Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells

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ABSTRACT The vascular endothelial growth factor (VEGF) family has recently expanded by the identification and cloning of three additional members, namely VEGF-B, VEGF-C, and VEGF-D. In this study we demonstrate that VEGF-B binds selectively to VEGF receptor-1/Flt-1. This binding can be blocked by excess VEGF, indicating that the interaction sites on the receptor are at least partially overlapping. Mutating the putative VEGF receptor-1/Flt-1 binding determinants Asp^{63}, Asp^{64}, and Glu^{67} to alanine residues in VEGF-B reduced the affinity to VEGF receptor-1 but did not abolish binding. Mutational analysis of conserved cysteines contributing to VEGF-B dimer formation suggest a structural conservation with VEGF and platelet-derived growth factor. Proteolytic processing of the 60-kDa VEGF-B$_{186}$ dimer results in a 34-kDa dimer containing the receptor-binding epitopes. The binding of VEGF-B to its receptor on endothelial cells leads to increased expression and activity of urokinase type plasminogen activator and plasminogen activator inhibitor 1, suggesting a role for VEGF-B in the regulation of extracellular matrix degradation, cell adhesion, and migration.

Vascular endothelial growth factor (VEGF) has been implicated as a key regulator of blood vessel formation (1). It is required for both vasculogenesis, where mesoderm-derived angioblasts form tubes, and for angiogenesis, where capillaries form by sprouting or intussusception from existing vessels (2). While vasculogenesis is restricted to embryonic development, angiogenesis continues to operate throughout life when neo-vascularization is required. Physiological angiogenesis is mainly restricted to the female reproductive cycle and wound healing, but the angiogenic machinery can also be recruited by pathological processes such as tumor growth (3).

VEGF exerts its functions through binding to two receptor tyrosine kinases, VEGFR-1/Flt-1 and VEGFR-2/KDR (1). These receptors are expressed almost exclusively on endothelial cells, although VEGFR-1 is also found in monocytes, where it mediates migration (4, 5). Targeted homozygous null mutations of both receptor genes result in arrest of embryonic development (6, 7). Disruption of the VEGFR-1 gene interferes with the organization of the vascular endothelium (6), whereas VEGFR-2 is required for endothelial cell differentiation and definitive hematopoiesis (7, 8). VEGF levels are critical for normal development, as inactivation of even one allele results in embryonic death (9, 10).

VEGF has been shown to regulate most steps of the angiogenic process, including endothelial cell degradation of extracellular matrix (ECM), migration, proliferation, and tube formation (1). In keeping with its ability to induce ECM degradation, VEGF increases the expression and activity of plasminogen activators, urokinase type plasminogen activator (uPA) and tissue type plasminogen activator (tPA) (11). These serine proteases convert plasminogen to plasmin and are thereby involved in tissue remodeling, cell invasion, and thrombolysis (reviewed in ref. 12). Whereas tPA is a fibrin-dependent intravascular enzyme, uPA functions as a receptor (uPAR)-bound cell surface activator. Both proteases are specifically inhibited by plasminogen activator inhibitor type 1 (PAI-1), the expression of which is also up-regulated by VEGF (11). This inhibition may serve to protect ECM from excessive proteolysis, as concerted expression of PAI-1 and uPA has been observed during physiological angiogenesis *in vivo* (13). Interestingly, both PAI-1 and uPA/uPAR have recently been implicated in regulation of cell adhesion and migration (12). uPAR and PAI-1 compete for binding to vitronectin, and PAI-1 regulates adhesion also directly by competing with integrin $\alpha_v\beta_3$ for vitronectin binding. Taken together, the uPA/uPAR/PAI-1 system may have a dual role: it can regulate proteolysis and cellular adhesiveness, the latter being independent of the enzymatic function.

The VEGF family of growth factors comprises at present five members—i.e., VEGF, placenta growth factor (PlGF) (14), VEGF-B/VRF (15, 16), VEGF-C/VRP (17, 18), and VEGF-D/FlGF (19, 20). While PlGF binds selectively to VEGFR-1 (21), VEGF-C and VEGF-D bind both VEGFR-3/Flt-4 and VEGFR-2 (17, 22). The corresponding receptor(s) for VEGF-B has not been reported. VEGF-B resembles PlGF in two aspects: it exists as two alternatively spliced forms, VEGF-B$_{167}$ and VEGF-B$_{186}$, which differ in their affinity for heparin and thus release and bioavailability, and it forms heterodimers with VEGF (15, 23), a property likely to alter its receptor specificity and biological effects. In contrast to PlGF, however, VEGF-B is widely expressed and is most prominent in heart and skeletal muscle (15).

Alanine-scanning mutagenesis of VEGF has implicated the negatively charged amino acid residues Asp^{63}, Glu^{64}, and Glu^{67} in VEGFR-1 binding (24). These acidic amino acid residues are conserved in VEGF-B and to lesser extent in PlGF.

