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Vascular Endothelial Growth Factor Stimulates Tyrosine Phosphorylation and Recruitment to New Focal Adhesions of Focal Adhesion Kinase and Paxillin in Endothelial Cells*

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Vascular endothelial growth factor (VEGF) stimulated the tyrosine phosphorylation of multiple components in confluent human umbilical vein endothelial cells (HUVECs) including bands of M_r 205,000, corresponding to the VEGF receptors Flt-1 and KDR, and M_r 145,000, 120,000, 97,000, and 65,000–70,000. VEGF caused a striking and transient increase in mitogen-activated protein (MAP) kinase activity and stimulated phospholipase C-γ tyrosine phosphorylation, but it had no effect on phosphatidylinositol 3′-kinase activity. VEGF caused a marked increase in tyrosine phosphorylation of p125 focal adhesion kinase (p125^{FAK}), which was both rapid and concentration-dependent. VEGF produced similar effects on p125^{FAK} in the endothelial cell line ECV.304. VEGF stimulated tyrosine phosphorylation of the 68-kDa focal adhesion-associated component, paxillin, with similar kinetics and concentration dependence to that for p125^{FAK}. Thrombin and the phorbol ester, phorbol 12-myristate 13-acetate, also increased p125^{FAK} tyrosine phosphorylation in HUVECs. The effect of VEGF on p125^{FAK} tyrosine phosphorylation was completely inhibited by the actin filament-disrupting agent cytochalasin D and was partially inhibited by the protein kinase C inhibitor GF109203X. Inhibition of the MAP kinase pathway using a specific inhibitor of MAP kinase kinase had no effect on p125^{FAK} tyrosine phosphorylation. VEGF stimulated migration and actin stress fiber formation in confluent HUVEC, and VEGF-induced p125^{FAK}/paxillin tyrosine phosphorylation was accompanied by increased immunofluorescent staining of p125^{FAK}, paxillin, and phosphotyrosine in focal adhesions in confluent cultures of HUVECs. These findings identify p125^{FAK} and paxillin as components in a VEGF-stimulated signaling pathway and suggest a novel mechanism for VEGF regulation of endothelial cell functions.

The endothelium lining the lumen of all blood vessels plays essential roles in the development and the function of the vasculature. It is central to angiogenesis, the maintenance of vascular tone and of vascular permeability, and is also involved in several disease states, particularly atherosclerosis and other vasculoproliferative disorders (1–3). A key regulator of endothelial cell functions is the 46-kDa secreted polypeptide growth factor, VEGF,[1] also known as vascular permeability factor (4–6). VEGF, which exists in at least four isoforms generated by alternative splicing from a single gene (7), is a major hypoxia-inducible angiogenic factor in tumors (8–10), and its expression is also up-regulated by hypoxia and by PDGF-BB, transforming growth factor-β, and basic fibroblast growth factor in arterial VSMC (11–13). In addition to its angiogenic activity, VEGF increases the permeability of vascular endothelium (14), stimulates migration of monocytes through endothelial monolayers (15, 16), and acts as a specific mitogen for endothelial cells (17).

Recent findings indicate that VEGF may have diverse effects in the cardiovascular system. Administration of VEGF protein and VEGF gene transfer inhibit intimal thickening following balloon angioplasty and improve blood flow in ischemic limbs, effects mediated through stimulation of endothelial cell regrowth and angiogenesis, respectively (18–20). VEGF is up-regulated in ischemic myocardium (21), and it has been proposed that VEGF may play a role in neovascularization of the advanced atherosclerotic plaque (10, 22, 23).

VEGF exhibits high affinity binding to two distinct protein tyrosine kinase receptors, the *fms*-like tyrosine kinase Flt-1 and KDR, the human homologue of Flk-1. Both receptors possess insert sequences within their catalytic domains and seven immunoglobulin-like domains in the extracellular regions and are related to the PDGF family of receptor protein-tyrosine kinases (24–27). Although expression of both VEGF receptor types occurs in adult endothelial cells including HUVECs, recent findings suggest that KDR and not Flt-1 is able to mediate the mitogenic and chemotactic effects of VEGF in endothelial cells (28, 29). The key targets for either VEGF receptor that mediate VEGF's diverse biological functions in endothelial cells remain incompletely understood, and to date studies of the downstream effectors and targets for the VEGF receptor have yielded varying results (29–31). Thus, VEGF has been reported to induce tyrosine phosphorylation of PLC-γ, of p120GAP, and of the Src homology 2 domain protein Nck in bovine aortic endothelial cells (30), while in porcine aortic endothelial cells transfected with KDR and Flt-1, VEGF had no effect on PLC-γ tyrosine phosphorylation or PI 3-kinase activity and only a weak effect on p120GAP tyrosine phosphorylation (29).

In addition to its mitogenic effects in endothelial cells, VEGF

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; anti-Tyr(P), anti-phosphotyrosine monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; MAP, mitogen-activated protein; mAb, monoclonal antibody; PI phosphatidylinositol; p85α, p85α subunit of PI 3-kinase; p120GAP, p120 GTPase-activating protein; p125^{FAK}, p125 focal adhesion kinase; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLC-γ, phospholipase C-γ; PAGE, polyacrylamide gel electrophoresis; VSMC, vascular smooth muscle cells.

also promotes the migration of endothelial cells, and it is increasingly recognized that endothelial cell migration plays an essential role in angiogenesis and vascular modeling (29, 32, 33). There is increasing evidence that $p125^{FAK}$ (34–36), a member of a growing family of nonreceptor protein-tyrosine kinases (37, 38), may play a key role in regulating the dynamic changes in actin cytoskeleton organization that are a prerequisite for cell migration (35, 36). $p125^{FAK}$ is associated in fibroblastic cells with focal adhesions, specialized subcellular structures that play a crucial role in mediating cell adhesion and motility, and its tyrosine phosphorylation is stimulated by β_1 and β_3 integrins (39–41) and by a variety of regulatory peptides and lipids that act through G-protein-coupled receptors (42–44). It has recently been demonstrated that $p125^{FAK}$ tyrosine phosphorylation is regulated by growth factor ligands for receptor protein-tyrosine kinases, including PDGF-BB, a potent chemoattractant for vascular smooth muscle cells (45–47). $P125^{FAK}$ tyrosine phosphorylation is also stimulated by other chemoattractants including hyaluronan and the T-lymphocyte chemokine, RANTES (regulated on activation normal T cell-expressed) (48, 49). Tyrosine phosphorylation of $p125^{FAK}$ is associated in several cell types with that of paxillin, a 68-kDa protein that co-localizes to focal adhesions (45, 49–52). Paxillin has been reported to associate with $p125^{FAK}$ and is a putative substrate for $p125^{FAK}$ (53, 54). Further evidence for the role of $p125^{FAK}$ in cell migration has come from studies in knockout mice and from overexpression. Murine $p125^{FAK}$ knockout embryos displayed disorganized mesenchymal tissue architecture, and embryonic mesodermal fibroblasts deficient in $p125^{FAK}$ exhibited a decreased rate of cell movement compared with wild-type cells (55). Overexpression of $p125^{FAK}$ in Chinese hamster ovary cells was found to be associated with increased cell migration (56).

The role of $p125^{FAK}$ in endothelial cell signal transduction pathways stimulated by VEGF is unknown. In the present paper, we investigated the tyrosine phosphorylation events stimulated by VEGF in human endothelial cells including $p125^{FAK}$ and paxillin tyrosine phosphorylation. We report here that tyrosine phosphorylation of $p125^{FAK}$ and paxillin are rapid events in the signal transduction pathways stimulated by VEGF, which may play a role in the migratory cell response to this factor.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVECs were obtained from Clonetics and were routinely cultured in the manufacturer's own medium supplemented with 2% fetal bovine serum. For experimental purposes, primary cultures of HUVECs were dispersed by treatment with 0.05% trypsin, 0.02% EDTA for 5 min at 37 °C and then replated in either 90- or 35-mm plastic dishes. Cultures were maintained in a humidified atmosphere containing 5% $C0_2$ and 90% air at 37 °C. For experimental purposes, cells were plated either in 33-mm Nunc Petri dishes at 10^5 cells/dish, or in 90-mm dishes at 2.5×10^5 cells/dish and used after 6–8 days or when the cells had formed a confluent monolayer. In some experiments, cells were rendered quiescent by incubation with M199 medium containing 1% FCS and without other supplements for 24 h. The human endothelial cell line ECV.304 (57) was maintained and propagated in M199 medium supplemented with 10% FCS. For some experiments, aortic medial VSMC were cultured from rabbit aortas by the tissue explant method as described (46). VSMC were grown to confluence in DMEM containing 20% FCS and were rendered quiescent by incubation for 40 h in DMEM containing 0.5% FCS.

