Vascular Endothelial Growth Factor Receptor-2 and Neuropilin-1
Form a Receptor Complex That Is Responsible for the Differential Signaling Potency of VEGF<sub>165</sub> and VEGF<sub>121</sub> <sup>*</sup>

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The two most abundant secreted isoforms of vascular endothelial growth factor A (VEGF<sub>165</sub> and VEGF<sub>121</sub>) are formed as a result of differential splicing of the VEGF-A gene. VEGF<sub>165</sub> and VEGF<sub>121</sub> share similar affinities at the isolated VEGF receptor (VEGFR)-2 but have been previously demonstrated to have differential ability to activate VEGFR-2-mediated effects on endothelial cells. Herein we investigate whether the recently described VEGF<sub>165</sub> isoform-specific receptor neuropilin-1 (Npn-1) is responsible for the difference in potency observed for these ligands. We demonstrate that although VEGFR-2 and Npn-1 form a complex, this complex does not result in an increase in VEGF<sub>165</sub> binding affinity. Therefore, the differential activity of VEGF<sub>165</sub> and VEGF<sub>121</sub> cannot be explained by a differential binding affinity for the complex. Using an antagonist that competes for VEGF<sub>165</sub> binding at the VEGFR-2-Npn-1 complex, we observe specific antagonism of VEGF<sub>165</sub>-mediated phosphorylation of VEGF-2 without affecting the VEGF<sub>121</sub> response. These data indicate that the formation of the complex is responsible for the increased potency of VEGF<sub>165</sub> versus VEGF<sub>121</sub>. Taken together, these data suggest a receptor-clustering role for Npn-1, as opposed to Npn-1 behaving as an affinity-converting subunit.

The product of the VEGF-A<sup>1</sup> gene is required for formation of the embryonic vasculature, because haploinsufficiency leads to embryonic lethality due to a failure in both angiogenesis and blood island formation (1). The effects of VEGF-A on development of the embryonic vasculature are mediated by an interaction with the VEGF receptor (VEGFR)-1 and -2 receptor tyrosine kinases (2–4), whereas effects on angiogenesis that occur in the adult animal appear to be mediated largely through VEGFR-2 (5–8), with VEGFR-1 playing a modulatory role (9–12).

VEGF-A exists in multiple protein isoforms with different heparin proteoglycan and extracellular matrix binding properties. These isoforms (VEGF<sub>165</sub>, VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>183</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub>) arise from alternate splicing of the VEGF-A gene. VEGF<sub>165</sub> is the predominant isoform and has heparin binding activity, whereas the VEGF<sub>121</sub> isoform is freely soluble and is devoid of heparin binding activity (13). Similarly, another VEGF superfamily member, placenta growth factor (PlGF), exists in three different isoforms that also exhibit differential heparin binding ability (14). Although VEGF<sub>165</sub> and VEGF<sub>121</sub> bind to VEGFR-2 with equal affinity, their activity in biochemical assays that rely on activation of VEGFR-2 is not equivalent (15–17), implying that their ability to activate VEGFR-2 is not solely dependent on VEGF<sub>165</sub> binding affinity.

Recently, neuropilin (Npn)-1 was identified as a receptor that binds VEGF<sub>165</sub> (18, 19), VEGF-E (20), PlGF<sub>152</sub> (21), and VEGF-B (22), whereas Npn-2 has been identified as a receptor that binds PlGF<sub>152</sub>, VEGF<sub>165</sub>, and VEGF<sub>145</sub> (23). Neither Npn binds VEGF<sub>121</sub>, and Npn-1 will not bind the non-heparin-binding form of PlGF, PlGF<sub>139</sub> (21, 23). Npn-1 and Npn-2 also bind various semaphorin ligands to mediate repulsive guidance activity in certain neuronal populations (24). The cytoplasmic domains of Npn-1 and Npn-2 are not required for semaphorin signaling (25), and they do not contain sequences predictive of enzymatic activity nor sequences predicted to be involved in coupling to intracellular signaling molecules. Nevertheless, the Npn-1 and Npn-2 proteins are obligate binding subunits of a semaphorin signaling receptor complex. Members of the plexin family appear to serve as the signaling subunits of the semaphorin receptor complex (26, 27). Potential signals that lie downstream of the neuropilin-plexin receptor complex include activation of a pertussis toxin-sensitive G-protein (28) and activation of Rac1, a small molecular mass G-protein involved in cytoskeletal rearrangement (26, 29). The carboxyl terminus of Npn-1 has been shown to bind to a PSD-95/Dlg/ZO domain containing a regulator of G-protein signaling protein (30, 31), although the role of this protein in semaphorin signaling has not been established.

Although the role of Npn-1 in mediating the neuronal effects of the semaphorins is being elucidated, much less is known about the role of Npn-1 in VEGF<sub>165</sub>-mediated angiogenesis. Targeted disruption of the Npn-1 gene in mice results in embryonic lethality, with the embryos exhibiting defects in the formation of brachial arch-derived vessels, impaired neural vascularization, and defects in vascular development in the yolk sac that occur in addition to expected defects in neuronal patterning (32, 33). Transgenic overexpression of Npn-1 results in embryonic lethality associated with excessive vascular formation, dilation, and hemorrhaging, as well as defects in skeletal morphogenesis and neuronal guidance (34, 35). Because the semaphorin III knockout mouse also exhibits cardiovascular abnormalities (36), it is not currently known whether the...
vascular effects attributed to the absence or the overexpression of Npn-1 are due to signaling alterations in the semaphorin or the VEGF systems. Because VEGF165 does not bind to Npn-1 (18, 23) and because mice engineered to express only the VEGF121 isoform do not exhibit deficiencies in the development of the embryonic vasculature (37), it is possible that Npn-1 might not be required for the development of the vasculature in response to VEGF-A. However, these same mice exhibit defects in cardiac vascularization and suffer from ischemic cardiomyopathy, implying that VEGF121 cannot substitute for VEGF165 in development of the cardiac vasculature during the period of postnatal cardiac development (37). These data suggest that the inability of VEGF121 to interact with Npn-1 may result in insufficient signaling to support the maturation of the cardiac vasculature.

