourette RP, Myles GM, Choi JL, Rohrschneider LR. EMBO J 1997;16:5880–5893. [PubMed: 9312046]
30. Meyer RD, Dayanir V, Majnoun F, Rahimi N. J. Biol. Chem 2002;277:27081–27087. [PubMed: 12023952]
31. Korff T, Augustin HG. J. Cell Biol 1998;143:1341–1352. [PubMed: 9832561]
32. Ji QS, Chattopadhyay A, Vecchi M, Carpenter G. Mol. Cell. Biol 1999;19:4961–4970. [PubMed: 10373546]
33. Klepeis VE, Cornell-Bell A, Trinkaus-Randall V. J. Cell Sci 2001;114:4185–4195. [PubMed: 11739651]
Fig. 1. *Role of individual tyrosine residues of VEGFR-2 in PLCγ1 activation.* Serum-starved semiconfluent PAE cells expressing CKR were either non-stimulated or stimulated with 40 ng/ml CSF-1 for the indicated times, washed, and lysed, and cell extracts were normalized for protein. Total cell lysates were subjected to Western blot analysis using anti-phospho-PLCγ1 antibody (A). The same membrane was reprobed with anti-PLCγ1 antibody (B). A schematic representation of tyrosine residues located in the cytoplasmic region of VEGFR-2 is shown in C. Serum-starved PAE cells expressing wild-type CKR, F799/CKR, or F1173/CKR were treated with CSF-1 for 10 min, washed, lysed, and subjected to Western blot analysis using anti-phospho-PLCγ1 (D). The same membrane was reprobed with anti-PLCγ1 antibody (E). Serum-starved PAE cells expressing wild-type CKR, F820/CKR, F949/CKR, F994/CKR, F1006/CKR, or F1221/CKR were treated with CSF-1, and total cell lysates were resolved by SDS-PAGE and blotted with anti-phospho-PLCγ1 antibody (F). The same membrane was reprobed with anti-PLCγ1 antibody for protein levels (G).
Fig. 2.
Mutation of tyrosine 1006 impairs the ability of VEGFR-2 to stimulate PLCγ1 activation, but not MAPK activation. Shown is a schematic representation of tyrosines 1006 and 1080 of VEGFR-2 (A). Serum-starved semiconfluent PAE cells expressing CKR, F1006/CKR, F1006/F1080/CKR were either non-stimulated or stimulated with 40 ng/ml CSF-1 for 10 min, washed, and lysed, and cell extracts were normalized for protein. Total cell lysates were subjected to Western blot analysis using anti-phospho-PLCγ1 antibody (B). The same membrane was reprobed with anti-PLCγ1 antibody (C). Serum-starved cells were stimulated with CSF-1 as described for B, immuno-precipitated with anti-VEGFR-2 antibody, and subjected to Western blot analysis using anti-phosphotyrosine antibody (D). The same membrane was reprobed with anti-VEGFR-2 antibody (E). Serum-starved cells were stimulated with CSF-1 as described for B, and total cell lysates were subjected to Western blot analysis using anti-phospho-MAPK (p44/42) antibody (F). The same membrane was reprobed with anti-MAPK antibody (G).
Fig. 3. **The C-SH2 domain of PLCγ1 associates with tyrosine 1006 of VEGFR-2.** Shown is a schematic presentation of PLCγ1 and GST fusion proteins containing the SH2 domains of PLCγ (A). Serum-starved semiconfluent PAE cells expressing CKR were stimulated with 40 ng/ml CSF-1, washed, and lysed, and cell extracts were normalized for protein. Total cell lysates were incubated with Sepharose-bound GST-N-SH2, GST-C-SH2, or GST-N+C-SH2. After extensive washing, the precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-VEGFR-2 antibody (B). Serum-starved semiconfluent PAE cells expressing CKR, F1006/CKR, and F1173/CKR were stimulated with 40 ng/ml CSF-1, washed, and lysed, and cell extracts were normalized for protein. Total cell lysates were incubated with either GST-C-SH2 or GST-C+N-SH2 as described for B and subjected to Western blot analysis using anti-VEGFR-2 antibody (C).
The presence of tyrosine 1006 of VEGFR-2 is required for inositol phosphate production and calcium flux. PAE cells expressing CKR, F1173/CKR, and F1006/CKR were incubated with 1μCi/ml myo-[H]inositol for 48 h in DMEM supplemented with 0.1% bovine serum albumin, and phospholipid production was measured by scintillation counting as described under “Materials and Methods” (A). Serum-starved PAE cells expressing CKR, F1173/CKR, and F1006/CKR were grown on glass coverslips and stimulated with CSF-1, and calcium flux was measured by confocal microscopy using Fluo-3/AM as a probe as described under “Materials and Methods” (B). IPs, inositol phosphates.
Fig. 5.
**Mutation of tyrosine 1006 enhances the ability of VEGFR-2 to stimulate cell proliferation.** Serum-starved PAE cells expressing wild-type CKR and tyrosine mutant CKRs were treated with different concentrations of CSF-1, and DNA synthesis was measured by \[^{3}H\]thymidine uptake. The results are expressed as the mean (cpm/well) ± S.D. of quadruplicates. The data are expressed as a ratio of stimulated versus non-stimulated samples (A). Serum-starved semiconfluent PAE cells expressing CKR or F1006/CKR were either non-stimulated or stimulated with 40 ng/ml CSF-1 for 20 or 30 min, washed, and lysed, and cell extracts were normalized for protein. Total cell lysates were subjected to Western blot analysis using anti-phospho-AKT antibody (B). The same membrane was reprobed with anti-AKT antibody (C).
Mutation of tyrosine 1006 abrogates the ability of VEGFR-2 to stimulate differentiation and tubulogenesis of PAE cells. PAE cells expressing CKR, F1173/CKR, and F1006/CKR were plated in six-well plates, serum-starved overnight, and either non-stimulated or stimulated with CSF-1 (20 ng/ml) for 24–30 h. Morphological changes associated with CSF-1 stimulation were viewed under an inverted microscope (magnification ×10) and photographed with the SPOT camera system (A). PAE cells expressing CKR, F1173/CKR, and F1006/CKR were prepared as spheroids and subjected to an in vitro angiogenesis/tubulogenesis assay as described under “Materials and Methods.” Sprouting and tubulogenesis were observed after 2 days under an inverted phase-contrast microscope, and pictures were taken using the SPOT camera system (B).
Fig. 7. 
Inhibition of PLCγ1 by pharmacological means inhibits the ability of CKR to stimulate tubulogenesis. PAE cells expressing CKR were prepared in spheroid forms and subjected to an in vitro angiogenesis/tubulogenesis assay as described for Fig. 6B. Spheroids were treated with different concentrations of the PLCγ1 inhibitor U73122 as indicated. Sprouting and tubulogenesis were observed after 2 days under an inverted phase-contrast microscope, and pictures were taken using the SPOT camera system.