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 Mr 205,000, corresponding to the VEGF receptors Flt-1 and KDR, and Mr 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 (p125FAK), which was both rapid and concentration-dependent. VEGF produced similar effects on p125FAK 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 p125FAK. Thrombin and the phorbol ester, phorbol 12-myristate 13-acetate, also increased p125FAK tyrosine phosphorylation in HUVECs. The effect of VEGF on p125FAK 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 had no effect on p125FAK tyrosine phosphorylation. VEGF stimulated migration and actin stress fiber formation in confluent HUVEC, and VEGF-induced p125FAK/paxillin tyrosine phosphorylation was accompanied by increased immunofluorescent staining of p125FAK, paxillin, and phosphotyrosine in focal adhesions in confluent cultures of HUVECs. These findings identify p125FAK 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...
also promotes the migration of endothelial cells, and it is increasingly recognized that endothelial cell migration plays an essential role in angiogenesis and vascular remodeling. There is increasing evidence that p125FAK 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 β1 and β2 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 p125FAK 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). Paxillin and other chemoattractants including hyaluronan and the T-lymphocyte chemokine, RANTES (regulated on activation normal T cell-expressed) (48, 49). Tyrosine phosphorylation of p125FAK is associated in several cell types with that of paxillin, a 68-kDa protein that colocalizes to focal adhesions (45, 49–52). Paxillin has been reported to associate with p125FAK and is a putative substrate for p125FAK (53, 54). Further evidence for the role of p125FAK in cell migration has come from studies in knockout mice and in overexpression. Murine p125FAK knockout embryos displayed disorganized mesenchymal tissue architecture, and embryonic mesodermal fibroblasts deficient in p125FAK exhibited a decreased rate of cell movement compared with wild-type cells (55). Overexpression of p125FAK in Chinese hamster ovary cells was found to be associated with increased cell migration (56).

The role of p125FAK 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 p125FAK and paxillin tyrosine phosphorylation. We report here that tyrosine phosphorylation of p125FAK 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% CO2 and 90% air at 37 °C. For experimental purposes, cells were plated either in 33-mm Nunc Petri dishes at 105 cells/dish or in 90-mm dishes at 2.5 × 105 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 vesicles were collected from rabbit aortas by the tissue explant method as described (46). Vesicles 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 × 105/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, unimplanted 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 on 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 value in the figure legends refers to the number of individual experiments.

Immunoprecipitations—Quiescent cultures of cells (approximately 106 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 Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and 1% Triton X-100 (lysis buffer). Lysates were clarified by centrifugation at 1 h for 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 lysate protein-A-agarose beads for a further 1 h or by incubating with 5 μg/l 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 125I-labeled sheep anti-mouse IgG or protein A as indicated.

Assays of PI 3-Kinase Activity—PI 3-kinase was determined by measuring phosphotyidylinositol phosphorylation in anti-phosphotyrosine immunoprecipitates as described (58, 59). Incubations 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-kine assay buffer and 10 μl of phosphotyridylinositol 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 addition of a final concentration of 1 μg/ml of [γ-32P]ATP, 100 μM ATP, and 10 mM MgCl2, 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 CHCl3 and methanol. Samples were vortexed for 20 s, and the phases were separated by centrifugation at 15,000 × g for 2 min. The lower CHCl3 phase was collected and washed with 80 μl of 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 prepsayed 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 H2O, CHCl3, N H4OH, 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 immunoblottedted with an antibody that specifically recognizes p42 and p44 MAP kinases (extracellular signal-regulated kinases 1 and 2) activated by phosphorylation at Tyr204 (60).