The ability of VEGF165 to bind to VEGFR-2 and Npn-1 through distinct regions of the VEGF165 molecule led to the assertion that VEGFR-2 and Npn-1 may signal as a co-receptor complex (18, 35). We demonstrate here that VEGFR-2 does indeed form a complex with Npn-1 in both heterologous systems as well as in cultured endothelial cells. We further demonstrate that, although the complex does not bind VEGF165 with an affinity that is greater than that exhibited at either VEGFR-2 or Npn-1 alone, formation of this complex can explain the differential properties that are observed in cultured endothelial cells for VEGF165-and VEGF121-mediated stimulation of VEGF-2 activation.

**EXPERIMENTAL PROCEDURES**

**Materials—** Carrier-free recombinant VEGF165 was purchased from R & D Systems Inc. (Minneapolis, MN). Na125I was purchased from Amersham Pharmacia Biotech. Heparin sulfate (catalog number 93999) and human recombinant VEGF165 (clone number A3294) were purchased from Sigma. PE-10 columns and protein G-Sepharose beads were purchased from Amersham Pharmacia Biotech. Donor calf serum, LipofectAMINE2000, and OptiMEM I were purchased from Life Technologies, Inc. Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). pJFE14 vector and hVEGFR-2/pJFE14 construct were obtained from Regeneron Pharmaceuticals (Tarrytown, NY). The pLTRMCSIRES-GFP and pLTR2hFLK1(full)IRES-GFP retroviral vectors were kindly provided by H. Nozawa (University of California, San Diego). pJFE14 vector and hVEGFR-2/pJFE14 construct were obtained from Regeneron Pharmaceuticals. The pBMN-Z-I-Neo vector used to construct hNpn-1 retroviral construct was obtained from Dr. Gary P. Nolan (Stanford University). The PGF152, Exon 6 peptide (amino acids 142–157) was synthesized by Synpep (Dublin, CA). All of the other reagents were obtained from commercial sources.

**Cell Culture—** Human umbilical vein endothelial cells (HUVECs) obtained from Cambrex (Walkersville, MD) were cultured in endothelial cell growth medium (Clonetics) and were used up to passage 3. Balb/c 3T3 A31 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in Balb/c growth medium (Dulbecco's modified Eagle's medium, 10% donor calf serum, 1% L-glutamine, 1% nonessential amino acids, 1% antimycotics, and 10% fetal bovine serum), QMXE packaging cells were cultured in COS-1 cell growth medium. All of the cells were grown at 37 °C in 5% CO2.

**Plasmid Construction—** For transient transfection in COS-1 cells, the hVEGFR-2 and hNpn-1 cDNAs were cloned into the mammalian expression plasmid pJFE14 containing the SV40 promoter (38). For stable expression in Balb/c 3T3 A31 cells, hVEGFR-2, GFP, and hNeuropilin-1 cDNAs were generated and subcloned into the pBMN-Z family of retroviral vectors. The pLTRMCSIRES-GFP (referred to as the Mock vector) and pLTR2hFLK1(full)IRES-GFP constructs are bi-cistronic modifications of the pBMN-Z vector, containing a GFP marker or containing GFP + hVEGFR-2. The hNpn-1 cDNA was subcloned into the pBMN-Z-I-Neo vector (OptiMEM I according to the manufacturer's instructions). The cells were plated 20 h after transfection.

**Creation of Stable Cells—** Retrovirus was generated by transfecting QMXE packaging cells with the appropriate retroviral construct (VEGF-A, VEGF-B, or Mock) using the same medium and methodology established for the COS-1 cells. Virus was generated overnight (33°C, 5% Balb/c 10 × 103 cells/100-mm dish were infected with viral supernatants containing 50 μg/ml of polybrene. Pools of infected Balb/c 3T3 A31 cells expressing hVEGFR-2 or GFP alone (Mock) were selected twice by fluorescence-activated cell sorting using the hVEGFR-2 specific monoclonal antibody 5J5A2 for the hVEGFR-2-containing cells or by GFP emission for the Mock cells. The hVEGFR-2 clone (D7R2) was generated by subjecting the hVEGFR-2 expressing cells to limiting dilution and identifying the SR-6 clones (39) which had been demonstrated to bind VEGF165 alone (40) or in Npn-1VEGFR-2 complexes (41). The VEGFR-2 rabbit polyclonal antibody (R2.2) was raised against the purified peptide sequence Ac-SKRRS-PSVSKTFEDILEPCC-amide (amino acids 1225–1246), unique to the carboxyl-terminal domain of VEGF-2, and affinity purified by QCB. The Npn-1 number 50 polyclonal antibody was the kind gift of Dr. Scott Ginty (Johns Hopkins University). This antibody was generated against amino acids 583–586 of rat neuropilin-1 (42). These residues correspond to the MAM domain and the extracellular juxtamembrane region and thus recognize only full-length Npn-1 protein. This antibody was sometimes substituted for the 5J5A2 antibody for immunoprecipitations (as indicated in the figure legends).

