The Adaptor Protein Shb Binds to Tyrosine 1175 in Vascular Endothelial Growth Factor (VEGF) Receptor-2 and Regulates VEGF-dependent Cellular Migration*

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Received for publication, November 20, 2003, and in revised form, March 10, 2004
Published, JBC Papers in Press, March 16, 2004, DOI 10.1074/jbc.M312729200

Previous studies have shown that the adaptor protein Shb is involved in receptor tyrosine kinase signaling. In this study, we demonstrate that Shb is phosphorylated in an Src-dependent manner upon vascular endothelial growth factor (VEGF) stimulation using porcine aortic endothelial cells expressing the human VEGF receptor 2 (VEGFR-2) (KDR). In co-immunoprecipitation experiments, we could detect an interaction between Shb and the VEGFR-2 in human telomerase-immortalized microvascular endothelial cells. Furthermore, in a glutathione S-transferase pull-down assay, the Src homology 2 domain of Shb was shown to interact with phosphorylated tyrosine 1175 in the C-terminal tail of VEGFR-2. VEGF-induced Shb phosphorylation was lost in porcine aortic endothelial cells expressing a chimeric murine VEGFR-2 (Flk-1) with a mutation at the corresponding position. Shb expression was specifically decreased by 80%, in a transient manner, by using the short interfering RNA technique. Reduced Shb expression led to a loss of stimulation of phosphatidylinositol 3-kinase, phosphorylation of focal adhesion kinase at tyrosine 576, the generation of focal adhesions, and stress fiber formation in response to VEGF. Furthermore, we show that VEGF-induced migration is inhibited in Shb short interfering RNA-treated cells. Our data demonstrate that Shb is important for VEGF signaling in endothelial cells. This is achieved by Shb binding to tyrosine 1175 in the VEGFR-2, which regulates VEGF-induced formation of focal adhesions and cell migration, of which the latter occurs in a phosphatidylinositol 3-kinase-dependent manner.

The adapter protein Shb has been shown previously to be involved in platelet-derived growth factor (PDGF),1 fibroblast growth factor (FGF), T cell, and interleukin (IL) 2-receptor signaling (1–4). Shb interacts directly with these receptors, initiating a specific cellular response depending on the growth factor stimulus. In this study we have investigated the role of Shb in vascular endothelial growth factor receptor 2 (VEGFR-2) signaling. Shb consists of a C-terminal Src homology 2 (SH2) domain, a central phosphotyrosine binding (PTB) domain, four central putative tyrosine phosphorylation sites, and a proline-rich N terminus (5–7). VEGF is a potent angiogenic factor that binds to VEGFR-2 (Flk-1/KDR) present on endothelial cells, evoking an intracellular signaling cascade leading to a number of physiological responses (8, 9). Gene inactivation of VEGFR-2 in mice leads to embryonal death at day 8.5–9.5, because of lack of endothelial cells (10). We have shown previously that Shb plays a number of important roles in endothelial cells. It can bind directly to the FGFR-1, via its SH2 domain, and regulate mitogen-activated protein kinase (MAPK) activation and mitogenicity (2). It can also bind directly to focal adhesion kinase (FAK), via its PTB domain, and regulate cell spreading (11) and tubular morphogenesis (12). Activation of VEGFR-2 is known to lead to cytoskeletal reorganization and increased cellular migration. This process has been shown to require the activation of phosphatidylinositol 3-kinase (PI 3-kinase) (8, 9). In addition, knockout experiments have demonstrated the importance of the cytoplasmic tyrosine kinase FAK for cellular migration (13).

In this study, we demonstrate that Shb is phosphorylated and binds directly to tyrosine 1175 upon VEGF stimulation in PAE/VEGFR-2 cells. By use of the siRNA technique, we find that Shb is required for VEGF-mediated PI 3-kinase activity, stress fiber formation, and cellular migration. Taken together, the data indicate that Shb plays a critical role in VEGF-2-mediated signaling.