Immunofluorescence 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 stained with 1:200 dilution of anti-paxillin antibody (Chemicon International). Cells were washed with washing buffer and incubated for 1 h at room temperature with secondary antibody: fluorescein isothiocyanate (FITC) conjugated against mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Coverslips were mounted on slides in mounting medium (Vector Laboratories, Burlingame, CA) and viewed using a Zeiss Axioshot microscope (Carl Zeiss, Thornwood, NY) equipped with a Metamorph video camera (Universal Imaging Co., West Chester, PA) and a personal computer. Images were recorded on enhanced video tape using a recording system (Beckman Instruments, Fullerton, CA).
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 p125FAK was a gift of Professor Thomas Parsons from Sigma, and PD98059 was obtained from Calbiochem. Wortmannin was obtained either from Cambridge Bioscience or from Sigma. PDGF-BB, Protein A-agarose, goat anti-rabbit IgG, and goat anti-mouse IgG were from Amersham, mouse IgG were from Oncogene Science, Inc. ECL reagents and horse-radish 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 Mr 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 Mr 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 nor an increase in PI 3-kinase activity measured in parallel anti-Tyr(P) immunoprecipitates (Fig. 2A). VEGF also failed to increase PI 3-kinase activity measured in immunoprecipitates prepared with a specific anti-p85α antibody (results not shown) nor an increase in PI 3-kinase activity measured in parallel anti-Tyr(P) immunoprecipitates (Fig. 2A). VEGF also failed to increase PI 3-kinase activity measured in parallel anti-Tyr(P) immunoprecipitates (Fig. 2A and results not shown). We also exami-
ined whether VEGF activated the MAP kinase cascade, a con-
vergent 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 activa-
tion of MAP kinase, which declined to near the control un-
stimulated level after 60 min (Fig. 2B, top). VEGF increased
activity of both p42 and p44 forms of MAP kinase corresponding
to extracellular signal-regulated kinases 1 and 2, respec-
tively, 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. 2B, bottom). VEGF also
stimulated tyrosine phosphorylation of PLC-γ. Western blot-
ing 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. 2C).

The results shown in Figs. 1 and 2 showed that VEGF stimu-
lated 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-phosphory-
lated in response to VEGF corresponded to p125FAK. Confluent
cultures of HUVECs were treated with 10 ng/ml VEGF for
different times, and anti-Tyr(P) immunoprecipitates were pre-
pared and blotted with antibody to p125FAK. As shown in
Fig. 3, VEGF markedly increased p125 FAK tyrosine phospho-
rylation. The effect of VEGF was rapid with a detectable in-
crease as early as 1 min (Fig. 3A). Although the effect of VEGF
was sustained at times of incubation up to 60 min, it was noted
in some experiments that p125FAK tyrosine phosphorylation
did not decline at times up to 1 h after the addition of VEGF.
Scanning of five independent experiments (shown in Fig. 4A)
showed that the half-maximum effect occurred after 1 min and
the maximum increase was at 10 min and that p125FAK tyro-
sine phosphorylation declined to approximately half the maxi-
mum level of phosphorylation after 2 h. The maximum mean
increase in p125FAK tyrosine phosphorylation stimulated by
VEGF after 10 min was 5-fold (n = 5) above control unstimu-
VEGF Stimulates FAK and Paxillin Tyrosine Phosphorylation

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. 3B). 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. 4B). At concentrations of VEGF above 10 ng/ml, p125 FAK tyrosine phosphorylation partially declined (Figs. 3B and 4B) but remained significantly above unstimulated levels at the highest concentration tested (25 ng/ml). As shown in Fig. 3C, 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/CAK2, 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 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. 6A). 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. 6A, 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. 6A).

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 pre-treated 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. 6B, GF209103X caused a partial reduction in VEGF-stimulated p125^FAK tyrosine phosphorylation (Fig. 6B). 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. 6B). It was also exam-
transverse filament bundles that crossed the cell. (Fig. 7)

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). This arrangement was disrupted by VEGF treatment. VEGF treatment stimulated an increase in actin filament formation and in particular increased the number of filamentous actin bundles. (Fig. 7)

mum effect at 10 ng/ml (Fig. 7). VEGF treatment stimulated an increase in 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.

plausible explanation for the results presented in Figs. 3–6 was that the VEGF-induced increases in p125FAK and paxillin immunoreactivity recovered by anti-Tyr(P) immunoprecipitation and/or the observed decline in p125FAK tyrosine phosphorylation at higher VEGF concentrations and longer times of treatment were due to VEGF-induced changes in the susceptibility of p125FAK 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 p125FAK 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 p125FAK. VEGF treatment for various times up to 60 min had no significant effect on the extraction of p125FAK 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 p125FAK and paxillin. It was, however, consistently noted that the Triton-insoluble fraction contained a major p125FAK-immunoreactive band of approximate M, 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 p125FAK (results not shown). No paxillin-immunoreactive species and of paxillin are indicated on the right, and the positions of molecular weight markers are indicated on the left.
VEGF Stabilizes FAK and Paxillin Tyrosine Phosphorylation

Fig. 9. VEGF stimulates p125FAK immunofluorescent staining of focal adhesions in HUVECs. Confluent cultures of HUVECs were either untreated (control, 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 p125FAK (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.