**Monoclonal Antibody Production—** For generation of the immungene for the 5J5A2 monoclonal antibody, the entire coding region of the VEGF-2 extracellular domain (amino acids 1–760) was expressed and produced as a fusion protein with a hexahistidine tag (6). The His6VEGFR-2 clone (D7R2) was generated by subjecting the hVEGFR-2 containing cells or by GFP emission for the Mock cells. The stable VEGFR-2 clonal cell line (Npn-1-2D) was generated by infecting Balb/c 3T3 A31 cells with hVEGFR-2/hNpn-1 stable cells (D7R2Npn-1-4D) were created by infecting the stable VEGFR-2 clonal cell line (D7R2) with hNpn-1 virus and selected in the presence of 1.5–2.0 mg/ml Geneticin. hVEGFR-2/hNpn-1 stable cells (D7R2Npn-1-4D) were created by infecting the stable VEGFR-2 clonal cell line (D7R2) with hNpn-1 virus and selected in the presence of 1.5–2.0 mg/ml Geneticin.

**Polycanal Antibody Production—** The Npn-1 rabbit polyclonal antibody (NP1ECD4) was raised against the purified peptide sequence Ac-DLDDKPKPEIKDTEGSC-amide (amino acids 814–830) and affinity purified by QCB, a division of BIOSOURCE International (Hopkinton, MA). The sequence used for generation of the NP1ECD antibody is from the extracellular juxtamembrane region of Npn-1, and this antibody was not recognized by the SR-6 clones (39) which had been demonstrated to bind VEGF165 alone (40) or in Npn-1VEGFR-2 complexes (41). The VEGFR-2 rabbit polyclonal antibody (R2.2) was raised against the purified peptide sequence Ac-SKRRS-PSVSKTFEDILEPCC-amide (amino acids 1225–1246), unique to the carboxyl-terminal domain of VEGF-2, and affinity purified by QCB. The Npn-1 number 50 polyclonal antibody was the kind gift of Dr. Scott Ginty (Johns Hopkins University). This antibody was generated against amino acids 583–586 of rat neuropilin-1 (42). These residues correspond to the MAM domain and the extracellular juxtamembrane region and thus recognize only full-length Npn-1 protein. This antibody was sometimes substituted for the 5J5A2 antibody for immunoprecipitations (as indicated in the figure legends).

**iodination of VEGF-165—** Iodination was performed using a modification of methods described previously (15, 43). Briefly, 5 μg of carrier-free human recombinant VEGF165 was suspended in 90 μl of Dulbecco's phosphate-buffered saline. To the reaction tube, 1 mCi of Na125I was added, followed by 40 μl of chloramine T (1 μg/ml in 0.5 M sodium phosphate buffer, pH 7.5), and incubated for 1 min. 50 μl of sodium metabisulfite (2 μg/ml in sodium phosphate buffer, pH 7.5) was added to stop the reaction. 500 μl of column elution buffer (0.5% BSA, 0.01% Tween 20 in Dulbecco's phosphate-buffered saline) was added to the reaction and transferred to pre-equilibrated PD-10 column for separation from unreacted iodine. The specific activity was corrected for column recovery and varied from 4,000 to 15,000 Ci/mmol.

**Radiolmmunoprecipitation—** 3 × 105 cells/100-mm dish were plated 24 h prior to the experiment. The cells were incubated with 300–700 pm [125I]VEGF165 for 4 h in 4°C binding buffer (minimum essential medium, 25 mM HEPES, 0.2% BSA) containing 1 μg/ml hirudin, 1 μg/ml heparin sulfate and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml antipain, 50 μg/ml benzamidine, 100 μg/ml soy bean trypsin inhibitor, 10 μg/ml benzamidine, 100 μg/ml pepstatin A, 0.1 mM phenyl melenyl fluoride).

Unbound ligand was removed by washing with ice-cold BSA-free binding buffer (minimum essential medium, 25 mM HEPES). Affinity labeling of the receptors was achieved by cross-linking with 15 mM disuccinimidyl glutarate in BSA-free binding buffer (15 min, 4°C). Excess disuccinimidyl glutarate was removed by washing with ice-cold Dulbecco's phosphate-buffered saline. The cells were lysed with RIPA buffer (20
mm Tris-HCl, pH 7.4, 100 mm NaCl, 1 mm EDTA, 10 mm NaI, 0.5% IGEFAI CA-630, 0.5% sodium deoxycholate, 1% BSA. Lysate was immunoprecipitated overnight at 4 °C with 10 μg of antibody (R2.2, NPIED4, or C-19), 0.1% SDS, and protein G-Sepharose beads. The samples were separated on 6% Novex SDS-polyacrylamide gel electrophoresis (PAGE) gels, and the images were analyzed on the Storm technology Inc., Lake Placid, NY; catalog number 05-321) and detected by ECL. Generation of competition curves and IC50 values were determined from analysis of the dose-response curves generated with GraphPad Prism.