EXPERIMENTAL PROCEDURES

Materials—Phosphate-buffered saline (PBS) for cell culture, bovine serum albumin (BSA), and gelatin were purchased from Sigma. Ham’s VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2; FAK, focal adhesion kinase; FGF, fibroblast growth factor; PI, phosphatidylinositol; PAE, porcine aortic endothelial; TIME, telomerase-immortalized microvascular endothelial; GST, glutathione S-transferase; SH2, Src homology 2; siRNA, short interfering RNA; IL2, interleukin 2; IL2R, IL2 receptor; PTB, phosphotyrosine binding; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; PLC-γ, phospholipase C-γ; CSF, colony-stimulating factor; EGF, epidermal growth factor; TBS, Tris-buffered saline; FGFR-1, fibroblast growth factor receptor 1; PDGFR, platelet-derived growth factor receptor; FACS, fluorescence-activated cell sorter.

* This work was supported by grants from the Juvenile Diabetes Foundation International, the JDRF-SRC-SDA joint program on stem cell research, the Swedish Medical Research Council Grant 31X-10822, the Swedish Diabetes Association, the Novo-Nordisk Foundation, the Association for International Cancer Research (to M. J. C. and L. C.-W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PAE, porcine aortic endothelial; TIME, telomerase-immortalized microvascular endothelial; GST, glutathione S-transferase; SH2, Src homology 2; siRNA, short interfering RNA; IL2, interleukin 2; IL2R, IL2 receptor; PTB, phosphotyrosine binding; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; PLC-γ, phospholipase C-γ; CSF, colony-stimulating factor; EGF, epidermal growth factor; TBS, Tris-buffered saline; FGFR-1, fibroblast growth factor receptor 1; PDGFR, platelet-derived growth factor receptor; FACS, fluorescence-activated cell sorter.
PBS containing 100 ng/ml VEGF for 10 min. Cells were washed with ice-cold PBS before agonist stimulation experiments. Cells were stimulated with or without addition of growth factors, prior to the experiments.

Serum-starved overnight in EBM MV2 media containing 1% FCS, with or without 50 ng/ml VEGF for 5 or 15 min. The cells were washed twice in ice-cold PBS and lysed in sample buffer, sonicated, and analyzed for Shb and actin expression by Western blotting.

Alternatively, 24 h post-transfection the cells were serum-starved in Ham’s F-12 medium with 0.2% BSA. The following day (48 h post-transfection), the cells were stimulated with either 50 ng/ml VEGF for 10 min or 10% FCS for 5 min. The cells were washed in ice-cold PBS and lysed in sample buffer, sonicated, boiled, and run on a 10% SDS-PAGE gel. Alternatively, PAE/VEGFR-2 cells were transfected with fluorescently labeled oligonucleotides, and after different time points, the cells were trypanotized and diluted with Ham’s F-12 media supplemented with 0.2% BSA. The samples were analyzed using a FACSICalibur.

Immunoprecipitation and Immunoblotting—PAE/VEGFR-2 cells were seeded on tissue culture dishes or 24-well plates and serum-starved overnight in Ham’s F-12 media supplemented with 0.2% BSA before agonist stimulation experiments. Cells were stimulated with or without 50 ng/ml VEGF for 10 min. Cells were washed with ice-cold PBS containing 100 μM Na2VO4 and lysed in 0.5 ml of Nonidet P-40 lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% 1% Triton X-100, 0.5% Nonidet P-40, 500 μM N-α-1,5% aprotinin, 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 μg/ml leupeptin, and 20 μM calpain inhibitor N-acetylcysteine-leucine-nerileucinal) for 10 min on ice before transfer to Eppendorf tubes. The lysates were centrifuged at 16,000 × g for 20 min at 4°C. The resulting supernatants were either mixed with sample buffer (2% SDS, 0.15 M Tris-HCl, pH 8.8, 10% glycerol, 5% 1-mercaptoethanol, and bromphenol blue) or incubated with antibodies against Shb on ice for 2 h before a final 1-h mixing with protein A-Sepharose. The antibody complex was washed three times in Nonidet P-40 lysis buffer and once in Tris-buffered saline (TBS). The beads were resuspended in sample buffer, boiled, and separated on SDS-PAGE gels. The gels were electrothermally transferred onto Immobilon membrane, which were blocked in TBS-T (0.1% Tween 20 in TBS) containing 5% BSA. The membranes were incubated with primary antibody, diluted in TBS-T containing 1.5% BSA, for 2 h, followed by washing in TBS-T. The membranes were then probed with the appropriate secondary horse-radish peroxidase-conjugated antibody, followed by development using the bound antibodies visualized by ECL. Before reprobing, the membranes were stripped in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM 1-mercaptoethanol at 50°C for 30 min.