Covalent dimerization of VEGF has been shown to be required for its biological activity (25), and recently the determination of the crystal structure verified the antiparallel arrangement of the two subunits covalently linked by two disulfide bridges between Cys^{51} and Cys^{60} (26). The eight conserved cysteine residues characteristic of the PDGF/VEGF growth factor family imply structural conservation between the members (for recent sequence alignment see ref. 20).

In this work we report that VEGF-B binds specifically to VEGFR-1. Mutation of the putative receptor-binding determinants to alanine residues reduced the affinity to VEGFR-1 but did not abolish receptor binding, and mutations in conserved cysteine residues predict that VEGF-B forms antiparallel dimers. Furthermore, we show that VEGF-B$_{186}$ is proteolytically processed, and we analyze the ability of VEGF-B to regulate the uPA/PAI-1 system in endothelial cells.

MATERIALS AND METHODS

Cell Culture and Materials. Sf9 cells were maintained in Sf-900 II SFM (GIBCO/BRL Life Technologies) supplemented with 0.1% pluronic f-68 for suspension growth, High Five cells (Invitrogen) in Ex-cell 400 medium (JHR Bioscience; Lenexa, KS), and Schneider 2 (S2) cells (Invitrogen) were grown in DES-medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). 293-EBNA, 293-T, and NIH 3T3-Flt1 cells (27) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. NIH 3T3-Flt1 cells were kept under continuous selection with 200 μg/ml neomycin. Bovine adrenal cortex-derived microvascular endothelial (BME) cells were kindly provided by M. B. Furie and S. C. Silverstein (28) and were grown in MEMα (GIBCO; Basel, Switzerland) supplemented with 15% donor calf serum on 1.5% gelatin-coated tissue culture flasks. Plasmin was purchased from Boehringer Mannheim, anti-VEGF (MAB 293) from R & D Systems, and human (h)VEGF$_{165}$ from R & D Systems or from Preprotech (Rocky Hill, NJ). Recombinant mouse (m)VEGF$_{164}$ and human basic fibroblast growth factor (bFGF) (155 amino acid form) were kind gifts from H. Weich (GBF, Braunschweig Germany) and P. Sarmientos (Farmitalia Carlo Erba, Milan), respectively.

Construction of Expression Plasmids and Generation of VEGF-B Mutants. The expression plasmid pIg-VEGFR-1 coding for the first five Ig-like domains of VEGFR-1 fused to human IgG1 Fc was constructed by ligating a *Hin*dIII fragment (coding for amino acids 1–549 of VEGFR-1) from pLTR-Flt1 into pIgplus vector (Ingenius; Novagen). Prior to the cloning the latter had been digested with *Xho*I and *Xba*I, blunted, and religated to correct the reading frame for the fusion protein production. For the spIg-VEGFR-2 construct, cDNA encoding the first four Ig-like domains of VEGFR-2 was amplified by PCR using human fetal lung cDNA library (CLONTECH) as a template. The primers 5′-ATGGTACCCCCAGGCTCA-GCATACAAAAAGAC-3′ and 5′-GCGTCTAGAGGGTG-GGACATACACAACCAG-3′were used and the amplified fragment was cleaved with *Kpn*I and *Xba*I and inserted into corresponding sites of signal pIg vector (Ingenius). mVEGF-B$_{186}$ cDNA (23) was cleaved by *Eco*RI and subcloned in pFASTBAC1 (GIBCO/BRL Life Technologies). A (His)$_6$ tag and an enterokinase site were introduced at the N terminus, devoid of signal sequence, using PCR with mVEGF-B$_{186}$ pSG5 as a template and the primers 5′-ATCGAGATCTTCATCA-CCATCACCATCACGGAGATGACGATGACAAACCTG-TGTCCCAGTTT-3′ and 5′-CAAGGGCGGGGCTTAGAG-ATCTAGCT-3′ (both containing *Bgl*II sites). The amplified fragment was cleaved with *Bgl*II and inserted into the *Bam*HI site, in frame with the signal sequence of gp67 of pAcGP67A (PharMingen). Human VEGF-B$_{186}$ cDNA was amplified by PCR using the forward primer 5′-GGAATTCCCCGCCCA-GGCCCCTGTC-3′ and the reverse primer 5′-GGAATTCA-ATGATGATGATGATGATGAGCCCCGCCCTTGGC-3′.

The amplified product containing a C-terminal (His)$_6$ tag was inserted into the *Eco*RI site of pPIC-9 (Invitrogen), in frame with the α mating factor signal sequence. The cysteine-to-serine mutants and the alanine mutants in mVEGF-B$_{167}$ pSG5 as well as mVEGF-B exon 1–5 mutant containing a C-terminal Kemptide motif (29), VEGF-B$_{kEx1–5}$ pSG5, were generated by M13-based *in vitro* single-stranded mutagenesis employing the helper phage M13KO7 (30) and the dut^- ung^- *Escherichia coli* strain RZ1032 (31). The primers 5′-ACGTAGATCTCCTG-TGTCCCAG-3′ and 5′-ACGTGAATTCTCAGCTGTCTG-GCTTCAC -3′ (introducing a stop codon after exon 5) were used to PCR-amplify the alanine mutants, which were subcloned in pMT/Bip/V5-HisC (Invitrogen). All constructs were verified by sequencing.