**RESULTS**

**VEGF165 and VEGF121 Exhibit Differential Potency in Stimulating VEGFR-2 Phosphorylation Despite Similar Affinity at VEGFR-2**—Although VEGF165 and VEGF121, have the same affinity for the isolated purified VEGFR-2 receptor (15), these two ligands exhibit a differential ability to activate the VEGFR-2 receptor in vitro (15–17). In native HUVEC, this differential activity manifests as an increased potency of VEGF165 relative to VEGF121. This difference in potency is characterized by an EC50 (concentration of agonist that provokes a response halfway between the base-line and maximal responses) for VEGF165 of 7.76 × 10−11 M (log EC50 = −10.11 ± 0.113, n = 14) and for VEGF121 of 2.45 × 10−9 M (log EC50 = −8.61 ± 0.091, n = 14) in stimulating phosphorylation of VEGFR-2, with a representative experiment illustrated in Fig. 1. The reported similarity in affinity between VEGF165 and VEGF121 at the isolated VEGFR-2 receptor is not an artifact of producing the protein as a receptor body (15), because when the full-length receptor is expressed in cells similar results are observed (Fig. 2A). In Fig. 2A, VEGFR-2 is overexpressed in COS-1 cells, and the ability of these two ligands to compete for [125I]VEGF165 binding is measured. In the COS-1 cells expressing the full-length receptor, the IC50 values for VEGF165 (IC50 = 6.34 × 10−10 M) and VEGF121 (IC50 = 3.12 × 10−10 M) are nearly identical, indicating similar affinity to VEGFR-2 when it is expressed in isolation. Surprisingly, VEGF121 has only limited ability to compete for [125I]VEGF165 binding in HUVEC (Fig. 2B, D). Despite the presence of functional VEGFR-2 in these cells (Fig. 1). Because the HUVEC are reported to contain Npn-1 (18), we examined whether the binding observed in HUVEC could be reproduced in the COS-1 cell system. As expected, VEGF121 does not compete in COS-1 cells where Npn-1 is expressed alone (Fig. 2C) but limited or no competition for binding is also observed in cells expressing Npn-1 in concert with VEGFR-2 (Fig. 2, B and D). The data from the COS-1 cells expressing defined receptor populations suggest that the relative inability of VEGF121 to compete for binding in HUVEC may be attributed to excess Npn-1 expression relative to VEGFR-2. We therefore explored the possibility that VEGFR-2 and Npn-1 form a co-receptor complex and that the preferential ability of VEGF165 to signal through this complex is responsible for the increased potency of VEGF165 relative to VEGF121 in the VEGFR-2 autophosphorylation assay. VEGFR-2 and Npn-1 Form a Co-receptor Complex—To demonstrate the potential for the VEGF-2-Npn-1 complex to form, we utilized an affinity labeling reciprocal immunoprecipitation experimental design. In this design, cells expressing each of the receptors in isolation or co-expressing both receptors are affinity-labeled with a high concentration of [125I]VEGF165 chemically cross-linked, and immunoprecipitated with antibodies.

**TABLE I**

| Cell Line | Type | Receptors | Clone | Log Kd ± S.E. | Kd | Bmax ± S.E. | n |
|-----------|------|-----------|-------|--------------|----|-------------|--|
| COS-1     | Transient | VEGFR-2 |       | −9.46 ± 0.15 | 3.39 × 10⁻¹⁰ | 13,670 ± 2,886 | 9 |
|           |       | VEGFR-2Npn-1 |     | −8.84 ± 0.00 | 1.43 × 10⁻⁹ | 245,700 ± 6,000 | 1 |
| Npn-1     |       | Mock     |       | −8.68 ± 0.17 | 2.09 × 10⁻⁹ | 151,950 ± 55,957 | 3 |
| Balb/c    | Stable | D7R2     |       | −8.95 ± 0.31 | 1.11 × 10⁻⁹ | 8,038 ± 2,080 | 4 |
|           |       | VEGFR-2Npn-1 |     | −9.43 ± 0.33 | 3.31 × 10⁻¹⁰ | 228,467 ± 150,999 | 5 |
|           |       | Npn-1-129 |       | −8.32 ± 0.13 | 4.17 × 10⁻¹⁰ | 143,506 ± 37,410 | 5 |
|           |       | Mock PGBMHG |     | −9.65 ± 0.13 | 2.22 × 10⁻¹⁰ | 48,825 ± 16,118 | 4 |
| HUVEC     | Native | VEGFR-2Npn-1 |     | −9.77 ± 0.12 | 1.69 × 10⁻⁹ | 49,450 ± 3,600 | 2 |

* Statistically greater than PGBMHG (p = 0.050).
specific for one member of the suspected receptor complex. If the complex forms, the receptor-specific antibody should more effectively precipitate the receptor against which it was generated, and additionally precipitate a band of the appropriate size corresponding to the alternate receptor in the complex. Fig. 3A demonstrates that the complex can indeed form in the presence of ligand in the COS-1 overexpression system. In cells expressing only VEGFR-2 (lane 1) a single band of ~240 kDa is observed. This band represents $[^{125}I]$VEGF<sub>165</sub> cross-linked to VEGFR-2. Using our VEGFR-2 specific antibody, in COS-1 cells co-expressing VEGFR-2 and Npn-1 we are able to immunoprecipitate not only affinity-labeled VEGFR-2, but an additional doublet of bands that runs at the predicted size (~140 kDa) for Npn-1 cross-linked to $[^{125}I]$VEGF<sub>165</sub> (Fig. 3A, left panel, lane 2). In cells overexpressing only Npn-1 (lane 3) the VEGFR-2 receptor is not present for the complex to form, and therefore we are unable to substantially detect the Npn-1 doublet. The data in lane 3 also indicate that the VEGFR-2 antibody does not cross-react with Npn-1, and the weak doublet detected in lane 3 may indicate formation of a complex between endogenous VEGFR-2 and the exogenous Npn-1. Interestingly, a weak doublet that corresponds to the size of the Npn-1 bands is also detected with the VEGFR-2 receptor-specific antibody in cells overexpressing VEGFR-2 (Fig. 3A, lane 1), which may indicate formation of a complex between endogenous Npn-1 (see Npn-1 Western data; Fig. 3B, right panel) in the presence of the exogenous VEGFR-2. The inability to detect these affinity-labeled bands in the COS-1 cells expressing empty vector (Fig. 3A, Mock, lane 4) provides a further indication that these bands are not nonspecific. The identity of the lower doublet is confirmed as being Npn-1 by the immunoprecipitation with the Npn-1-specific antibody (Fig. 3A, lane 7). The inability to detect bands in VEGFR-2 cells alone (lane 5) demonstrates that when Npn-1 is not overexpressed, the complex with endogenous Npn-1 and overexpressed VEGFR-2 is not detected because no affinity-labeled VEGFR-2 receptor is detected in cells immunoprecipitated by the Npn-1 specific receptor antibody. Lane 5 also demonstrates that the Npn-1 receptor-specific antibody does not cross-react with the VEGFR-2 receptor. When both receptors are co-expressed (Fig. 3A, lane 6) and immunoprecipitated using the Npn-1 receptor-specific antibody, a band corresponding to the size of affinity-labeled VEGFR-2 is apparent, in addition to the affinity-labeled Npn-1 doublet. COS-1 cells expressing Npn-1 alone immunoprecipitated with the Npn-1 antibody confirm the identity of the doublet as being due to $[^{125}I]$VEGF<sub>165</sub> binding to Npn-1 (lane 7). The inability to detect the doublet in COS-1 cells expressing empty vector (Fig. 3A, lane 8) again demonstrates the specificity of the immunoprecipitated bands. Finally, we observe high molecular mass bands either in cells expressing VEGFR-2 alone (Fig. 3A, lane 1), in cells expressing Npn-1 alone (lane 7), or in cells co-expressing these receptors (lanes 2 and 6). Because it is difficult to estimate the molecular masses of the bands in this region of the gel, and the individual receptor homodimers are of comparable molecular masses, we cannot identify the precise nature of these bands in cells co-expressing both VEGFR-2 and Npn-1.