Preparation of Fusion Protein and Pull-down Experiments—Fusion proteins corresponding to the SH2 domain and p55 Shb with the SH2 domain deleted (GST-ShbΔSH2) were produced, as described (5, 18). PAE/VEGFR-2 cells were seeded on plastic dishes and grown to near confluence, serum-starved, and stimulated with either nothing (control) or 50 ng/ml VEGF for 10 min and lysed in Nonidet P-40 lysis buffer. Lysates from 106 cells were centrifuged at 16,000 × g at 4°C for 20 min. Supernatant was collected and mixed with 10 μg of fusion protein (GST control), GST-Shb ΔSH2, or GST-ShbΔSH2 fusion proteins coupled to glutathione-Sepharose, and either 50 mM HEPES or phosphoantibody was added to a final concentration of 20 mM. Samples were mixed for 60 min at 4°C. Phosphorylated and nonphosphorylated peptides corresponding to tyrosine 1175 in the VEGFR-2 (KDR) and phospho-MAPK was kindly provided by Cell Signaling. The anti-actin antibody was from Santa Cruz Biotechnology. The mouse monoclonal anti-paxillin antibody (PI3520) was from Pharmingen. TNB blocking solution was from PerkinElmer Life Sciences. Goat anti-mouse 488 secondary antibody was from Molecular Probes, and fluoromount G was purchased from Southern Biotechnology Associates Inc., Birmingham, AL. PAE cells expressing chimeric VEGFR-2 (extracellular domain of human CSF-1R/c-Fms fused with the transmembrane and cytoplasmic domains of murine VEGFR-2 [GK1]) and PAE cells expressing chimeric VEGFR-2 with a point mutation at tyrosine 1173 (murine) in the cytoplasmic domain were obtained and cultured as PAE cells (see above) (15). Telomerase-immortalized human microvascular endothelial (TIME) cells (17) were transfected with fluorescently labeled oligonucleotides, and after different time points, the cells were trypanotized and diluted with Ham’s F-12 media supplemented with 0.2% BSA. The samples were analyzed using a FACSCalibur.

PI 3-Kinase Assay—Cells were transfected with scrambled or Shb1 siRNA as above. At 48 h, the cells were serum-starved for 1 h, after which they were stimulated for 10 min with 50 ng/ml VEGF or kept unstimulated in lysing buffer. The cells were fixed in 3% paraformaldehyde in PBS for 10 min, and permeabilized using aceton at −20°C for 3 min. The slides were air-dried, and nonspecific interactions were blocked by incubating with 1 h with 10% BSA in PBS. Fluorescently labeled antibodies were added, and after a 1-h incubation, the slides were mounted. The samples were examined using the ×40 lens in a Nikon microscope. Alternatively, the cells were transfected in tissue culture dishes and plated on collagen-coated slides 24 h after transfection. After an additional 18 h culture period, the cells were serum-starved for 4 h prior to stimulation with 50 ng/ml VEGF for 0, 60, and 120 min. The cells were fixed by washing with 37 mM H3PO4, 1.5 mM CaCl2, and 3.2 mM MgCl2 containing 0.2% Triton X-100. For fluorescent staining, the fixed cells were incubated in TNB blocking reagent, followed by incubation with primary anti-paxillin antibody diluted in blocking buffer, and followed by incubation with the secondary goat anti-mouse
Vegf Stimulates Shb Phosphorylation

Untransfected PaE cells and PaE cells stably transfected with Vegfr-2 were lysed, and the proteins were separated on an SDS-PAGE gel and probed for the presence of Vegfr-2 (A). Pae/Vegfr-2 cells were stimulated with 50 ng/ml Vegf for 10 min and lysed, and Western blotting was carried out with phosphotyrosine (4G10) and Vegfr-2 antibodies (B). Pae/Vegfr-2 cells were stimulated with 50 ng/ml Vegf for 1, 5, or 25 min or kept unstimulated (control). The cells were lysed and immunoprecipitated with anti-Vegfr-2 antibody. Western blot analysis was performed with antibodies directed against phosphotyrosine and Shb. Similar results were obtained in three independent experiments (C). Human TIME cells were seeded on fibronectin for 2 h and stimulated or not (control) with 50 ng/ml Vegf for 10 min. The cells were lysed, immunoprecipitated with an anti-Vegfr-2 antibody, and the proteins separated on an SDS-PAGE gel. The membrane was probed with anti-Shb antibody (D). Western blot analysis was also performed on total cell lysate and probed for the presence of Shb (D).