Protein Expression and Purification. For production of recombinant baculoviral protein in Sf9 and High Five cells, mVEGF-B recombinant plaques were purified and amplified (32), and the corresponding expressed proteins as well as hVEGF-B$_{186}$ expressed in *Pichia pastoris* (strain GS115) were purified by using Ni-NTA Superflow resin (Qiagen). For ligand competition assay, High Five cells were infected with mVEGF-B$_{186}$ pFASTBAC1 virus or with a mock virus, and the media were harvested 48 hr after infection and immediately used or frozen at $-70°C$. The S2 cells were transfected and the expression was induced according to the supplier. The conditioned media were collected 72 hr after induction.

Antibodies. Purified m(His)$_6$VEGF-B$_{186}$ protein was used for immunization of rabbits according to standard procedures. The obtained antiserum and the antiserum to mVEGF-B N-terminal peptide (23) were affinity purified with m(His)$_6$VEGF-B$_{186}$ covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia). For quantitative immunoblots, media from infected or transfected insect cells were electrophoresed together with 1–30 ng of purified m(His)$_6$VEGF-B$_{186}$ as a standard and detected by using the affinity-purified antibodies.

Transfections, Immunoprecipitations, and Soluble Receptor Binding. 293-T cells were transfected with hVEGF$_{165}$pSG5, mVEGF-B$_{167}$pSG5, VEGF-B$_{kEx1–5}$ pSG5, mVEGF-B$_{186}$ pSG5, VEGFR-1 pIg, and VEGFR-2 pIg by using calcium phosphate precipitation. VEGFR-3 EC-Ig pREP7 (a kind gift from K. Pajusola, Biotechnology Institute, Helsinki), and hVEGF-CΔNΔC (His)$_6$pREP7 (33) were similarly expressed in 293-EBNA cells. Cells expressing the growth factors were metabolically labeled 48 hr after transfection with 100 μCi/ml Pro-mix L-[^{35}S] (Amersham) for 5–6 hr (1 μCi = 37 kBq), and the media were collected. Heparin was added to the labeling medium of VEGF-B$_{167}$ and VEGF at 10 μg/ml, unless otherwise stated. Metabolically labeled media (except from the VEGF transfection) were immunodepleted of endogenous expressed VEGF and heterodimers by absorption for 2 hr with 2 μg/ml VEGF antibody MAB 293 on staphylococcal protein A-Sepharose. Media of the cells expressing receptor Igs were replaced 48 hr after transfection by DMEM containing 0.1% BSA and incubated for an additional 12 hr. About 50 ng of receptor-Ig fusions and the corresponding volume of media from mock-transfected cells were absorbed to protein A-Sepharose. The metabolically labeled growth factors were incubated with the receptor-Igs for 3 hr at $+4°C$ and washed with ice-cold binding buffer (PBS/0.5% BSA/0.02% Tween 20/1 mM phenylmethylsulfonyl fluoride) three times and twice with PBS containing 1 mM phenylmethylsulfonyl fluoride. For competition studies 2 μg of recombinant hVEGF$_{165}$ was added to the binding reaction. Equal volumes of media containing the metabolically labeled factors were immunoprecipitated with the affinity-purified N-terminal peptide VEGF-B antibody, VEGF-C antiserum 882 (33), or VEGF MAB 293 for 2 hr, washed twice with ice-cold 10 mM Tris·HCl, pH 8.0/1% Triton X-100/25 mM EDTA/1 mM phenylmethylsulfonyl fluoride and twice with PBS containing 1 mM phenylmethylsulfonyl fluoride, and analyzed by SDS/PAGE.

Analysis of Competition for Binding to Cell Surface Receptors. Recombinant hVEGF$_{165}$ was labeled with Na^{125}I by using the Iodo-Gen reagent (Pierce) and purified by gel filtration on PD-10 columns (Pharmacia) to a specific activity of 1.0×10^5 cpm/ng. For binding analysis, NIH 3T3-Flt1 cells were seeded in 24-well plates coated with 0.2% gelatin, grown to confluence, washed twice with ice-cold binding buffer (DMEM/0.5 mg/ml BSA/10 mM Hepes, pH 7.4) and incubated in triplicate with 0.5 ng/ml ^{125}I-VEGF in binding buffer containing increasing amounts of unlabeled VEGF or media from VEGF-B- or mock-infected insect cells. After incubation for 2 hr at +4°C, the cells were washed three times with ice-cold binding buffer and twice with PBS containing 0.5 mg/ml BSA and lysed in 0.5 M NaOH. The solubilized radioactivity was measured with a γ counter. The nonspecific binding was less than 5% in all experiments.