It is noteworthy that more intense labeling of VEGFR-2 is observed upon co-expression with Npn-1 (Fig. 3A, lane 2 versus lane 1) and that more intense labeling of Npn-1 is observed upon co-expression with VEGFR-2 (Fig. 3A, lane 6 versus lane 7). This is because co-expression results in increased expres-
Fig. 2. Examination of the binding affinity of VEGF<sub>165</sub> (squares) and VEGF<sub>121</sub> (circles) in cells expressing various receptor complements. Cells with various VEGF receptor backgrounds were incubated in the presence of 382 pM (COS-1, where the average $K_d = 339$ pM or 2 nM for VEGFR-2 and Npn-1, respectively; see Table I) or 287 pM (HUVEC, where the average $K_d = 169$ pM; see Table I) $[^{125}]$VEGF<sub>165</sub>, and increasing concentrations of cold VEGF<sub>165</sub> or VEGF<sub>121</sub> to equilibrium (4 h) at 4 °C as described under “Experimental Procedures.” A, COS-1 cells expressing VEGFR-2 ($IC_{50}$ VEGF<sub>165</sub> = 634 pM, VEGF<sub>121</sub> = 312 pM). B, endogenous HUVEC cells known to express both VEGFR-2 and Npn-1 ($IC_{50}$ VEGF<sub>165</sub> = 79.5 pM, VEGF<sub>121</sub> = no displacement). C, COS-1 cells expressing Npn-1 ($IC_{50}$ VEGF<sub>165</sub> = 1090 pM, VEGF<sub>121</sub> = no displacement). D, COS-1 cells expressing both VEGFR-2 and Npn-1 ($IC_{50}$ VEGF<sub>165</sub> = 2890 pM, VEGF<sub>121</sub> = no displacement). The $IC_{50}$ values were calculated by fitting the data to a four-parameter logistic equation (Prism software). The data points represent the averages ± S.E. of triplicate determinations. This experiment has been repeated twice with similar results.

Fig. 3. Affinity labeling and immunoprecipitation of the VEGFR-2/Npn-1 receptor complex. A, COS-1 cells transiently overexpressing the indicated receptors were affinity-labeled at 4 °C for 4 h with a tracer concentration greater than the $K_d$ ([125I]VEGF<sub>165</sub>) to allow for better labeling of the receptors. The cells were then lysed, immunoprecipitated (IP) using the receptor-specific antibody listed (for VEGFR-2, R2.2; for Npn-1, C-19), separated by SDS-PAGE, and developed using the Storm System (Molecular Dynamics). The left panel demonstrates that the Npn-1 doublet is co-precipitated with affinity-labeled VEGFR-2 when the two receptors are co-expressed. The right panel demonstrates that VEGFR-2 is co-precipitated in the complex when an Npn-1 specific antibody is used. In both panels, a high molecular mass band is observed that may represent the VEGFR-2-Npn-1 complex (see text). This experiment has been repeated three times with similar results. B, Western blot of matched lysates depicts the relative receptor expression level achieved (20 μg of total protein was loaded per lane). Blotting antibodies are R2.2 for VEGFR-2 and the Npn-1 number 30 antibody for Npn-1.

sion of both VEGFR-2 and Npn-1 protein versus that obtained when either receptor is expressed alone (Fig. 3B) and is therefore not an indication of increased binding affinity at the receptor complex (see also Table I).

Fig. 4 demonstrates the ability of the VEGFR-2-Npn-1 complex to form in endogenous cells, as similar results are found when the reciprocal immunoprecipitations, described above, are performed in HUVEC cells. The VEGFR-2-specific antibody immunoprecipitates affinity-labeled VEGFR-2 along with a triplet of bands (~120–140 kDa), of which two bands correspond
represent an endogenous soluble form of Npn-1 because soluble asterisk precipitates, and this band may represent the VEGFR-2 by the Npn-1 antibody (see text). Similar to what is observed in Fig. 3, immunoprecipitated (top lane) using the receptor-specific antibody listed (for VEGFR-2, R2.2; for Npn-1, C-19), separated by SDS-PAGE, and developed using the Storm system (Molecular Dynamics). The left lane demonstrates that a triplet of affinity-labeled bands were co-precipitated with affinity-labeled VEGFR-2. The right lane confirms the identity of the top two bands of the triplet as being Npn-1 because an identical doublet is immunoprecipitated using an Npn-1 specific antibody (right panel). The asterisk represents the Npn-1 band not immunoprecipitated by the Npn-1 antibody (see text). Similar to what is observed in Fig. 3, a high molecular mass band is also present in the HUVEC immunoprecipitates, and this band may represent the VEGFR-2-Npn-1 complex (see text). This experiment has been repeated three times with similar results.