Results

Shb Is Phosphorylated upon Vegf Stimulation—Previous studies have demonstrated that the adapter protein Shb is phosphorylated in response to a number of different agonists, i.e. FGF-2 (19), nerve growth factor (20), PDGF (1), and IL2 (4). The aim of this study was to determine whether Shb has a role in Vegf signaling. For this purpose we utilized PaE cells stably transfected with the human Vegfr-2 (KDr) cDNA (16). Expression of Vegfr-2 in these cells was confirmed by Western blotting (Fig. 1A). Stimulation of Pae/Vegfr-2 cells with Vegf resulted in a prominent phosphorylated protein of 220 kDa, which corresponds to the activated Vegfr-2 (Fig. 1B). Pae/Vegfr-2 cells were then stimulated with Vegf, and the phosphorylation status of Shb was analyzed. Shb was rapidly phosphorylated in response to Vegf stimulation (Fig. 1C), peaking at 5 min. A phosphorylated band of 220 kDa, probably corresponding to the Vegfr-2, was co-immunoprecipitated with Shb. To demonstrate further an interaction between Shb and the Vegfr-2, we utilized telomerase-immortalized human microvascular endothelial (time) cells. These cells were stimulated or not with Vegf, lysed, immunoprecipitated with anti-Vegfr-2 (KDr) antibody, and probed with Shb antibody. The experiment demonstrated that Shb co-immunoprecipitated with the activated Vegfr-2 (Fig. 1D), suggesting that Shb and Vegfr-2 interact in a Vegf-dependent manner.

Vegf-Induced Shb Phosphorylation Is Src-Dependent—We have shown previously that FGF-induced Shb phosphorylation is Src-dependent (11). Therefore, the potential role of Src in Vegf-induced Shb phosphorylation was investigated by use of the Src family selective kinase inhibitor PP2. Pae/Vegfr-2 cells were either pre-treated with PP2 or kept untreated before stimulation with or without Vegf. The cells were lysed and immunoprecipitated with Shb antibody, and Western blot analysis was performed. Vegf-induced Shb phosphorylation was prevented by prior incubation with PP2 (Fig. 2A). Furthermore, inhibition of Src activity did not prevent the association of Shb with a phosphotyrosyl protein migrating at 220 kDa (Fig. 2A), because the amount of co-immunoprecipitated 220-kDa product correlated with the amount of total Shb in the immunoprecipitates. The membrane was also probed with an antibody directed against phosphorylated tyrosine 1175 of the Vegfr-2, which confirmed that Shb and phosphorylated Vegfr-2 co-immunoprecipitate (Fig. 2A). Analysis of total cell lysate from the same experiment revealed that PP2 did not directly affect the phosphorylation status of the Vegfr-2 (Fig. 2B). Our data clearly show that Src family kinase activity is necessary for Vegf-stimulated Shb phosphorylation but not for the direct association of Shb and Vegfr-2 in Pae/Vegfr-2 cells.