Zymography and Reverse Zymography. Confluent monolayers of BME cells in 35-mm gelatin-coated tissue culture dishes were washed twice with serum-free medium, and cytokines were added in serum-free medium containing trasylol (200 Kunitz inhibitory units/ml). Fifteen hours later, cell extracts were prepared and analyzed by zymography and reverse zymography as previously described (34, 35).

RNA Preparation, *in Vitro* Transcription, and Northern Blot Hybridization. Cytokines were added to confluent monolayers of BME cells to which fresh complete medium had been added 24 hr previously. Total cellular RNA was prepared after indicated times by using Trizol reagent (Life Technologies, Basel, Switzerland). Northern blotting, UV cross-linking, methylene blue staining of filters, *in vitro* transcription, hybridization, and post-hybridization washes were as previously described (35). ^{32}P-labeled cRNA probes were prepared from bovine uPA (36), human tPA (37), and bovine PAI-1 (35) cDNAs as previously described (35, 38).

RESULTS

VEGF-B Binds Selectively to VEGFR-1. To investigate VEGF-B binding to VEGFR-1, -R-2, and -R-3, expression plasmids for mVEGF-B$_{167}$ and mVEGF-B$_{186}$ were transfected into 293-T cells. Conditioned medium from metabolically labeled transfected cells was depleted of endogenous VEGF and possible VEGF-VEGF-B heterodimers, and VEGF-B was precipitated by using VEGFR-Ig fusion proteins bound to protein A-Sepharose. Both VEGF-B splice isoforms specifically bound to VEGFR-1-Ig but not to VEGFR-2-Ig or VEGFR-3-Ig (Fig. 1*A*). The latter two receptor-Ig fusion proteins were functional as indicated by their ability to bind VEGF and VEGF-C, respectively. Interestingly, polypeptides of 32 and 16 kDa were precipitated from the mVEGF-B$_{186}$ conditioned medium with both anti-VEGF-B antibodies and by VEGFR-1-Ig. While the 32-kDa band corresponds to the glycosylated full-length form of mVEGF-B$_{186}$ (23), the 16-kDa form is likely to arise by proteolytic processing (discussed below). The binding of these two forms as well mVEGF-B$_{167}$ to VEGFR-1 was abolished by excess recombinant hVEGF (Fig. 1*B*), thus confirming the specificity of the interaction and suggesting that the receptor binding sites for VEGF and VEGF-B are, at least, partially overlapping.

We next examined the ability of VEGF-B to bind cell-surface-expressed VEGFR-1. In keeping with the data obtained with soluble receptors, conditioned medium from mVEGF-B$_{186}$ baculovirus-infected but not mock-infected
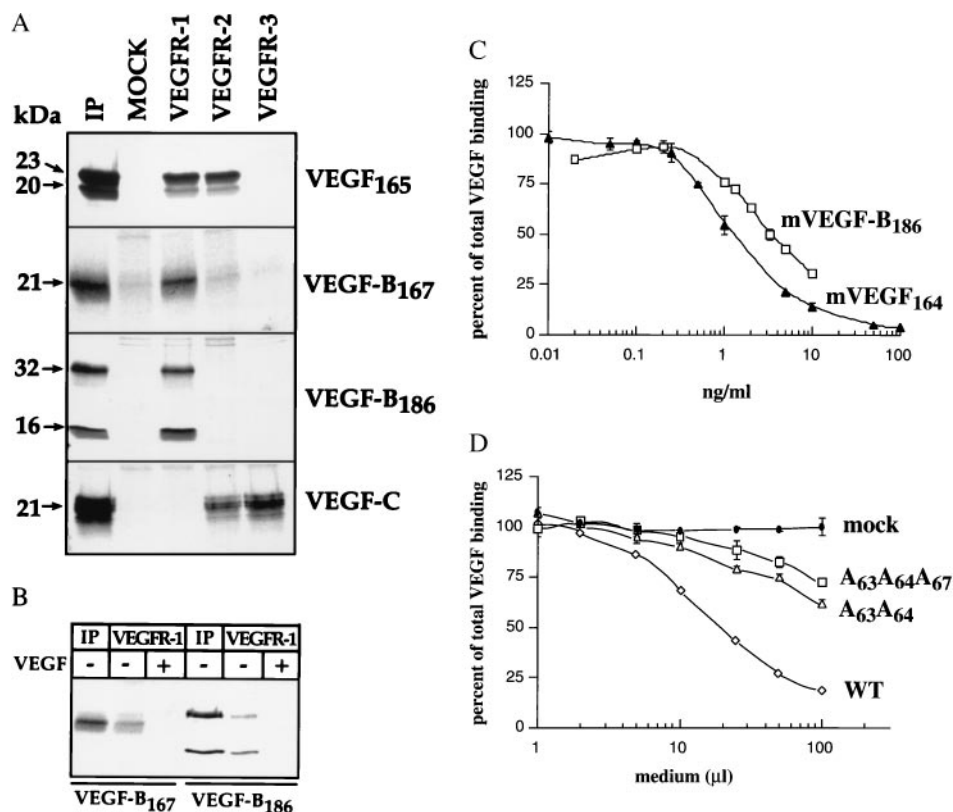