Assays of Cell Migration—Cell migration was measured in a modified Boyden chemotaxis chamber (NeuroProbe Inc., Cabin John, MD) essentially as described (46). Test chemoattractants were diluted in DMEM supplemented with 1% (w/v) bovine serum albumin (Sigma) and placed in the bottom wells of the chamber. Polycarbonate filters with 8-μm pores (Polyfiltronics) were preincubated in a 0.1% solution of collagen type I (Sigma) and placed between the chemoattractants and the upper chambers. Cells were trypsinized and washed twice in M199 and resuspended in M199 containing 1% (w/v) bovine serum albumin to give a final cell concentration of 3×10^5/ml. 15,000 cells were placed into each well in the upper chamber, and the chemotaxis chambers were routinely incubated at 37 °C for 6 h. After the incubation, unmigrated cells were removed from the upper side of the filters, and migrated cells were stained with Pro-Diff (Braidwood Laboratories, Beckenham, Kent, UK). Filters were mounted onto microscope slides, and stained cells were counted at × 200 magnification in four fields/well. In each individual experiment, chemotaxis was performed in four separate wells for each concentration of a given test substance under a specified condition. Each *n* value in the figure legends refers to the number of individual experiments.

Immunoprecipitations—Quiescent cultures of cells (approximately 10^6/immunoprecipitation) were washed twice with M199 medium, treated with peptide factors in 1 ml of this medium as indicated, and lysed at 4 °C in 1 ml of a solution containing 10 mM Tris/HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and 1% Triton X-100 (lysis buffer). Lysates were clarified by centrifugation at 15,000 × *g* for 10 min and precleared by incubation with albumin-agarose for 1 h at 4 °C. After removal of albumin-agarose by brief (10-s) centrifugation, the supernatants were transferred to fresh tubes for immunoprecipitation. Immunoprecipitations were routinely performed by incubating lysates with 1 μg/ml antibody as indicated for 3 h at 4 °C. Immunocomplexes were collected either by incubating lysates with protein A-agarose beads for a further 1 h or by incubating with 5 μg/lysate anti-mouse IgG for 1 h followed by a 1-h incubation with protein A-agarose beads. Immunoprecipitates were washed three times with lysis buffer, and proteins were extracted with 2 × SDS-PAGE sample buffer. Phosphotyrosyl proteins were immunoprecipitated with the anti-Tyr(P) mAb Py20. Immunoprecipitates were washed three times with lysis buffer and further analyzed by Western blotting.

Western Blotting—Treatments of quiescent cultures of cells with factors, cell lysis, and immunoprecipitations were performed as described above. After SDS-PAGE, proteins were transferred to Immobilon membranes (Millipore Corp.). Membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 3–5 h in phosphate-buffered saline, 0.05% Tween-20 containing either anti-Tyr(P) or protein-specific antibodies (1 μg/ml of each) as indicated. Immunoreactive bands were visualized either by chemiluminescence using horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG and ECL™ reagent or using ^{125}I-labeled sheep anti-mouse IgG or protein A as indicated.

Assays of PI 3-Kinase Activity—PI 3-kinase was determined by measuring phosphatidylinositol phosphorylation in anti-phosphotyrosine immunoprecipitates as described (58, 59). Immunoprecipitates were washed three times with lysis buffer, once in 50 mM Hepes, pH 7.5, and once in PI 3-kinase assay buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA). Immunoprecipitates were preincubated in 25 μl of PI 3-kinase assay buffer and 10 μl of phosphatidylinositol for 20 min at 4 °C. In some experiments, inhibitors of PI 3-kinase were also added to immunoprecipitates for this preincubation period. Reactions were initiated by the addition of 15 μl of assay mixture containing 10 μCi of [γ-^{32}P]ATP, 100 μM ATP, and 10 mM $MgCl_2$, and incubations were routinely performed for 10 min at room temperature. Reactions were terminated by the addition of 100 μl of 1 N HCl followed by the addition of 200 μl of a 1:1 mix of $CHCl_3$ and methanol. Samples were vortexed for 20 s, and the phases were separated by centrifugation at 15,000 × *g* for 2 min. The lower $CHCl_3$ phase was collected and washed with 80 μl of a 1:1 mix of 1 N HCl and methanol, and the phases were separated by centrifugation as before. The lower phase was collected and applied to LK6D6 silica gel TLC plates (Whatman), which had been presprayed with 1% potassium oxalate and allowed to dry prior to sample application. TLC plates were routinely developed for 45 min using a 29.2:180:10.8:140 mixture of H_2O, $CHCl_3$, NH_4OH, and methanol, respectively. Developed TLC plates were dried and exposed to x-ray film for 1–3 days.

MAP Kinase Assay—Cells were treated with factors as indicated, washed rapidly twice with ice-cold PBS, and immediately extracted by the addition of 100 μl of boiling 2 × SDS-PAGE sample buffer. Cell extracts were collected by scraping, heated to 95 °C for 10 min, and run on 12.5% acrylamide SDS-PAGE gels. Following transfer to Immobilon membranes, proteins were immunoblotted with an antibody that specifically recognizes p42 and p44 MAP kinases (extracellular signal-regulated kinases 1 and 2) activated by phosphorylation at Tyr^{204} (60).

Immunofluorescent Staining—HUVECs were cultured on glass coverslips and allowed to grow to confluence. Following treatments, cells were washed three times with ice-cold PBS and then fixed in 3% paraformaldehyde in PBS for 30 min at 4 °C. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and

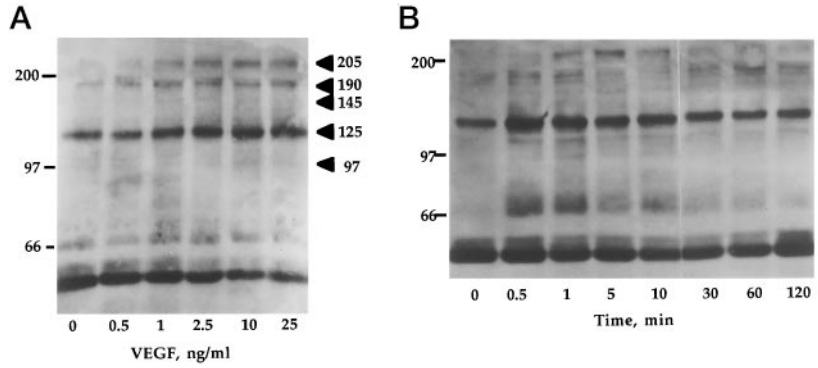

FIG. 1. **VEGF stimulation of tyrosine phosphorylation in HUVECs.** Confluent cultures of HUVECs were washed with DMEM and incubated in 1 ml of the same medium and in the presence of either different concentrations of VEGF for 10 min (*A*) or with 10 ng/ml VEGF for different times (*B*). Cells were lysed, and anti-Tyr(P) immunoprecipitates were prepared and immunoblotted with Py20. Protein bands of 205, 145, 125, 97, and 68 kDa are indicated by *arrowheads*. The positions of molecular weight markers are indicated on the *left*. The results shown are representative of three independent experiments.