Shb Binds Directly to Tyrosine 1175 in the Vegfr-2—The Shb adapter protein SH2 domain has been shown previously to bind to the consensus motif Y(V/I)L (6). This corresponds to tyrosine 766 in the Fgfr-1 (2) and tyrosine 720 in the PDGF receptor-α (1). In the human Vegfr-2 (KDr), tyrosine 1175 has the sequence YIVL, suggesting that it may be a potential Shb-binding site. To investigate this further, Pae/Vegfr-2 cells were stimulated with Vegf, lysed, and mixed with GST fusion proteins either encompassing the SH2 domain of Shb or p55 Shb with a deletion of the SH2 domain. The potential binding was competed with the addition of 20 mM free phosphotyrosine or phosphorylated or unphosphorylated (control) peptides at 0.1 (data not shown) or 0.2 mM directed against the tyrosine 1175 site in the Vegfr-2 (Fig. 3A). The experiment confirmed that the Shb SH2 domain bound to tyrosine 1175 in the Vegfr-2 in a phosphotyrosine-dependent manner. Furthermore, Shb with an SH2 domain deletion also appeared to bind to the Vegfr-2, although this association was only partly phosphotyrosine-dependent.
To confirm further the role of tyrosine 1175 in regulating Shb activation, we utilized PAE cells expressing a chimeric receptor composed of the extracellular domain of the human colony-stimulating factor 1 (CSF-1) receptor and the intracellular domain of the murine VEGFR-2 (Flk-1) (21). The mutation at tyrosine 1173 was mutated to phenylalanine (Y1173F). Activation of the chimeric receptor with CSF-1 resulted in receptor phosphorylation (Fig. 3C). Analysis of Shb phosphorylation revealed that although phosphorylation of the chimeric receptor resulted in Shb phosphorylation, activation of the Y1173F mutant receptor did not stimulate phosphorylation (Fig. 3B). Analysis of the degree of activation/phosphorylation of other intracellular signaling proteins revealed that although the receptor could evoke a normal activation of p44/42 MAPK and actin (Fig. 3B), it was not able to stimulate the phosphorylation of FAK at position tyrosine 576 (Fig. 3C). Taken together, these results strongly suggest that Shb binds to the pYIVL site in the VEGFR-2 (Flk-1/KDR).

**Gene Silencing by siRNA Specifically Lowers Shb Expression**—To determine the physiological role of Shb in VEGFR-2 signaling, we decided to utilize siRNA technology using a synthetic oligonucleotide RNA duplex. We selected two sequences directed against different parts of the Shb gene (Shb1 and Shb6) and a control (scrambled) sequence. PAE/VEGFR-2 cells were transfected with a fluorescein-labeled oligonucleotide, and FACS analysis was carried out, which revealed a transfection efficiency of at least 80% (Fig. 4A). Furthermore, Shb protein expression was specifically decreased upon treatment with the siRNA technique in a transient fashion. By using the Shb1 sequence, we managed to decrease Shb expression by 80% (24 h), 70% (48 h) and 40% (72 h post transfection) compared with a scrambled sequence (Fig. 4B). Another sequence, Shb6, caused a weaker inhibition of Shb expression. The blot was also probed for actin content in order to confirm the specificity of the Shb sequences. Our data show successful transient inhibition of Shb protein expression in PAE/VEGFR-2 cells using the siRNA technique.

**VEGF-induced Phosphorylation of FAK Tyrosine 576 Is Decreased in siRNA Shb1-treated Cells**—In order to determine the consequences of Shb protein reduction, different intracellular signaling pathways were examined. Because Shb has been shown to bind and regulate FAK phosphorylation (11), we investigated the phosphorylation status of FAK under these conditions. We found that VEGF-induced phosphorylation of FAK tyrosine 576 was reduced in the siRNA Shb1-treated cells by 40% (Fig. 5A). In contrast, in the untransfected (results not
shown) and in the scrambled oligonucleotide-treated cells, VEGF increased FAK tyrosine 576 phosphorylation by 20%. FCS caused increased FAK phosphorylation in all cells, indicating that in the siRNA Shb1-treated cells, FAK tyrosine 576 could still be phosphorylated. The membrane was stripped and reprobed for actin content to ensure equal loading. Similar results were obtained in three independent experiments.

**Reduced Shb Expression Leads to Decreased Formation of Stress Fibers and Focal Adhesions in Response to VEGF Stimulation**—In addition to FAK, PLC-γ, and MAPK, it was of interest to investigate the role of Shb in VEGF-induced PI 3-kinase activation, because it was shown previously (6) that the SH3 domain of the p85 subunit of PI 3-kinase could associate in vitro with proline-rich motifs of Shb. Cells treated with Shb1 siRNA failed to increase their PI 3-kinase activity in response to VEGF stimulation, unlike the scrambled control (Fig. 5C). When probing the Shb immunoprecipitations with an antibody recognizing the p85 subunit of PI 3-kinase, no evidence for an association between Shb and PI 3-kinase was obtained (results not shown). Thus, Shb is required for PI 3-kinase activation in response to VEGF, although no evidence for a direct association between Shb and PI 3-kinase was obtained.