FIG. 1. VEGF-B binds selectively to VEGFR-1. (*A*) Metabolically labeled VEGF$_{165}$, mVEGF-B$_{167}$, mVEGF-B$_{186}$, or VEGF-C was precipitated by using receptor-Ig fusion proteins bound to protein A-Sepharose, or immunoprecipitated with specific antibodies as described in the text. Approximately 50 ng of each receptor-Ig was used. The precipitates were analyzed by SDS/PAGE in reducing conditions. (*B*) mVEGF-B$_{167}$ and mVEGF-B$_{186}$ were bound to VEGFR-1-Ig as above in the absence (−) or presence (+) of 2 μg of recombinant hVEGF$_{165}$. (*C*) Displacement of ^{125}I-hVEGF$_{165}$ from NIH 3T3/VEGFR-1 cells by mVEGF-B$_{186}$ or mVEGF$_{164}$. (*D*) Displacement of ^{125}I-hVEGF$_{165}$ from NIH 3T3/VEGFR-1 cells by conditioned medium containing equal amounts of mVEGF-B$_{Ex1-5}$ wild-type (WT) or mutant forms. Mean ± SD of triplicate samples in one of three independent experiment is shown.

High Five cells competed for ^{125}I-VEGF binding to NIH 3T3-Flt-1 cells (Fig. 1*C*). The half-maximum inhibitory concentration, IC$_{50}$, for mVEGF-B$_{186}$ was estimated to be 3 ng/ml by quantitative immunoblotting, whereas recombinant mVEGF$_{164}$ competed for ^{125}I-hVEGF binding at an IC$_{50}$ of 1.5 ng/ml (Fig. 1*C*).

The negatively charged amino acid residues Asp^{63}, Glu^{64}, and Glu^{67} in VEGF have been shown to be important for VEGFR-1 binding (24). To analyze whether the corresponding residues in VEGF-B are the major determinants in the VEGFR-1 interaction, Asp^{63}, Asp^{64}, and Glu^{67} were mutated to alanine residues in VEGF-B$_{167}$ and in the truncated Ex1–5 variant. Both mutants of VEGF-B$_{167}$ retained binding to VEGFR-1-Ig (data not shown); however, the corresponding Ex1–5 mutants showed markedly reduced receptor affinities when competing for ^{125}I-hVEGF binding to NIH 3T3-Flt-1. Interestingly, the effect of mutating all three negatively charged residues was more severe than mutations in the first two (Fig. 1*D*).

Proteolytic Processing of VEGF-B$_{186}$. We have previously demonstrated that mVEGF-B$_{186}$ is modified by O-linked glycosylation, resulting in the apparent molecular mass of 32 kDa of the secreted protein (23). When mVEGF-B$_{186}$ was expressed in 293-T cells, a 16-kDa band appeared in addition to the 32-kDa form (Fig. 1A). This band was also observed in conditioned medium from transfected COS cells labeled for a longer period. Under nonreducing conditions mVEGF-B$_{186}$ migrated as three different dimeric polypeptides of 34, 48, and 60 kDa (Fig. 2). The 34-kDa band migrates slightly slower than the product of the mVEGF-B$_{kEx1–5}$ construct encoded by exons 1–5, indicating that the putative cleavage site is present in the beginning of the sequence encoded by exon 6A (23). The 48-kDa band may represent a heterodimer between a processed and a full-length monomer. The three different dimeric polypeptides were able to bind VEGFR-1, and the processed subunits showed enhanced receptor binding (Fig. 2). Interestingly, plasmin treatment of unprocessed mVEGF-B$_{186}$ expressed in COS cells resulted in an N-terminal 15-kDa fragment capable of interacting with VEGFR-1-Ig (data not shown). This finding suggested that, similarly to VEGF, the plasmin cleavage product contains the receptor-binding epitopes.