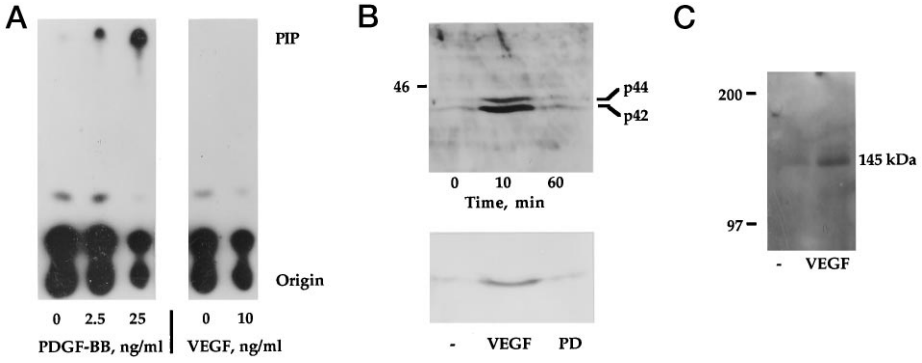

FIG. 2. **Effect of VEGF on PI 3-kinase activity, MAP kinase activation, and PLC-γ tyrosine phosphorylation in HUVECs.** *A*, confluent cultures of HUVECs were stimulated with 10 ng/ml VEGF for 10 min and then lysed. Anti-Tyr(P) immunoprecipitates were prepared, and PI 3-kinase activity in immunoprecipitates was assayed as described under "Experimental Procedures." In parallel, anti-Tyr(P) immunoprecipitates were prepared from VSMC treated either in the absence or presence of 25 ng/ml PDGF-BB for 10 min. Approximately equivalent numbers of HUVECs and VSMC were used in these experiments. The results shown are representative of two independent experiments. The positions of the origin and of phosphatidylinositol phosphate are indicated. *B, top*, cells were treated for the indicated times with 10 ng/ml VEGF. Whole cell extracts were then prepared and analyzed by SDS-PAGE followed by immunoblotting with an antibody specific for the activated tyrosine-phosphorylated form of MAP kinase. *Bottom*, cells were either untreated (*lanes 1* and *2*) or pretreated for 1 h with 10 μM PD98059 (*lane 3*). The cells were then treated for 10 min with either VEGF (*lanes 2* and *3*) or an equivalent volume of solvent (*lane 1*). Cells treated with VEGF alone also received an equivalent volume of solvent. Cell extracts were prepared, and MAP kinase activity was determined by Western blotting as described under "Experimental Procedures." *C*, confluent HUVECs were treated for 10 min with 10 ng/ml VEGF, and anti-Tyr(P) immunoprecipitates were then prepared and immunoblotted with a specific antibody to PLC-γ. The position of the major 145-kDa PLC-γ-immunoreactive band detected is indicated on the *right*, and positions of molecular weight markers are indicated on the *left*.

then washed three times in PBS. Fixed and permeabilized cells were incubated with primary antibody for 1 h at room temperature, washed three times in PBS, and then incubated for 45 min at room temperature with a secondary antibody conjugated to FITC. Cells were finally washed three times (5 min each wash) in PBS. Coverslips were mounted onto microscope slides using Vectashield mounting medium. Filamentous actin was stained with FITC-phalloidin in PBS (1 μg/ml) for 20 min at room temperature. Immunofluorescent staining was observed and photographed using a Zeiss Axiophot epifluorescence microscope fitted with a × 63 (numerical aperture 1.4, oil) objective lens.

Materials—Recombinant VEGF was obtained either from Upstate Biotechnology, Inc. or from R & D Systems. Cytochalasin D was from Sigma. Wortmannin was obtained either from Cambridge Bioscience or from Sigma, and PD98059 was obtained from Calbiochem. The BC3 polyclonal antibody to $p125^{FAK}$ was a gift of Professor Thomas Parsons (University of Virginia). Py20 anti-Tyr(P) mAb and mAbs to $p125^{FAK}$, paxillin, Pyk2, p85α, and PLC-γ were from Transduction Laboratories, Inc. 4G10 anti-Tyr(P) mAb was from TCS biologicals Ltd. Antibody to the activated phosphorylated form of p42/p44 MAP kinase was purchased from New England Biolabs Inc. The U13 antibody to p85α was a gift of Mike Waterfield (Ludwig Institute of Cancer Research, London). PDGF-BB, Protein A-agarose, goat anti-rabbit IgG, and goat anti-mouse IgG were from Oncogene Science, Inc. ECL™ reagents and horseradish peroxidase-conjugated anti-mouse IgG were from Amersham, UK. All other reagents used were of the purest grade available.

RESULTS

Treatment of cultured HUVECs stimulated the tyrosine phosphorylation of multiple protein bands as detected by anti-Tyr(P) blots of anti-Tyr(P) immunoprecipitation. The major bands phosphorylated were of M_r 205,000, 185,000, 145,000, 125,000, 100,000, and 68,000 (Fig. 1). The predicted molecular mass of both KDR and Flt-1 VEGF receptors is approximately 150 kDa, but due to glycosylation of the extracellular domain, these proteins characteristically migrate in SDS-PAGE gels as bands of M_r 205,000. The effects of VEGF on PI 3-kinase activity, MAP kinase activation, and PLC-γ tyrosine phosphorylation were subsequently investigated. VEGF induced neither tyrosine phosphorylation of the p85α PI 3-kinase subunit as judged by immunoblot of anti-Tyr(P) immunoprecipitates with a specific p85α mAb (results not shown) nor an increase in PI 3-kinase activity measured in parallel anti-Tyr(P) immunoprecipitates (Fig. 2*A*). VEGF also failed to increase PI 3-kinase activity measured in immunoprecipitates prepared with a specific anti-p85α antibody (results not shown). It was verified in parallel assays that PDGF-BB induced PI 3-kinase activity in VSMC as measured either in anti-Tyr(P) or anti-p85α immunoprecipitates (Fig. 2*A* and results not shown). We also exam-

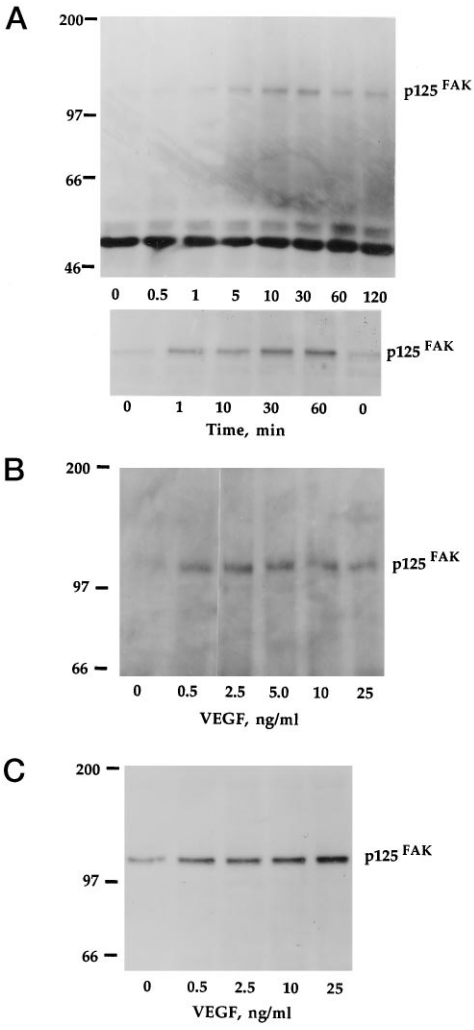

FIG. 3. **VEGF stimulates p125^{FAK} tyrosine phosphorylation in HUVECs.** Confluent cultures of HUVECs were washed with DMEM and incubated in 1 ml of the same medium and in the presence of either 10 ng/ml VEGF for different times (*A*) or with different concentrations of VEGF for 10 min (*B*). In some experiments, confluent cultures of ECV.304 cells were treated for 10 min with the indicated concentrations of VEGF (*C*). All cells used were lysed, and anti-Tyr(P) immunoprecipitates were prepared and immunoblotted with antibody to p125^{FAK}. The position of p125FAK is indicated on the *right*, and positions of molecular weight markers are indicated on the *left*. The results shown are representative of five (*A*), six (*B*), and two (*C*) independent experiments.