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**Figure 5.** VEGF-induced phosphorylation of FAK tyrosine 576 and activation of PI 3-kinase is decreased in siRNA Shb1-treated cells. PAE/VEGFR-2 cells were transfected with either Shb1 or scrambled sequence or kept untransfected (control). The cells (also including untransfected PAE cells) were then stimulated with 50 ng/ml VEGF for 10 min, 5% FCS for 5 min, or nothing (control) and lysed. The lysates were subjected to Western blot analysis and blotted for the presence of phosphorylated FAK (Tyr-576) (A), phosphorylated PLC-γ (Tyr-783) (B), and phosphorylated MAPK (C). Quantification of signal ratios (p-FAK/FAK) in A is indicated. The membranes were also probed for the total amounts of FAK and actin. C, PI 3-kinase activity was assessed by the phosphorylation of phosphatidylinositol after transfection with scrambled and Shb1 siRNA. PI 3-kinase activity was determined after stimulation for 10 min with 50 ng/ml VEGF. Lysates were immunoprecipitated for phosphotyrosine or analyzed by Western blotting for total MAPK as a control for loading. The incorporation of 32P into PI 3-phosphate is shown. The results in A–C are representative of two independent experiments.
tion to increased numbers of focal adhesions, these were commonly larger and more distinct. The Shb1 siRNA-treated cells, however, were not able to respond to VEGF over the 1–4-h time with increased formation of focal adhesions (Fig. 6B). Our data suggest that Shb is important for VEGF-induced formation of stress fibers and focal adhesions.

Fig. 6. Decreased Shb expression by siRNA leads to reduced stress fiber formation and generation of focal adhesions upon VEGF stimulation. PAE/VEGFR-2 cells were transfected with either Shb1 or scrambled sequence or kept untransfected (results not shown). A, the cells were then stimulated with 50 ng/ml VEGF for 5 or 15 min or kept unstimulated (Control). The slides were fixed in paraformaldehyde and stained with rhodamine phalloidin. The samples were examined using the ×40 lens in a Nikon microscope. Bar represents 10 μm. The results are representative of three independent experiments. B, the cells were stimulated or not with 50 ng/ml VEGF for 1 or 4 h. Staining for paxillin as a marker for focal adhesions is shown. The experiment was performed twice.
VEGF-induced Migration Is Perturbed by siRNA Shb1 Treatment of PAE/VEGFR-2 Cells—To define further the physiological role of Shb in the VEGF-2 response, we decided to study cellular migration/chemotaxis. PAE/VEGFR-2 cells were transfected with siRNA Shb1, maintained for 48 h, and then plated on a collagen-coated membrane in a Boyden chamber. Stimulation with VEGF and FCS lead to an approximate doubling in migration in untransfected and control oligonucleotide-treated cells (Fig. 7A). In the siRNA Shb1-treated cells, VEGF failed to induce migration; however, FCS could still evoke a migratory response. This suggests that Shb is required for VEGF-2-mediated migration in endothelial cells.

To address the potential role of PI 3-kinase in Shb-dependent VEGF-induced cell migration, PAE/VEGFR-2 cell migration was determined in the presence of the PI 3-kinase inhibitor LY294002 (Fig. 7B). VEGF-induced cell migration was completely inhibited in the presence of this inhibitor of PI3-kinase, suggesting a role of PI 3-kinase in the Shb-dependent effect.

**DISCUSSION**

In this study we have examined the role of the adaptor protein Shb in VEGF signaling. Shb has been shown previously to participate in PDGF receptor-α and -β, TrkA, T cell receptor, and IL2R signaling. In addition, we have shown recently that Shb plays an important role in FGFR-1 signaling in endothelial cells (2, 11, 12). Because VEGF is a potent regulator of angiogenesis in endothelial cells, we were interested in the potential role of Shb in transducing VEGF-2 signals. Our data show that stimulation of PAE/VEGFR-2 cells with VEGF leads to an association between VEGF-2 and Shb and increased Shb phosphorylation. Upon treatment with PP2, an Src family kinase inhibitor, the VEGF-induced Shb phosphorylation is completely abolished. Similar data were obtained with respect to FGFR-1 signaling in immortalized brain endothelial cells, where FGF-2-induced Shb phosphorylation was Src-dependent (11). It has been shown recently (22) that Src is associated with the VEGF-2, although the role of Src in VEGF signaling remains obscure. Taken together, it would appear that Src may phosphorylate Shb in response to a number of agonists.