in size to the doublet that is immunoprecipitated by the Npn-1 antibody in affinity-labeled COS-1 cells (Fig. 3A, lanes 6 and 7). The third band (Fig. 4, left panel, asterisk, and Fig. 7A, lane 1, asterisk) of the triplet observed in the HUVEC cells might represent an endogenous soluble form of Npn-1 because soluble Npn-1 is competent to bind [125I]VEGF165 (40) and may also form a complex with VEGFR-2 (41). This band is not observed in the COS-1 system (Fig. 3) because the Npn-1 cDNA used for these experiments would only produce the full-length receptor. In the anti-Npn-1 immunoprecipitates from HUVEC cells (Fig. 4, right panel, and Fig. 7A, lane 4), a doublet of the size seen in the COS-1 cells (Fig. 3A) is once again observed. The triplet seen in the anti-VEGFR-2 immunoprecipitates is not observed here because the Npn-1 antibody used for these experiments would not be predicted to immunoprecipitate the soluble form of Npn-1 (see “Experimental Procedures”). Similar to Fig. 3, high molecular mass bands are also observed with both antibodies, which might suggest the formation of a heteromeric receptor complex.

To confirm the identity of the bands in the affinity labeling experiments and to examine the possibility of ligand-independent complex formation, we utilized a reciprocal immunoprecipitation Western blot analysis experimental design, in either the presence or the absence of VEGF165, in both the HUVEC cells and COS-1 overexpression systems. As observed in the affinity labeling experiments (Fig. 3A), Fig. 5A demonstrates that we are able to immunoprecipitate Npn-1 with our VEGFR-2-specific antibody only in COS-1 cells that co-express VEGFR-2 in concert with Npn-1 (Fig. 5A, left panels). Conversely, we are also able to immunoprecipitate VEGFR-2 with the Npn-1-specific antibody only in COS-1 cells co-expressing VEGFR-2 with Npn-1 (Fig. 5A, right panels). The ability to detect the co-receptor complex in COS-1 cells appears to be independent of the presence of VEGF165 ligand.

Western blot analysis of COS-1 and HUVEC cells demonstrates that the expression levels of VEGFR-2 and Npn-1 are substantially lower in the HUVEC cells relative to the COS-1 cells (Fig. 5B). The lower expression levels of the receptors, coupled with the limitation imposed by the efficiency of the receptor-specific antibodies, precludes detection of the receptor complex using the reciprocal immunoprecipitation Western technique in HUVEC cells. Detection of the co-receptor complex in the endogenous HUVEC system is only possible in the presence of ligand because of the sensitivity afforded by the use of radioiodinated ligand (compare Fig. 5A with Fig. 4). For this reason, we are unable to determine whether a ligand-independent complex exists in HUVEC cells. Although the COS-1 system indicates a potential for a ligand-independent complex to form in endogenous systems, we cannot exclude the possibility that the ligand-independent complex observed in the COS-1 cells is driven by the higher receptor expression levels obtained in the COS-1 cell system.

Formation of the VEGFR-2-Npn-1 Complex Does Not Result in an Increase in Ligand Binding Affinity—With the formation of the co-receptor complex demonstrated, we investigated the mechanism by which the complex increased the signaling potency of VEGF165. The simplest explanation would be that Npn-1 acts as an affinity converter and that the affinity of VEGF165 for VEGFR-2 is increased in the presence of Npn-1. Although the COS-1 system is an effective tool to determine whether the complex can form, a transient expression system does not ensure that co-expression of both receptors occurs in every cell. To avoid this caveat, we produced stable cell lines overexpressing VEGFR-2 on an Npn-1 background. We chose the Balb/c 3T3 A31 cell line in which to produce our stable cell lines because these cells are reported to have endothelial cell characteristics (45). Fig. 6A illustrates that the endogenous Balb/c 3T3 A31 cells contain detectable amounts of VEGF binding sites (vector control, PGBMGH) that can be explained by the expression of detectable amounts of Npn-1 (Fig. 6B). Not surprisingly, overexpression of human Npn-1 on this background increases the number of binding sites relative to vector control (p = 0.050, Bmax = 143,306 ± 37,419, n = 5, in Npn-1(2) versus 48,825 ± 16,118, n = 4 in PGBMGH) without a change in binding affinity (Table I). When VEGFR-2 is overexpressed on the Npn-1 background, there is a small but statistically insignificant increase in binding site number with no change in binding affinity (Fig. 6A and Table I, D7R2). The lack of a statistically significant increase in Bmax when VEGFR-2 is overexpressed on the Npn-1 background (Table I and Fig. 6A) can be attributed to the formation of the VEGFR-2-Npn-1 complex, which the VEGF165 ligand perceives as indistinguishable from free VEGFR-2 or Npn-1. We attempted to further increase Npn-1 expression in the D7R2 cells but achieved only marginal success, as evidenced by the Western analysis in Fig. 6B and a lack of statistically significant increase in Bmax versus that in the parent D7R2 cells (Table I). Nevertheless, these data clearly indicate that binding affinity to Npn-1 is not increased in the presence of VEGFR-2, because we could not discern a subpopulation of higher affinity binding sites when the data were fit to a one-site versus two-site binding model (data not shown). Furthermore, the inability to detect a high affinity subpopulation is independent of the ratio of VEGFR-2 to Npn-1 because these ratios are quite different in the COS-1 and Balb/c cells (compare Western analyses in Figs. 3B, 5B, and 6B).