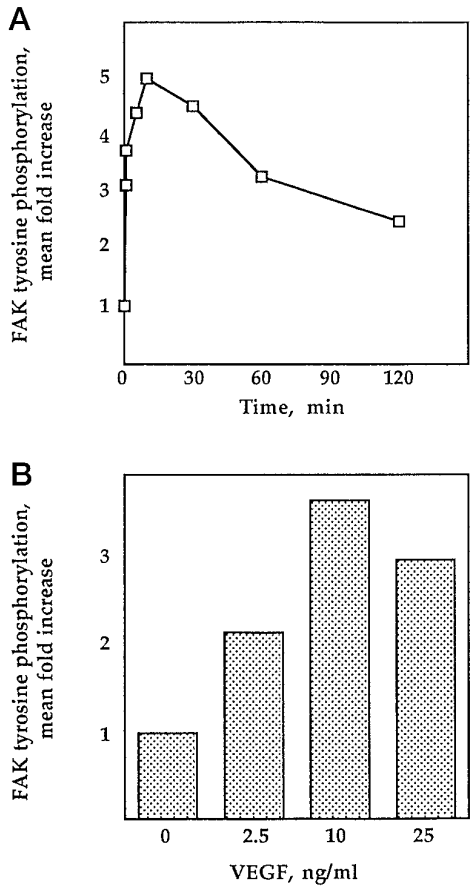

FIG. 4. **Semiquantification of time dependence and concentration dependence of VEGF-stimulated tyrosine phosphorylation.** Confluent cultures of HUVECs were washed with DMEM and incubated in 1 ml of the same medium and in the presence of either 10 ng/ml VEGF for different times (*A*) or with different concentrations of VEGF for 10 min (*B*). Autoradiograms obtained from these experiments similar to those shown in Fig. 2 were scanned, and p125^{FAK}-immunoreactive bands were quantified by scanning densitometry using an LKB Ultroscan XL densitometer. The values shown represent the mean -fold increases (A, $n = 5$; B, $n = 6$) in p125^{FAK} tyrosine phosphorylation above control unstimulated levels.

ined whether VEGF activated the MAP kinase cascade, a convergent pathway in the action of many growth factors. Western blotting of HUVEC extracts with an antibody specific for the activated tyrosine-phosphorylated form of MAP kinase showed that a 10-min treatment with VEGF caused a striking activation of MAP kinase, which declined to near the control unstimulated level after 60 min (Fig. 2*B*, *top*). VEGF increased activity of both p42 and p44 forms of MAP kinase corresponding to extracellular signal-regulated kinases 1 and 2, respectively, although it was consistently noted that activation of p42 MAP kinase was more prominent than that of the p44 form. VEGF stimulation of MAP kinase was completely inhibited by the specific MAP kinase kinase inhibitor PD98059 (61, 62), indicating that activation of MAP kinases by VEGF occurs through the kinase cascade that mediates activation of MAP kinase by other growth factors (Fig. 2*B*, *bottom*). VEGF also stimulated tyrosine phosphorylation of PLC-γ. Western blotting of anti-Tyr(P) immunoprecipitates with a specific PLC-γ mAb revealed a striking increase in a major 145-kDa band in HUVECs treated with 10 ng/ml VEGF for 10 min (Fig. 2*C*).

The results shown in Figs. 1 and 2 showed that VEGF stimulated the tyrosine phosphorylation of multiple protein bands, activated p42/p44 MAP kinases, stimulated PLC-γ tyrosine phosphorylation and failed to activate PI 3-kinase. It was next investigated whether the 125-kDa band tyrosine-phosphorylated in response to VEGF corresponded to p125^{FAK}. Confluent cultures of HUVECs were treated with 10 ng/ml VEGF for different times, and anti-Tyr(P) immunoprecipitates were prepared and blotted with a specific p125^{FAK} mAb. As shown in Fig. 3, VEGF markedly increased p125^{FAK} tyrosine phosphorylation. The effect of VEGF was rapid with a detectable increase as early as 1 min (Fig. 3*A*). Although the effect of VEGF was sustained at times of incubation up to 60 min, it was noted in some experiments that p125^{FAK} tyrosine phosphorylation declined after 30 min. In other experiments, however (Fig. 3*A*, *bottom*), VEGF-induced p125^{FAK} tyrosine phosphorylation did not decline at times up to 1 h after the addition of VEGF. Scanning of five independent experiments (shown in Fig. 4*A*) showed that the half-maximum effect occurred after 1 min and the maximum increase was at 10 min and that p125^{FAK} tyrosine phosphorylation declined to approximately half the maximum level of phosphorylation after 2 h. The maximum mean increase in p125^{FAK} tyrosine phosphorylation stimulated by VEGF after 10 min was 5-fold ($n = 5$) above control unstimu-

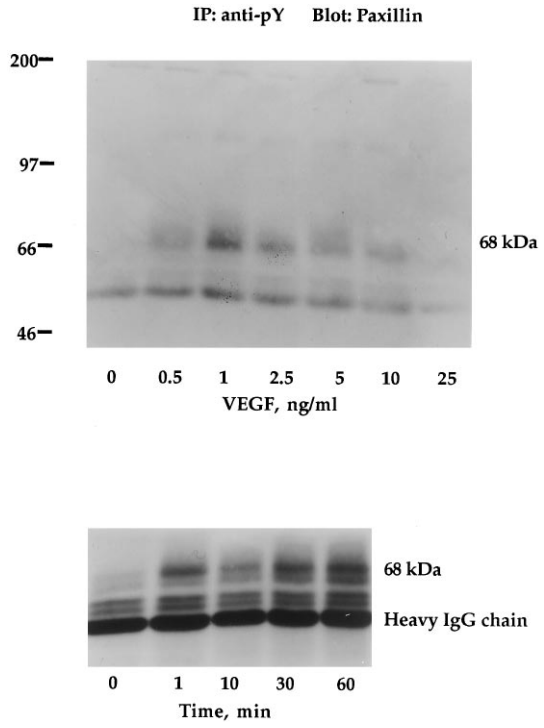

FIG. 5. **VEGF stimulates paxillin tyrosine phosphorylation in HUVECs.** Confluent cultures of HUVECs were washed with DMEM and incubated in 1 ml of the same medium and in the presence of either different concentrations of VEGF for 10 min (*top*) or with 10 ng/ml VEGF for different times (*bottom*). Cells were lysed, and anti-Tyr(P) immunoprecipitates were prepared and immunoblotted with antibody to paxillin. The position of paxillin (68 kDa) and of IgG heavy chain are indicated. The positions of molecular weight markers are indicated on the *left*. The results shown are representative of three independent experiments.

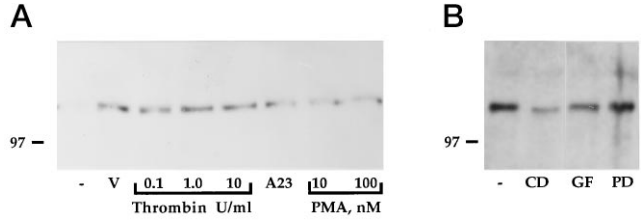

FIG. 6. **Effects of thrombin, PKC activation, MAP kinase kinase inhibition, and disruption of the actin cytoskeleton on p125^{FAK} tyrosine phosphorylation.** *A*, confluent HUVECs were treated for 10 min without (−) or with 10 ng/ml VEGF, thrombin at the concentrations shown, 100 nM A23187 (A23), or phorbol 12-myristate 13-acetate at the concentrations shown. Cells were lysed, and anti-Tyr(P) immunoprecipitates were prepared and immunoblotted with antibody to p125^{FAK}. The autoradiogram shown is representative of three independent experiments. The positions of molecular weight markers are indicated on the *left*. *B*, confluent cultures of HUVECs were pretreated for 1 h with 2 μM cytochalasin D (*CD*), or 3 μM GF109203X (*GF*), or 10 μM PD98059 (*PD*) or with an equivalent volume of Me_2SO (−). In each case, cells were then treated with 10 ng/ml VEGF for a further 10 min. Cells were lysed, and anti-Tyr(P) immunoprecipitates were prepared and immunoblotted with anti-p125^{FAK} antibody. The result shown is representative of three independent experiments. The positions of molecular weight markers are indicated on the *left*.

lated levels (Fig. 4*A*).