We also observed an association between Shb and the VEGFR-2 in the human TIME cells, a microvascular endothelial cell line, suggesting that Shb is activated in response to VEGF-2 phosphorylation in a number of endothelial cells. Our data implicate the tyrosine 1175 pYIVL in the VEGFR-2 as a phosphotyrosine-dependent binding site of the Shb SH2 domain. The Shb SH2 domain has been shown previously to bind tyrosine 766 in the FGFR-1 (2), tyrosine 720 in the PDGF α-receptor (1), tyrosine 510 in the IL2Rβ, and the ITAM motif in the T cell receptor (18). Use of PAE cells expressing the mutant chimeric CSF1/VEGFR-2 (Flk-1) further implicate tyrosine 1175 as the binding site for Shb. Most interesting, in the FGFR-1, both Shb and PLC-γ can bind to the same site at tyrosine 766 (2, 23). In the case of the VEGF-2, PLC-γ has been reported previously to bind to tyrosine 1175 (24). Thus, in the case of the angiogenic tyrosine kinase receptors VEGFR-2 and FGFR-1, both Shb and PLC-γ have the potential to bind to the same site (2, 25). In addition, Shb and PLC-γ can associate with each other (3). However, it appears that Shb and PLC-γ do not compete for the common binding sites in FGFR-1 (2) or in the VEGF-2, as shown in the siRNA experiment, where VEGF-stimulated PLC-γ phosphorylation was not altered upon lowered Shb expression. Another adaptor protein, Sck, has also been shown to interact with tyrosine 1175 in the VEGFR-2 (26). This would suggest that this site interacts with multiple signaling molecules.

However, unlike the situation with the FGFR-1 (2), Shb does not appear to regulate MAPK activity in response to VEGFR-2 activation, as shown in both the PAE CSF1/VEGFR-2 Y1173 cells and in the Shb siRNA knockdown. Therefore, we conclude that Shb is able to differentially modulate intracellular signaling from different receptor tyrosine kinases. This could be explained by the unique involvement of FRS2 in FGFR-1 signaling. This adaptor protein is required for FGFR-1-dependent MAPK activation, a process that involves Shb (2). However, by using both PAE-KDR and TIME cells, we could not detect FRS2 activation in response to VEGF (data not shown), suggesting that the VEGF-2 couples to MAPK independently of FRS2 and Shb.

In addition to the binding of the Shb SH2 domain GST fusion protein to tyrosine 1175 in the VEGFR-2, we also observed an interaction between the GST-Shb fusion protein with the SH2 domain deleted and phosphorylated VEGFR-2. The nature of this interaction remains uncertain, because 20 nm free phosphotyrosine only partly inhibited this association. However, because this fusion protein also contains the proline-rich domain of Shb, in addition to the PTB domain, it is possible that the interaction with the VEGFR-2 is not direct and is instead mediated by another protein, interacting with both Shb and the VEGF-2.

We have used the siRNA technique to specifically silence Shb expression in PAE/VEGFR-2 cells. siRNA was originally discovered in Caenorhabditis elegans (27). Shb knockdown using Shb siRNA resulted in a loss of PI 3-kinase activation in response to VEGF. Furthermore, the Y1173F murine VEGFR-2 mutant displayed reduced PI 3-kinase activation in response to VEGF (15, 21). Thus, Shb-dependent binding to 1175/1173 appears important for the PI 3-kinase response. It is not clear how this effect is exerted. We have reported previously that the SH3 domain of p85 PI 3-kinase can interact in vitro with the proline-rich motifs of Shb but have presently no evidence for a direct association between Shb and PI 3-kinase. Alternatively, the effect could be indirect, via another mediator such as FAK or VE-cadherin (28, 29). The participation of FAK could contribute to this response, because we observed that FAK tyrosine 576 phosphorylation was reduced in the cells with suppressed Shb content upon VEGF stimulation. A similar decrease in FAK activation was observed in response to activation of the murine VEGFR-2 (Flk-1) mutant chimeric Y1173F receptor. However, the effect of Shb on FAK phosphorylation under these conditions is small and unlikely to explain the full effect of Shb knockdown on PI 3-kinase activation.