Mutational Analysis of Conserved Cysteine Residues. To examine the contribution of the conserved cysteine residues for VEGF-B dimerization, Cys^{51} (Cys 2) and Cys^{60} (Cys 4) were mutated to serine residues individually (C2S and C4S) or in combination (C2S,C4S) (Fig. 3*A*). The mutants and wild-type VEGF-B$_{167}$ were either immunoprecipitated with the affinity-purified N-terminal VEGF-B antibody and analyzed under nonreducing and reducing conditions, or bound to VEGFR-1-Ig (Fig. 3*B*). All mutants were expressed in approximately
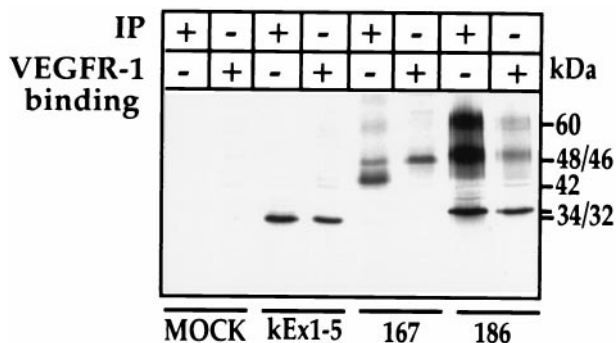
FIG. 2.   VEGF-B$_{186}$ is proteolytically processed. Metabolically labeled VEGF-B$_{kEx1–5}$, VEGF-B$_{167}$, and VEGF-B$_{186}$ were immunoprecipitated (IP) with the affinity-purified N-terminal VEGF-B antibody or bound to VEGFR-1-Ig. The bound ligands were analyzed by SDS/PAGE in nonreducing conditions.
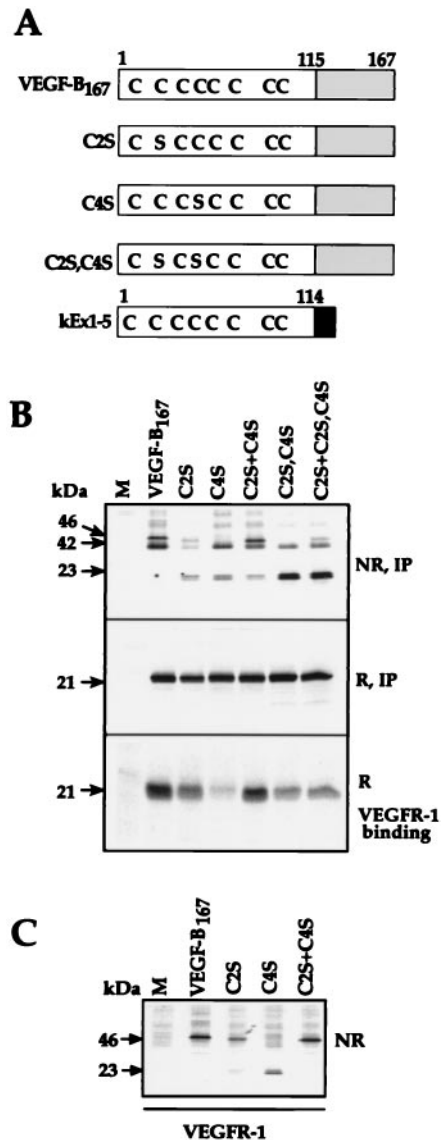
FIG. 3.   Mutational analysis of conserved cysteine residues in VEGF-B$_{167}$. (*A*) Schematic illustration of wild-type and mutant forms of VEGF-B$_{167}$. (*B*) The cysteine mutants were transfected individually or in combination, labeled in the presence of 50 μg/ml heparin, subjected to immunoprecipitation (IP) or VEGFR-1 Ig binding, and analyzed by SDS/PAGE in reducing (R) and nonreducing (NR) conditions. (*C*) Some of the VEGF-B$_{167}$ mutants were expressed as above and labeled in the presence of 10 μg/ml heparin. The conditioned media were incubated with VEGFR-1-Ig and the bound ligands were subjected to SDS/PAGE under nonreducing conditions.

similar amounts (Fig. 3*B Middle*). Wild-type VEGF-B$_{167}$ migrated under nonreducing conditions as two bands, 42 and 46 kDa; however, only the 46-kDa form bound to VEGFR-1-Ig (see Figs. 2 and 3 *B* and *C*). The 42-kDa band may correspond to dimers joined together by aberrant disulfide bonding, since such a polypeptide doublet is not seen in VEGF-B$_{186}$ or VEGF-B$_{kEx1–5}$, which lack the additional eight cysteine residues found in the C-terminal part of VEGF-B$_{167}$. The mutant C4S gave rise to monomers and some dimers migrating at 42 kDa, which were unable to bind to VEGFR-1-Ig. Surprisingly, the C2S mutant, although partially monomeric, could still form dimers capable of receptor binding. Cotransfection of the single mutants (C2S+C4S) resulted in increased amounts of the receptor-binding 46-kDa form, indicating that they complement each other by establishing a disulfide link between the nonmutated cysteine residues, probably in the same way as

shown for VEGF (25). Cotransfection of a single mutant with the double mutant failed to complement. However, both C4S and C2S,C4S showed residual receptor binding (Fig. 3C), which could be explained by the interaction of VEGFR-1-Ig with the monomers.