The effect of VEGF on p125^{FAK} tyrosine phosphorylation was also potent and concentration-dependent with a detectable increase as low as 0.5 ng/ml (Fig. 3*B*). In six independent experiments, the maximum increase in p125^{FAK} tyrosine phosphorylation was induced by 10 ng/ml VEGF, and a half-maximum increase was obtained at a concentration of 2.5 ng/ml (Fig. 4*B*). At concentrations of VEGF above 10 ng/ml, p125^{FAK} tyrosine phosphorylation partially declined (Figs. 3*B* and 4*B*) but remained significantly above unstimulated levels at the highest concentration tested (25 ng/ml). As shown in Fig. 3*C*, VEGF also induced a concentration-dependent increase in p125^{FAK} tyrosine phosphorylation in confluent cultures of the human endothelial cell line ECV.304. It was tested whether tyrosine phosphorylation of the major 125-kDa phosphotyrosyl band seen in anti-Tyr(P) blots of anti-Tyr(P) immunoprecipitates (Fig. 1) could be accounted for by Pyk2/CAKβ, a recently identified p125^{FAK}-related protein-tyrosine kinase (37, 38). VEGF did not stimulate tyrosine phosphorylation of Pyk2 as judged by immunoblotting of anti-Tyr(P) immunoprecipitates with specific Pyk2 antibody (results not shown).

To examine whether the 68-kDa focal adhesion-associated protein paxillin was also tyrosine-phosphorylated in HUVECs in response to VEGF, anti-Tyr(P) immunoprecipitates prepared from VEGF-treated cells were immunoblotted with a specific anti-paxillin antibody. Fig. 5 shows that VEGF increased paxillin tyrosine phosphorylation in HUVECs with a concentration dependence and kinetics similar to that obtained for p125^{FAK}. Compared with p125^{FAK}, paxillin tyrosine phosphorylation exhibited a more marked decline at higher concentrations (above 10 ng/ml) of VEGF. Scanning densitometry showed, however, that VEGF-stimulated paxillin phosphotyrosine content remained above the basal level even at the highest VEGF concentration (25 ng/ml) tested. The effect of 10 ng/ml VEGF on paxillin tyrosine phosphorylation was also rapid with a detectable increase as early as early as 1 min after the addition of VEGF, reached a maximum by 30 min, and was sustained for up to 60 min after the addition of VEGF. In three independent experiments the maximum mean increase in paxillin tyrosine phosphorylation was 5-fold above control levels.

The effects on p125^{FAK} tyrosine phosphorylation of other factors in HUVECs was also investigated. Thrombin, like VEGF, increases endothelial permeability (63, 64), and p125^{FAK} tyrosine phosphorylation is increased in thrombin-activated platelets (65, 66) and in thrombin-treated mesangial cells (67). Thrombin treatment of confluent HUVECs produced an increase in p125^{FAK} tyrosine phosphorylation in HUVECs comparable with the effect of VEGF (Fig. 6*A*). Since VEGF has been reported to activate phospholipase C-γ it was also examined whether the p125^{FAK} tyrosine phosphorylation pathway could be stimulated by agents that directly activate the signaling events distal to phospholipase C-γ activation. As shown in Fig. 6*A*, treatment with the biologically active phorbol ester, phorbol 12-myristate 13-acetate, which directly activates PKC, caused a weak stimulation of p125^{FAK} tyrosine phosphorylation compared with the effects of either VEGF or thrombin. A similarly weak effect was obtained with the Ca^{2+} ionophore A23187 (Fig. 6*A*).

The role of the PKC pathway in mediating VEGF-induced p125^{FAK} tyrosine phosphorylation was further examined using the selective PKC inhibitor, GF109203X. HUVECs were pretreated with the PKC inhibitor GF109203X at a concentration (3 μM) that completely blocks PKC activation in several cell types including Swiss 3T3 cells (43, 68). As shown in Fig. 6*B*, GF209103X caused a partial reduction in VEGF-stimulated p125^{FAK} tyrosine phosphorylation (Fig. 6*B*). Semiquantification by scanning densitometry showed that in three independent experiments the mean reduction in VEGF-stimulated p125^{FAK} tyrosine phosphorylation consequent upon pretreatment with GF109203X was approximately 40% (n = 3). In accord with findings in other cell types (43, 46, 65), it was verified in parallel cultures that disruption of the actin filament network in HUVECs by a 1-h pretreatment with 2 μM cytochalasin D completely inhibited VEGF stimulation of p125^{FAK} tyrosine phosphorylation (Fig. 6*B*). It was also exam-

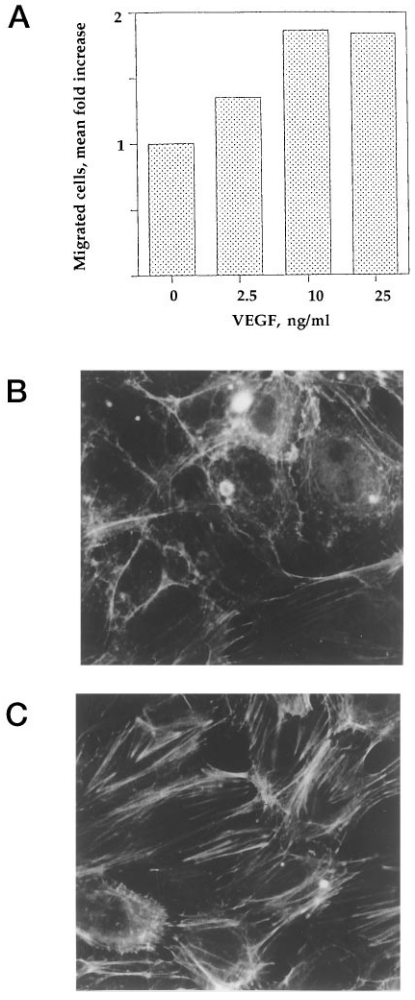

FIG. 7. **VEGF stimulation of actin filament formation and cell migration in HUVECs.** *A*, the effect of the indicated concentrations of VEGF on directed HUVEC migration was examined as described under "Experimental Procedures." Confluent cultures of HUVECs were treated in the absence (*B*) or presence (*C*) of 10 ng/ml VEGF for 30 min. Cells were fixed, permeabilized, incubated with FITC-phalloidin, and photographed as described under "Experimental Procedures." The results shown are representative of three independent experiments.