FAK is phosphorylated in vitro by Src at a number of sites: Tyr-407, Tyr-576, Tyr-577, Tyr-861, and Tyr-925. Phosphorylation of tyrosine 576, in the FAK kinase domain, enhances the activity of FAK (30). Therefore, it is possible that Shb is required for VEGF-mediated activation of FAK by allowing Src to phosphorylate tyrosine 576. Indeed, we have shown previously (11) that the PTB domain of Shb can bind directly to FAK and regulate its activity in response to FGFR-1 activation in endothelial cells. Taken together, it appears that Shb regulates FAK activity both in response to FGFR-1 and VEGF-2 activation. The regulation of FAK tyrosine 576 phosphorylation by the Shb knockdown appears to have functional consequences, because these cells failed to respond to VEGF stimulation with an increased formation of focal adhesions. VEGF has been shown previously to increase the formation of focal adhesions (31), an effect that occurs progressively over a 4-h period (32). Taken as a whole, our data indicate deregulated FAK activity in response to VEGF upon Shb reduction.

PI 3-kinase activation has been shown to be important for cell migration in response to a number of agonists including VEGF (9). This effect involves the Rho/ Rac family of monomeric G-proteins, which regulate stress fibers and membrane ruffles.
Analysis of stress fiber formation following Shb siRNA treatment showed reduced actin stress fibers in response to VEGF. We have shown previously that FGF-2 stimulation of immortalized brain endothelial cells overexpressing Shb leads to an increase in stress fiber formation and the formation of irregular, densely stained patches (12), suggesting that Shb

![Fig. 7. Decreased Shb expression by siRNA inhibits VEGF-induced migration. A, PAE/VEGFR-2 cells were transfected with either Shb1 or scrambled sequence or kept untransfected (control). The cells were kept in Ham’s F-12 supplemented with 10% FCS for 48 h post-transfection, when the cells were trypsinized, counted, and seeded out on a collagen-coated membrane in a Boyden chamber. After 4 h of incubation at 37 °C, the cells were fixed in ethanol, stained with Giemsa, and washed in H2O. The non-migrating cells, on the upper surface, were wiped off, and the migrated cells were mounted on a coverglass using EUKITT and counted. Results are expressed as percentage of basal response (mean ± S.E., n = 3) in three independent experiments. * indicates significantly different compared with basal conditions; p < 0.05 using Student’s t test for unpaired data. B, PAE/VEGFR-2 cell migration in response to 20 ng/ml VEGF was tested in the absence or presence of the PI 3-kinase inhibitor LY294002 (30 μM). Means ± S.E. for three observations are given. * indicates p < 0.05 when tested with a paired Student’s t test against VEGF alone.](image-url)

(33). Analysis of stress fiber formation following Shb siRNA treatment showed reduced actin stress fibers in response to VEGF. We have shown previously that FGF-2 stimulation of immortalized brain endothelial cells overexpressing Shb leads to an increase in stress fiber formation and the formation of irregular, densely stained patches (12), suggesting that Shb
plays an important role in regulating the cytoskeleton. This could have consequences for cell migration, because reducing Shb expression by siRNA also prevents the ability of endothelial cells to migrate in response to VEGF. Besides the importance of the PI 3-kinase in this process, presently confirmed by the inhibitory effect of the PI 3-kinase inhibitor LY294002, FAK activity could be of significance, because cells from FAK-deficient mice display reduced motility and spreading (13, 34). Furthermore, FAK is known to be critical for the focal adhesion assembly and disassembly required for directional migration or chemotaxis, although we presently do not directly confirm an involvement of FAK in VEGF-induced migration (35). Therefore, by regulating PI 3-kinase and FAK activation in concert, Shb could influence cellular migration. Indeed, cell migration is an important in vivo physiological function of VEGF, as endothelial cells must migrate and proliferate to form new capillaries in the angiogenic response. The ability of Shb to regulate this process suggests that modulators of Shb signaling may have a therapeutic potential as anti-angiogenic therapy.

We conclude that after VEGF stimulation, the VEGFR-2 is phosphorylated, Src associates with the receptor, and the SH2 domain of Shb binds to phosphorylated tyrosine 1175 in the C-terminal tail of the VEGFR-2. Src phosphorylates Shb, which allows the subsequent activation of PI 3-kinase and phosphorylation of FAK at tyrosine 766 in the kinase domain. This will then regulate focal adhesion/stress fiber formation allowing the spatial and temporal orchestration of the migratory response in endothelial cells.

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