VEGF-B Increases uPA and PAI-1 Synthesis. The competition analysis using purified recombinant $(His)_6VEGF-B_{186}$ indicated that only a minor portion of the protein is biologically active, since the native unpurified $VEGF-B_{186}$ competed more effectively with ^{125}I-hVEGF for binding to VEGFR-1-expressing cells. Despite this, we tried to address some of the biological responses to VEGF-B. By Northern blot analysis, we found that $VEGF-B_{186}$ (50 ng/ml) increased the steady-state levels of uPA and PAI-1 mRNAs in BME cells (Fig. 4A). The

FIG. 4. Recombinant $hVEGF-B_{186}$ increases the expression and activity of uPA and PAI-1 in BME cells. (*A*) Replicate filters, containing 5 μg per lane of total cellular RNA prepared from confluent monolayers of BME cells incubated in the presence of 50 ng/ml $hVEGF-B_{186}$ for the indicated times, were hybridized with ^{32}P-labeled cRNA probes as described in the text. RNA integrity and uniformity of loading were determined by staining the filters with methylene blue after transfer and cross-linking (*Bottom*); 28S and 18S ribosomal RNAs are shown. (*B*) Cell extracts prepared from BME cells, incubated in the presence of $hVEGF-B_{186}$, VEGF, or bFGF at the indicated concentrations for 15 hr, were subjected to zymography (*Upper*) and reverse zymography (*Lower*) as described in the text.

kinetics of PAI-1 induction was more rapid and transient than of uPA, in agreement with findings reported for bFGF and VEGF (11, 35, 39). $VEGF-B_{186}$ also induced PAI-1 but not uPA mRNA in bovine aortic endothelial cells which, like BME cells, express VEGFR-1 as judged by reverse transcriptase-coupled PCR analysis using specific primers (data not shown). By zymography and reverse zymography, $VEGF-B_{186}$ induced a dose-dependent increase in uPA and PAI-1 activity in BME cells (Fig. 4B). In contrast to VEGF (11), $VEGF-B_{186}$ did not increase tPA activity. The apparent lack of induction of PAI-1 activity by VEGF, used as a control, may reflect the rapid sequestration of PAI-1 into a complex with VEGF-induced tPA; this complex is observed by zymography of the culture supernatant of VEGF-treated cells (data not shown).

DISCUSSION

In this study we demonstrate that VEGF-B specifically binds to VEGFR-1 and that endothelial cells respond to VEGF-B by increased expression and activity of uPA and PAI-1. Mutations of the conserved cysteine residues indicate that VEGF-B forms, similarly to VEGF, antiparallel covalent dimers. We also show that $VEGF-B_{186}$ is proteolytically cleaved, resulting in an N-terminal fragment, which contains the cystine knot motif as well as the receptor-binding epitopes.

VEGF-B is the third ligand identified for VEGFR-1, the others being VEGF and PlGF. The VEGF-binding determinant has been localized to the second Ig-like domain on VEGFR-1, whereas full VEGF binding requires the context of the first and especially the third Ig-like domain (40–42). The first three Ig-like domains of VEGFR-1 are also sufficient for VEGF-B binding (unpublished data), which is in agreement with the finding that VEGF and VEGF-B compete for VEGFR-1 binding. Charged amino acid to alanine scan mutagenesis led Keyt *et al.* (24) to propose that the VEGFR-1-binding epitope in VEGF involves a stretch of acidic residues (Asp^{63}, Glu^{64}, and Glu^{67}) that are located in loop II. These acidic amino acid residues are conserved in VEGF-B and partially also in PlGF. Our data indicate that these residues are contributing to the receptor affinity; however, they might not be the major determinants for VEGFR-1 binding. While this manuscript was in preparation, the crystal structure of VEGF in complex with the second Ig-homology domain of VEGFR-1 was reported (42). The VEGF–VEGFR-1 domain 2 interface is dominated by hydrophobic contacts, and many VEGF residues found to be important for VEGFR-2 binding (26) are also buried in the interface with VEGFR-1 (42). Asp^{63} is involved in a polar interaction, whereas Glu^{64} and Ile^{43} may be in contact with the third domain of VEGFR-1. Further studies are needed to elucidate the amino acid residues in VEGF-B critical for VEGFR-1 binding.

VEGF-B belongs to a growth factor superfamily containing a cystine knot motif. In addition to the disulfide bridges in the cystine knot in VEGF-B, two disulfide bridges join the two antiparallel monomers into a dimer. When Cys^{51} and Cys^{60} were mutated into serine residues, VEGF-B dimer formation and VEGFR-1 binding were severely reduced. Coexpression of the single mutants complemented the dimerization and receptor-binding defects.