The limitation to this analysis is that the Balb/c 3T3 A31
cells do not allow us to compare the binding affinity at VEGFR-2 to that of Npn-1 when each are expressed alone. However, the binding to VEGFR-2 expressed alone in the COS-1 cells (3.39 \times 10^{-10} \text{ M}) is similar to that observed in the D7R2 cells (2.91 \times 10^{-10} \text{ M}), despite the much higher Npn-1 background in the D7R2 cells (Table I and Figs. 3B, 5B, and 6B). Furthermore, the affinity at Npn-1 in the Balb/c cells (4.17 \times 10^{-10} \text{ M}) is similar to that at VEGFR-2 expressed in the COS-1 cells (Table I). This indicates that the affinity of VEGF_{165} is similar at either receptor, making it even less likely that we missed detection of a subpopulation of high affinity sites that is created upon co-expression of the two receptors.

Access to the VEGFR-2-Npn-1 Complex Can Explain the Increased Potency of VEGF_{165} versus VEGF_{121} for Activation of VEGFR-2—To demonstrate that the enhanced potency of VEGF_{165} could be explained by enhanced signaling through the VEGFR-2-Npn-1 complex versus VEGFR-2 alone, we chose to specifically antagonize the binding of VEGF_{165} to Npn-1 to see whether the potency of VEGF_{165} in the anti-phosphotyrosine assay would be reduced to match that of VEGF_{121}. A potential Npn-1 antagonist has been identified by Migdal et al. (21), who examined the effects of peptides generated from the Exon 6 and Exon 7 portions of PlGF152 on the binding of [125I]VEGF_{165} in HUVEC cells. These authors concluded that a peptide consisting of the first 16 amino acid residues from Exon 6 of PlGF_{152} was sufficient to block the binding of [125I]VEGF_{165} to a 120-kDa band whose characteristics were consistent with that of Npn-1. Fig. 7A demonstrates the ability of this peptide to compete specifically for [125I]VEGF_{165} binding in HUVEC cells. With no competitor (lane 1), [125I]VEGF_{165} binding to the
VEGFR-2 band and the Npn-1 triplet in HUVEC cells is detectable by immunoprecipitation with a VEGFR-2-specific antibody. An excess of cold VEGF165 (30 nM) completely abrogates the labeling of both the VEGFR-2 band and the Npn-1 triplet (lane 2), and the PlGF152 Exon 6 peptide competes for binding to the Npn-1 triplet with a proportional effect on the labeling of the VEGFR-2 band (lane 3). Furthermore, when HUVEC cells are affinity-labeled with [125I]VEGF165 and immunoprecipitated with an Npn-1-specific antibody, the PlGF152 Exon 6 peptide completely abrogates the labeling of the Npn-1 triplet (lane 6). These data are consistent with the PlGF152 Exon 6 peptide being able to compete for binding of VEGF165 to Npn-1 and to the VEGFR-2-Npn-1 complex in the native HUVEC cells. The ability of the PlGF152 Exon 6 peptide to bind preferentially to Npn-1 is demonstrated in Fig. 8 where the peptide is able to compete for [125I]VEGF165 binding in COS-1 cells expressing Npn-1 but does not compete effectively for binding to VEGF-2 when it is expressed in the absence of Npn-1.

Because the PlGF152 Exon 6 peptide is able to compete for VEGF165 binding to Npn-1, we hypothesized that if Npn-1 is responsible for the increased potency of VEGF165, preventing the formation of the VEGFR-2-Npn-1 complex by blocking the binding of VEGF165 to Npn-1 should reduce the signaling potency of VEGF165. Furthermore, because VEGF121 does not have access to the Npn-1 receptor but activates VEGFR-2, the PlGF152 Exon 6 peptide should have no effect on the signaling potency of VEGF121. Fig. 8 demonstrates that in the absence of the PlGF152 Exon 6 peptide, VEGF165 and VEGF121 demonstrate significantly different signaling potencies in native HUVEC (VEGF165 EC50 = 283 pM; VEGF121 EC50 = 1.84 nM). However, in the presence of 100 μM PlGF152 Exon 6 peptide, the signaling potency of VEGF165 is dramatically reduced (VEGF165 + PlGF152 Exon 6 peptide EC50 = 1.06 nM), whereas the signaling potency of a ligand that does not have access to Npn-1 and that signals solely through VEGFR-2 (VEGF121) is unaffected (VEGF121 + PlGF152 Exon 6 peptide EC50 = 2.38 nM). From these data, we conclude that the ability of VEGF165 to bind to the VEGFR-2-Npn-1 complex is responsible for the differential potency of VEGF165 relative to VEGF121 in stimulating activation of VEGFR-2.

**DISCUSSION**

Neuropilin-1 has been identified as an isoform-specific receptor for VEGF165 (18), the VEGF-E isoform VEGF152 (20), and both splice isoforms of VEGF-B (22). In the case of VEGF165, it was hypothesized that Npn-1 acts as a potentiator of ligand binding to VEGFR-2, thereby explaining the differential activity of VEGF165 versus VEGF121 in various endothelial cell signaling assays (18).