To further examine the functional significance of VEGF-stimulated p125FAK tyrosine phosphorylation, the localization of p125FAK in HUVECs was examined by immunofluorescent staining. In control, unstimulated cells (Fig. 9A), p125FAK immunostaining was predominantly diffuse with weak cytoplasmic staining and some staining putatively of the nuclear or juxtanuclear region. Anti-p125FAK staining of focal adhesions, which appear as characteristically elongated dashes or short streaks, was generally weak in unstimulated cells, and p125FAK-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-p125FAK immunostaining of focal adhesions and a large increase in the number of focal adhesions per cell (Fig. 9B). The increase in p125FAK immunostaining of focal adhesions was also evident after treatment with VEGF for 10 min (results not shown) and was sustained after 1 h (Fig. 9C) and for up to 4 h (results not shown). VEGF-induced p125FAK 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 p125FAK 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. 10A). Similar to the results obtained with p125FAK, VEGF treatment stimulated a noticeable increase in paxillin staining of focal adhesions (Fig. 10B). 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 p125FAK. 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 p125FAK 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. 10C). VEGF induced a marked increase in the immunofluorescent staining of focal adhesions and filaments and an increase in the number of immunostained structures (Fig. 10D). The overall pattern of anti-Tyr(P) immunostaining of cytoskeletal structures was very similar in appearance to that observed with p125FAK 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 p125FAK and paxillin. The effect of VEGF was both rapid and concentration-dependent. The concentration dependence for VEGF-induced p125FAK/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 p125FAK/paxillin pathway.

The tyrosine phosphorylation of p125FAK 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 p125FAK 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 p125FAK 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 p125FAK pathway in HUVECs by VEGF is critically dependent on the integrity of the actin filament network. Second, VEGF-stimulated p125FAK tyrosine phosphorylation and focal adhesion-associated p125FAK 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 p125FAK/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 receptors is responsible for mediating VEGF activation of the PI 3-kinase (72, 73). Phosphorylation of Tyr 925 is induced by PI 3-kinase (72, 73).

Stimulation of the p125FAK pathway in HUVECs was accompanied by a marked VEGF-induced increase in the localization of both p125FAK and paxillin to focal adhesions and filamentous structures. It is most likely that the filamentous immunofluorescent staining produced by p125FAK, paxillin, and anti-phosphotyrosine antibodies is due to decoration of actin filaments. The fact that VEGF treatment also stimulated the formation of focal adhesions tends to support the former possibility, namely that immunolocalization of p125FAK/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 (70) 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 p125FAK/paxillin pathway.
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 p125FAK, suggesting that some components in HUVECs may remain constitutively associated in nascent focal adhesions. Furthermore, the presence of considerable diffuse cytoplasmic staining of p125FAK 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 p125FAK to these structures, we do not rule out the possibility that active recruitment of p125FAK (and possibly other components) to nascent focal adhesions may also occur. VEGF-induced immunolocalization of p125FAK 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 p125FAK becomes translocated to the actin cytoskeleton in thrombin-stimulated platelets (66), possibly reflecting differences either in the way that p125FAK associates with the actin cytoskeleton or in the manner that VEGF in HUVECs and thrombin in platelets affect the interaction of p125FAK with the actin cytoskeleton.

It was noteworthy that the Triton-insoluble HUVEC fraction was enriched in a 55-kDa p125FAK-immunoreactive species, which was poorly detected in the Triton-soluble fraction. Several variant species of FAK have been reported, including a species truncated at the carboxyl terminus (86, 87). Since the antibody used for detection of this fragment is directed to a region of the p125FAK molecule comprising amino acid residues 354–533, it is unlikely that the 55-kDa species represents a larger version of p41/p43FRNK 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 p125FAK or is nonspecifically recognized by antibodies to p125FAK 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 stimulates p125FAK 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 p125FAK. Tyrosine phosphorylation of components of the epithelial and endothelial cell adhesions 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 p125FAK and paxillin to intercellular junctions, since components of focal adhesions and of the endothelial adhesions junctions are both linked to the actin cytoskeleton, it is attractive to speculate that tyrosine phosphorylation of components of focal adhesions and of adhesions 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. 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 p125FAK–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 p125FAK tyrosine phosphorylation suggests that this pathway, possibly in conjunction with tyrosine phosphorylation-mediated disruption of adhesions 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 p125FAK can suppress “anoikis,” a subset of apoptosis induced in epithelial and endothelial cells (91, 92), raises the intriguing possibility that the p125FAK pathway may also participate in VEGF-induced signaling related to cell survival. Regardless of the precise functional role(s) of the p125FAK  pathway in the endothelium, these results identify p125FAK 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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