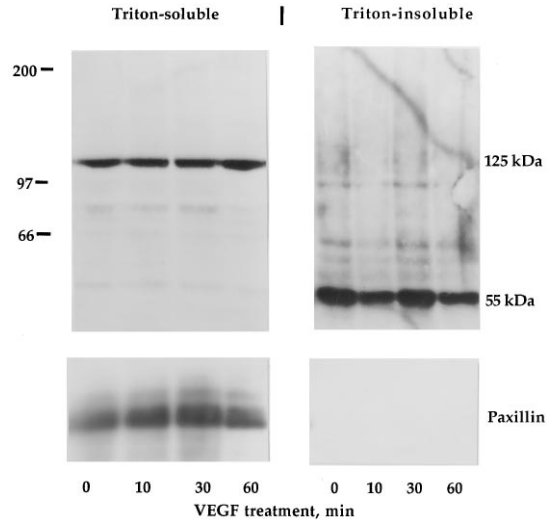

FIG. 8. **Effect of VEGF on Triton solubility of p125^{FAK}.** Confluent cultures of HUVECs were treated for the times indicated with 10 ng/ml VEGF. The cells were then lysed with 1% Triton X-100 under identical conditions to those used for immunoprecipitations described under "Experimental Procedures." Cell lysates were centrifuged at top speed in a microcentrifuge for 10 min. The supernatant was removed and was used as the Triton-soluble fraction. The pellet was retained and used as the Triton-insoluble fraction. The Triton-soluble fraction was adjusted to 1 × SDS-PAGE sample buffer, and the Triton-insoluble fraction was extracted into 1 × SDS-PAGE sample buffer. Each fraction was adjusted to the same total volume, and equal volumes of each fraction were loaded onto SDS-PAGE gels. Following SDS-PAGE and transfer to membranes, Western analysis was performed using specific antibody either to p125^{FAK} (*top*) or paxillin (*bottom*). The autoradiogram shown is representative of two independent experiments. The positions of 125- and 55-kDa p125FAK-immunoreactive species and of paxillin are indicated on the *right*, and the positions of molecular weight markers are indicated on the *left*.

ined whether inhibition of the MAP kinase cascade using the specific MAP kinase kinase inhibitor, PD98059, had any effect on p125^{FAK} tyrosine phosphorylation. The results showed that inhibition of MAP kinase activation by pretreatment for 1 h with 10 μM PD98059 had no inhibitory effect on VEGF stimulation of p125^{FAK} tyrosine phosphorylation (Fig. 6*B*).

VEGF stimulated the directed migration of HUVECs with a half-maximal effect at approximately 2.5 ng/ml and a maximum effect at 10 ng/ml (Fig. 7*A*). Since p125^{FAK} has been implicated in the regulation of actin cytoskeleton organization, we tested whether VEGF induced changes in the actin filament network. Staining of confluent cultures of HUVECs with FITC-phalloidin showed that in control unstimulated cells filamentous actin was characteristically organized in cortical arrays (Fig. 7*B*). VEGF treatment stimulated an increase in actin filament formation and in particular increased the number of transverse filament bundles that crossed the cell. (Fig. 7*C*).

We subsequently examined whether VEGF-induced signaling through the p125^{FAK} pathway was concomitant with changes in the association of p125FAK and paxillin with the actin cytoskeleton. First, we examined whether VEGF changed the distribution of p125^{FAK} and paxillin between Triton-soluble and Triton-insoluble compartments in HUVECs. Indeed, one plausible explanation for the results presented in Figs. 3–6 was that the VEGF-induced increases in p125^{FAK} and paxillin immunoreactivity recovered by anti-Tyr(P) immunoprecipitation (and/or the observed decline in p125^{FAK} tyrosine phosphorylation at higher VEGF concentrations and longer times of treatment) were due to VEGF-induced changes in the susceptibility of p125^{FAK} and paxillin to extraction by 1% Triton X-100. This was tested by performing direct Western blot analysis of 1% Triton X-100 lysates with antibody to p125^{FAK} and paxillin without prior immunoprecipitation. As shown in Fig. 8 (*top*), the conditions under which lysates were normally prepared for immunoprecipitation resulted in the extraction of virtually the entire pool of cellular immunoreactive p125^{FAK}. VEGF treatment for various times up to 60 min had no significant effect on the extraction of p125^{FAK} as judged using either an anti-FAK mAb directed against a portion of it comprising residues 354–533 (Fig. 8) or the BC3 polyclonal anti-FAK antibody, which specifically recognizes the carboxyl-terminal noncatalytic region of FAK (results not shown). Very similar results were obtained for paxillin (Fig. 8, *bottom*). In contrast, the Triton-insoluble fraction contained very little or no immunoreactive p125^{FAK} and paxillin. It was, however, consistently noted that the Triton-insoluble fraction contained a major p125^{FAK}-immunoreactive band of approximate M_r 55,000, which was either absent or much more weakly recognized in the Triton-soluble fraction. The 55-kDa band was seen in Triton-insoluble extracts most strongly by the anti-FAK mAb directed against amino acid residues 354–533 (Fig. 8, *top*), very weakly by BC3, and was also recognized by an antibody that specifically recognizes the amino terminus of p125^{FAK} (results not shown). No paxillin-immunoreactive species were found in the Triton-insoluble fraction.

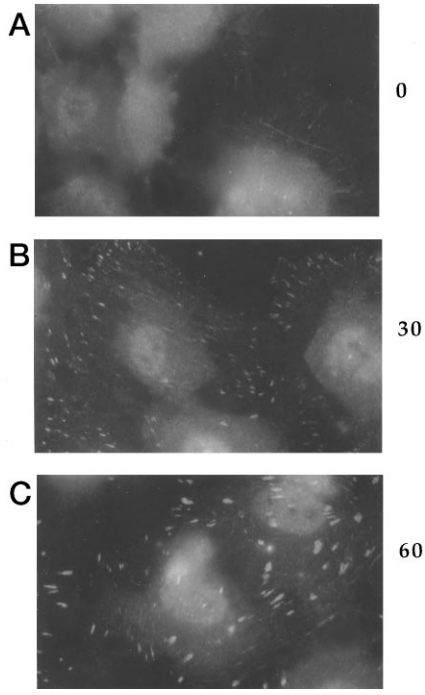

FIG. 9. **VEGF stimulates p125^{FAK} immunofluorescent staining of focal adhesions in HUVECs.** Confluent cultures of HUVECs were either untreated (controls, *panel A*) or treated with 10 ng/ml VEGF for 30 min (*panel B*) or 60 min (*panel C*). Cells were fixed, permeabilized, and incubated with antibody to p125^{FAK} (*A–C*). Immunofluorescent staining was performed and was observed and photographed as described under "Experimental Procedures." The photographs shown were taken using a × 63 apochromat objective (numerical aperture 1.4, oil). The results shown are representative of at least 20 different fields observed in each experiment and of six similar independent experiments.

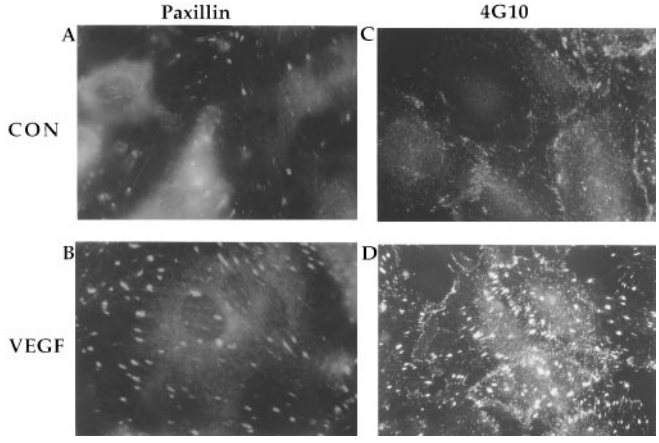

FIG. 10. **VEGF stimulates paxillin and phosphotyrosine immunofluorescent staining of focal adhesions in HUVECs.** Confluent cultures of HUVECs were either untreated (controls, *panels A* and *C*) or treated with 10 ng/ml VEGF for 60 min (*panels B* and *D*). Cells were fixed, permeabilized, and incubated with antibody to paxillin (*A* and *B*) or with 4G10 anti-Tyr(P) mAb (*C* and *D*). Immunofluorescent staining was performed and was observed and photographed as described under "Experimental Procedures." The photographs shown were taken using a × 63 apochromat objective (numerical aperture 1.4, oil). The photographs shown are representative of at least 20 different fields observed in each experiment and of five (paxillin) and three (4G10) similar independent experiments.