The observed proteolytic processing of $VEGF-B_{186}$ may regulate receptor affinity, but is perhaps not important for bioavailability of the protein, since this isoform is readily secreted from cells. However, the apparently increased affinity of the processed form needs to be confirmed by using recombinant $VEGF-B_{186}$ forms. Recently, VEGF-C was shown to undergo proteolysis, with trimming of both N- and C-terminal extensions of VEGF-C, which resulted in an increased affinity for VEGFR-3 and activation of a VEGFR-2-binding property (33). Also the generation of an active fragment of $VEGF_{189}$ has been reported to require proteolysis (43).

VEGF and VEGF-B are likely to have only partially overlapping biological roles due to their different receptor binding
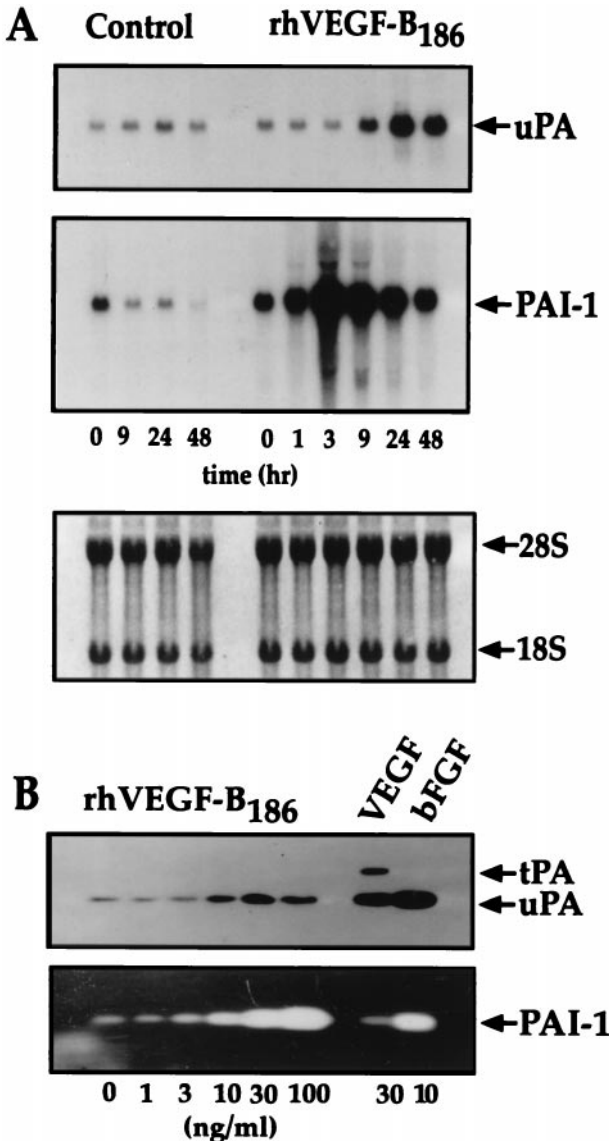
specificities. Results of gene targeting experiments have indicated that VEGFR-2 is required for the development of endothelial cells (7), and VEGFR-1 plays a role in vascular organization (6). Functional differences between the two VEGFRs have been observed also when receptor-transfected cells were used: whereas VEGFR-2-expressing fibroblasts respond to VEGF by proliferation (44), VEGFR-1-expressing cells fail to do so (45). In keeping with this observation, we have not been able to demonstrate any mitogenic effects by using purified recombinant tagged VEGF-B, although one cannot exclude the possibility that the amount of active protein in these preparations is not sufficient to trigger a response. It is possible that the previously reported stimulation of DNA synthesis by VEGF-B$_{167}$ conditioned medium from 293-EBNA cells (15) is due to heterodimer formation with the endogenous VEGF, similar to what has been reported for PlGF·VEGF heterodimers (46, 47). It is also possible that the binding of VEGF-B to VEGFR-1 enables VEGF to bind primarily to VEGFR-2 and to elicit a mitogenic response. In fact, PlGF has been shown to potentiate the activity of low concentrations of VEGF (21).

In this report we show that endothelial cells respond to VEGF-B by inducing the expression and activity of uPA and PAI-1. Interestingly, PAI-1 expression preceded that of uPA by several hours, similar to results with VEGF (11, 39). The concerted expression of PAI-1 and uPA may serve to protect the ECM from extensive proteolysis. Alternatively, PAI-1 may function independently of its role as an inhibitor. With regard to this latter possibility, it is of interest that PAI-1 has been shown to compete with integrin $\alpha_v\beta_3$ for vitronectin binding (48), enabling it to regulate cell–matrix interactions. PAI-1 and uPA may thus regulate both proteolysis and cellular migration during different stages of angiogenesis, and these dual functions are likely to be synergistic.

In this study we have identified VEGF-B as the second VEGFR-1-specific ligand. VEGF-B has a broad tissue expression pattern as opposed to PlGF, suggesting that it might be a functional homologue of PlGF in tissues others than the placenta. It might be involved in endothelial ECM degradation and adhesion, but *in vivo* studies will be necessary to characterize the physiological role of VEGF-B in angiogenesis and vascular maintenance.

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