To further examine the functional significance of VEGF-stimulated p125^{FAK} tyrosine phosphorylation, the localization of p125^{FAK} in HUVECs was examined by immunofluorescent staining. In control, unstimulated cells (Fig. 9*A*), p125^{FAK} immunostaining was predominantly diffuse with weak cytoplasmic staining and some staining putatively of the nuclear or juxtanuclear region. Anti-p125^{FAK} staining of focal adhesions, which appear as characteristically elongated dashes or short streaks, was generally weak in unstimulated cells, and p125^{FAK}-immunoreactive focal adhesions were relatively sparse and poorly defined. In contrast, treatment of HUVECs for 30 min with 10 ng/ml VEGF caused a striking increase in anti-p125^{FAK} immunostaining of focal adhesions and a large increase in the number of focal adhesions per cell (Fig. 9*B*). The increase in p125^{FAK} immunostaining of focal adhesions was also evident after treatment with VEGF for 10 min (results not shown) and was sustained after 1 h (Fig. 9*C*) and for up to 4 h (results not shown). VEGF-induced p125^{FAK} immunostaining occurred both at the cell edges, where, frequently, clusters of short streaks of staining were aligned in parallel, and in the cell interior. It was noteworthy that p125^{FAK} staining in the putative nuclear region consistently exhibited a discrete granular or dotty configuration characteristically interspersed with areas in which staining was absent. It was verified that incubation with the FITC-conjugated secondary antibody alone produced no staining above background.

VEGF also caused a less marked increase in paxillin immunofluorescent staining of focal adhesions in HUVECs (Fig. 10, *A* and *B*). Control cells exhibited strong diffuse paxillin immunofluorescent staining in the putative perinuclear region, and strongly stained focal adhesion-like structures were apparent but relatively few in number (Fig. 10*A*). Similar to the results obtained with p125^{FAK}, VEGF treatment stimulated a noticeable increase in paxillin staining of focal adhesions (Fig. 10*B*). Paxillin mAb immunostaining was present both in focal adhesions and in filamentous arrays aligned in parallel both at the cell periphery and in the cell interior. A VEGF-induced increase in paxillin immunostaining of focal adhesions was evident as early as 10 min after the addition of VEGF and was sustained for up to 4 h (the longest time examined). VEGF also induced some increase in immunofluorescent staining of vinculin in focal adhesions, although the increase was less marked than that of p125^{FAK}. Immunolocalization of vinculin also showed considerable staining of intercellular junctions in confluent cultures of HUVECs, consistent with the association of vinculin with the endothelial adherens junction (69) (results not shown).

VEGF-induced changes in p125^{FAK} and paxillin immunofluorescent staining of focal adhesions in HUVECs were paralleled by similar changes in tyrosine phosphorylation in focal adhesions as determined by immunofluorescent staining with the 4G10 anti-Tyr(P) mAb. Control untreated cells exhibited weak focal adhesion and filamentous staining (Fig. 10*C*). VEGF induced a marked increase in the immunofluorescent staining of focal adhesions and filaments and an increase in the number of immunostained structures (Fig. 10*D*). The overall pattern of anti-Tyr(P) immunostaining of cytoskeletal structures was very similar in appearance to that observed with p125^{FAK} and paxillin antibodies. A VEGF-induced increase in 4G10 anti-Tyr(P) immunostaining of focal adhesions and actin filaments was also evident after 10 min (results not shown), and similar results were obtained with a different anti-Tyr(P) mAb, Py20 (results not shown).

DISCUSSION

The results presented here show that in cultures of HUVECs, VEGF induced a striking increase in the tyrosine phosphorylation of the focal adhesion-associated proteins p125^{FAK} and paxillin. The effect of VEGF was both rapid and concentration-dependent. The concentration dependence for VEGF-induced p125^{FAK}/paxillin tyrosine phosphorylation is similar to that observed for the effects of VEGF on mitogenesis in endothelial

cells (29) and in the present paper also correlated with the concentration dependence for the chemotactic response of HUVECs to VEGF. Since HUVECs are known to express both KDR and Flt-1 receptors, it is unclear at present which of these receptors is responsible for mediating VEGF activation of the $p125^{FAK}$/paxillin pathway.

The tyrosine phosphorylation of $p125^{FAK}$ and paxillin was noticeably biphasic with respect to both time of treatment with VEGF and to VEGF concentration. It is plausible that the decline in $p125^{FAK}$ tyrosine phosphorylation could result from disruption of the actin cytoskeleton at higher VEGF concentrations. In this context it is noteworthy that VEGF has been reported to induce disorganization of actin stress fibers in Balb/c 3T3 cells (70). For several reasons, however, our results argue against this interpretation. First, the finding that VEGF stimulation of $p125^{FAK}$ tyrosine phosphorylation was completely abolished by the actin-depolymerizing agent cytochalasin D indicates that, in accord with previous findings in other cell types, activation of the $p125^{FAK}$ pathway in HUVECs by VEGF is critically dependent on the integrity of the actin filament network. Second, VEGF-stimulated $p125^{FAK}$ tyrosine phosphorylation and focal adhesion-associated $p125^{FAK}$ and paxillin immunofluorescent staining remained above control, unstimulated levels at all of the VEGF concentrations examined and for prolonged times of treatment. Third, VEGF at high concentrations did not cause any noticeable perturbations in HUVEC actin filament organization (results not shown). We therefore conclude that the decline in $p125^{FAK}$/paxillin tyrosine phosphorylation at higher VEGF concentrations, particularly noticeable in the case of paxillin, is unlikely to be related to any disruptive effect of VEGF on the actin cytoskeleton similar to the effects of high concentrations of PDGF in Swiss 3T3 cells (45). Alternatively, the decline in tyrosine phosphorylation after longer times of treatment and at higher concentrations may be related to internalization and down-regulation of VEGF receptors (71). Consistent with this possibility, tyrosine phosphorylation of the 205-kDa band corresponding to the VEGF receptor also exhibited a decline at longer times of treatment.

The major site of tyrosine phosphorylation of $p125^{FAK}$ is Tyr^{397}. Tyr^{397} is both a site of autophosphorylation and is required for association of $p125^{FAK}$ with the Src homology 2 domains of both Src family kinases and of the p85α subunit of PI 3-kinase (72, 73). Phosphorylation of Tyr^{925} is induced by cell attachment to fibronectin and is a binding site for the adaptor protein Grb-2 (74). $p125^{FAK}$ can also be tyrosine-phosphorylated by Src family kinases at amino acids Tyr^{407}, Tyr^{576}, and Tyr^{577}, and in addition to Tyr^{397}, Tyr^{576}, and Tyr^{577} may also be necessary for maximum enzymatic activity (75). The putative substrates and sites for $p125^{FAK}$-catalyzed tyrosine phosphorylation are still poorly understood, but a specific residue in paxillin, Tyr^{118}, is phosphorylated by $p125^{FAK}$ *in vitro* (76). Since Tyr^{118} is located in one of the consensus binding sites in paxillin for the adaptor protein Crk (77), it is possible that phosphorylation at this site may serve to modulate association of paxillin with Crk. Identification of the sites of $p125^{FAK}$ and paxillin phosphorylation induced by VEGF is likely to be valuable in elucidating the effects of VEGF on associations between $p125^{FAK}$/paxillin and other signaling molecules.

The mechanism(s) that mediated VEGF stimulation of the $p125^{FAK}$ pathway were also investigated. PI 3-kinase has recently been implicated in the regulation of $p125^{FAK}$. Studies with mutant receptors have shown a requirement for the PI 3-kinase binding motif in PDGF-induced cell migration and membrane ruffling (78–80). Furthermore, $p125^{FAK}$ can associate with both the Src homology 2 and 3 domains of p85α (66, 73) and it was recently reported that stimulation of $p125^{FAK}$ tyrosine phosphorylation by PDGF in Swiss 3T3 cells requires PI 3-kinase (81). The Flt-1 VEGF receptor has been shown to interact in a yeast two-hybrid system with p85α via a specific residue in the Flt-1 cytoplasmic domain, Tyr^{1213} (82), but to date, however, there is no direct evidence that either KDR or Flt-1 can associate with p85α *in vivo*. The results presented here show that VEGF neither stimulates PI 3-kinase nor p85α tyrosine phosphorylation in HUVECs, thus arguing against the involvement of this pathway in VEGF stimulation of $p125^{FAK}$/paxillin tyrosine phosphorylation. It is not precluded that VEGF may be acting through a distinct PI 3-kinase pathway possibly independent of p85α tyrosine phosphorylation, which we were unable to detect using available reagents. The role of VEGF stimulation of the MAP kinase cascade was also investigated. Recent reports showing that integrin engagement leads to activation of MAP kinases (83, 84) raise the possibility that this pathway may mediate stimulation of $p125^{FAK}$ tyrosine phosphorylation. The finding that a specific inhibitor of this pathway had no effect on VEGF-induced $p125^{FAK}$ tyrosine phosphorylation suggests that VEGF activates the $p125^{FAK}$ pathway independently of MAP kinase activation. This finding is consistent with the report that integrin-mediated activation of MAP kinase is dependent on Ras activation and can be dissociated from adhesion-dependent activation of $p125^{FAK}$, cell spreading and focal adhesion, and stress fiber formation (85). We conclude that VEGF regulation of the p125FAK pathway can be dissociated from activation of both PI 3-kinase and MAP kinase. The fact that VEGF has been reported to stimulate PLC-γ implicated PKC activation as a possible mediator of VEGF stimulation of the $p125^{FAK}$ pathway in HUVECs. Previous findings show that bombesin stimulation of $p125^{FAK}$/paxillin tyrosine phosphorylation in Swiss 3T3 cells cannot be accounted for by either the mobilization of Ca^{2+} or PKC (43). The findings presented here show that direct activation of PKC by the biologically active phorbol ester phorbol 12-myristate 13-acetate had a weak effect on $p125^{FAK}$ tyrosine phosphorylation relative to those of either VEGF or thrombin, and a selective PKC inhibitor only partially inhibited VEGF-stimulated $p125^{FAK}$ tyrosine phosphorylation. These results suggest that while PKC may partially contribute to VEGF stimulation of the $p125^{FAK}$ pathway, it is likely that other signaling pathways are involved. Identification of the relevant signal transduction events involved warrants further investigation.

Stimulation of the $p125^{FAK}$/paxillin pathway in HUVECs was accompanied by a marked VEGF-induced increase in the localization of both $p125^{FAK}$ and paxillin to focal adhesions and filamentous structures. It is most likely that the filamentous immunofluorescent staining produced by $p125^{FAK}$, paxillin, and anti-phosphotyrosine antibodies is due to decoration of actin filaments. The fact that VEGF treatment also stimulated an increase in tyrosine phosphorylation in focal adhesions is consistent with the notion that an increase in $p125^{FAK}$/paxillin tyrosine phosphorylation occurs concomitantly and is possibly a prerequisite for VEGF-induced localization of these components to focal adhesions. Increased immunofluorescent staining of focal adhesions could reflect either *de novo* formation of focal adhesions and/or VEGF-stimulated recruitment of $p125^{FAK}$ and paxillin to nascent focal adhesions. The fact that formation of focal adhesions has been shown to occur concomitantly with stress fiber formation (35, 36) and that VEGF also increased stress fiber formation in HUVECs tends to support the former possibility, namely that immunolocalization of $p125^{FAK}$/paxillin in focal adhesions reflects assembly of focal adhesions. It should be emphasized, however, that previous studies have been performed largely in immortalized Swiss 3T3

cells, and results obtained in these cells may not be readily applicable to primary cultures of other cell types. Immunofluorescent staining of the focal adhesion component, vinculin, showed some increase in response to VEGF, but this appeared to be less marked than that of $p125^{FAK}$, suggesting that some components in HUVECs may remain constitutively associated in nascent focal adhesions. Furthermore, the presence of considerable diffuse cytoplasmic staining of $p125^{FAK}$ and paxillin, as well as some more discrete staining possibly of the nuclear and perinuclear regions, suggests the existence of substantial non-cytoskeletal-associated pools of these molecules, which could provide a source for recruitment. While focal adhesion formation remains the most likely explanation for increased immunofluorescent localization of $p125^{FAK}$ to these structures, we do not rule out the possibility that active recruitment of $p125^{FAK}$ (and possibly other components) to nascent focal adhesions may also occur. VEGF-induced immunolocalization of $p125^{FAK}$ and paxillin to focal adhesions was not accompanied by an apparent translocation of these components to the Triton-insoluble fraction of VEGF-stimulated HUVECs. This contrasts with a previous report that $p125^{FAK}$ becomes translocated to the actin cytoskeleton in thrombin-stimulated platelets (66), possibly reflecting differences either in the way that $p125^{FAK}$ associates with the actin cytoskeleton or in the manner that VEGF in HUVECs and thrombin in platelets affect the interaction of $p125^{FAK}$ with the actin cytoskeleton.

It was noteworthy that the Triton-insoluble HUVEC fraction was enriched in a 55-kDa $p125^{FAK}$-immunoreactive species, which was poorly detected in the Triton-soluble fraction. Several variant species of FAK have been reported, including a widely expressed $p41/p43^{FRNK}$ and a species truncated at the amino terminus (86, 87). Since the antibody used for detection of this fragment is directed to a region of the $p125^{FAK}$ molecule comprising amino acid residues 354–533, it is unlikely that the 55-kDa species represents a larger version of $p41/p43^{FRNK}$ that comprises only the noncatalytic carboxyl-terminal domain (86). It is also unlikely that p55 is simply a product of proteolytic breakdown, since extractions were performed in the presence of protease inhibitors and p55 was more weakly detected in the Triton-soluble fraction that contained almost all of the immunoreactive parent 125-kDa species. Determination of whether p55 represents a novel variant of $p125^{FAK}$ or is nonspecifically recognized by antibodies to $p125^{FAK}$ will require further experimental work.

The signaling pathways through which VEGF elicits its diverse biological effects in target cells have remained elusive. VEGF has previously been reported to stimulate the directed migration of endothelial cells (29), but the mechanisms involved have not previously been investigated. The findings that VEGF stimulated $p125^{FAK}$ and paxillin tyrosine phosphorylation and promoted their recruitment to focal adhesions are consistent with a role for these components in VEGF stimulation of endothelial cell migration. It is likely that the stimulation of endothelial cell motility, particularly *in vivo*, involves a more extensive network of signaling events distal to $p125^{FAK}$. Tyrosine phosphorylation of components of the epithelial and endothelial cells adherens junction, including cadherins and catenins, is associated with loss of integrity of intercellular adhesions and increased cell motility (87–89). Although we found no evidence for localization of $p125^{FAK}$ and paxillin to intercellular junctions, since components of focal adhesions and of the endothelial adherens junctions are both linked to the actin cytoskeleton, it is attractive to speculate that tyrosine phosphorylation of components of focal adhesions and of adherens junctions may be functionally integrated through a common program of signaling events in the migration of endothelial cells. Consistent with this possibility, we found that VEGF induces tyrosine phosphorylation of the vascular endothelium-specific cadherin-5 and of β-catenin in HUVECs.[2] Further experimental work is necessary to fully delineate the pathways and components mediating the regulation of the actin cytoskeleton network by VEGF.

VEGF regulation of the $p125^{FAK}$/paxillin pathway may have other implications for the function of endothelial cells. Since VEGF and thrombin increase the permeability of endothelial cell monolayers, the finding that both of these factors stimulate $p125^{FAK}$ tyrosine phosphorylation suggests that this pathway, possibly in conjunction with tyrosine phosphorylation-mediated disruption of adherens junction integrity, is involved in the regulation of endothelial permeability. VEGF has recently been shown to act as a survival factor for endothelial cells by preventing apoptosis (90). In this context, recent findings suggesting that $p125^{FAK}$ can suppress "anoikis," a subset of apoptosis induced in epithelial and endothelial cells (91, 92), raises the intriguing possibility that the $p125^{FAK}$ pathway may also participate in VEGF-induced signaling related to cell survival. Regardless of the precise functional role(s) of the $p125^{FAK}$ pathway in the endothelium, these results identify $p125^{FAK}$ and paxillin as components in a signal transduction pathway in the action of VEGF that may be a point of convergence in the regulation of several key endothelial cell functions, all of which are critically dependent upon interactions between the cell surface and the actin cytoskeleton.

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