[Fig. 6] The affinity of VEGF165 to VEGFR-2 is not increased in the presence of Npn-1. A: Balb/c 3T3 A31 cells stably expressing the indicated receptors are incubated to equilibrium with increasing concentrations of [125I]VEGF165 in the presence or the absence of a saturating dose of unlabeled VEGF165 (30 nM). The curves depicted represent specific binding, where the error bars are the standard error of the mean for triplicate determinations. The binding parameters for VEGFR-2 and Npn-1 are as follows: squares, D7R2-Npn-1 cells, Bmax = 98,750 ± 3,436 sites/cell, Kd = 119 ± 15 pM; circles, PGBMGH (Mock) cells, Bmax = 38,990 ± 3,135 sites/cell, Kd = 123 ± 36 pM; triangles, Npn-1(2) cells, Bmax = 78,390 ± 3,381 sites/cell, Kd = 184 ± 25 pM; diamonds, D7R2 cells, Bmax = 60,800 ± 2,080 sites/cell, Kd = 145 ± 17 pM. The errors associated with the curve fitting parameters represent approximate errors in the estimates for the curves. B: Western blot analysis of matched lysates demonstrating relative receptor levels (20 μg of total protein was loaded per lane). The blotting antibodies are R2.2 and Npn-1 number 30 for VEGFR-2 and Npn-1, respectively. The binding experiment has been repeated at least three times with similar results (Table 1). The Western blot analysis has been repeated at least six times with similar results.
engineered to co-express Npn-1 with VEGFR-2. Affinity labeling experiments are not accurate indicators of receptor-ligand binding affinity because the results can be biased by changes in cross-linking efficiency of the ligand to the receptor that result from conformational alterations upon ligand binding to the receptor component within the complex versus that observed when the receptor component is expressed alone. Additionally, the amount of radioligand observed to cross-link a receptor component can be influenced by alterations in receptor expression between cell lines that express the receptor alone versus those that co-express multiple receptor components. Indeed, we observe a similar increase in binding to VEGFR-2 in COS-1 cells transiently expressing VEGFR-2 in concert with Npn-1 versus that observed in COS-1 cells that express VEGFR-2 alone, but this increased binding to VEGFR-2 can be explained by increased expression of VEGFR-2 in the presence of Npn-1 that often occurs in this system (Fig. 3).

Further support for the contention that formation of the VEGFR-2-Npn-1 receptor complex does not result in an alteration in VEGF binding affinity comes from a detailed analysis of the saturation isotherms in cells expressing Npn-1 alone or in concert with VEGFR-2 (Fig. 6 and Table I). The binding affinity of [125I]VEGF 165 is similar in cells expressing Npn-1 alone or VEGFR-2 alone, and there is no increase in affinity observed upon co-expression of VEGF-2 on an Npn-1 background (Fig. 6 and Table I). These data demonstrate that formation of the VEGFR-2-Npn-1 co-receptor complex in the presence of ligand (Figs. 3, 4, and 7) does not result in formation of a subpopulation of high affinity binding sites. In this respect, the VEGFR-2-Npn-1 receptor complex is similar to that observed for the glial cell line-derived neurotrophic factor receptor a subunit and the Ret receptor tyrosine kinase (RTK) co-receptor complex, where ligand binding is also observed to the non-receptor tyrosine kinase a subunit in the absence of the RTK, and co-expression of the a subunit with the RTK does not result in an increase in ligand binding affinity (46). Hence, Npn-1 does not appear to function as an affinity converter for VEGF in concert with VEGFR-2, as has been observed for other multi-component receptor systems (47). This is in contrast to the role played by Npn-1 in the regulation of ligand binding affinity of Sema3A to the Npn-1 Plexin1 co-receptor complex (26).

Despite a lack of effect on ligand binding affinity, Npn-1 appears to be responsible for the discrimination of signaling efficiency elicited by VEGF in response to VEGFR versus VEGF 121. Previous attempts at blocking Npn-1 involvement in VEGF signaling utilized a recombinant version of the Npn-1-binding domain of VEGF (16, 18). This glutathione S-transferase-Exon 7–8 fusion protein competes for [125I]VEGF binding to both VEGFR-2 and Npn-1, (18) and reduces signaling in response to both VEGF 165 and VEGF 121 (16). Because VEGF 231 does not bind to Npn-1 (18, 23), it is possible that the antagonistic effect of this protein is due to direct antagonism of signaling through VEGFR-2. Indeed, the results of the deletion
analysis on the activity of this fusion protein (16) is inconsistent with the known structure of the heparin-binding region of VEGF165 (48, 49), suggesting that this reagent may not represent the Npn-1-binding domain of VEGF165 and, by inference, that the protein is not an Npn-1 antagonist. Similar to what was observed for the glutathione S-transferase-Exon 7–8 protein, we also find that a peptide derived from the Exon 6 region of PlGF152 (21) can compete for the binding of [125I]VEGF165 to Npn-1, with a proportional competition for [125I]VEGF165 binding to VEGFR-2 in HUVEC cells (Fig. 7A). The proportional decrease in binding to VEGFR-2 in HUVEC probably represents a decrease in binding to VEGFR-2 in the VEGF2-Npn-1 complex because this concentration of the PlGF152 Exon 6 peptide does not substantially compete for binding at VEGFR-2 when it is expressed in the absence of Npn-1 (Fig. 7B). Notably, this peptide only antagonizes the signaling of VEGF165 and does not affect VEGF121-mediated phosphorylation of VEGFR-2 (Fig. 8). Together, these data suggest that a blockade of binding to the VEGFR-2-Npn-1 complex can antagonize VEGF signaling in an isoform-specific manner and that isoform-specific binding to the VEGFR-2-Npn-1 complex can explain the increased potency of VEGF165 versus VEGF121 in HUVEC. These data stand in contrast to that observed for PlGF152 and PlGF152, where access to the complex of VEGFR-1 with Npn-1 (41) does not appear to confer a signaling advantage for the Npn-1-binding PlGF isoform (PlGF152) (21).

If formation of the VEGFR-2-Npn-1 complex does not result in a higher affinity state of the receptor, how is the increased potency of VEGF165 relative to VEGF121 explained? In Fig. 9 we propose a mechanism wherein formation of the VEGF2-Npn-1 complex can serve to potentiate signaling through VEGFR-2. VEGFR-2 is thought to bind to VEGF121 across the dimer interface, at the opposite poles of the ligand dimer (50–52) (Fig. 8A). VEGF165 binds to VEGFR-2 and Npn-1 through distinct epitopes, with the VEGF-2 binding epitope similar to that observed with VEGF121, and the Npn-1 binding epitope occurring symmetrically in the Exon-7–8 regions of the VEGF121 dimer (Fig. 9B). Unlike VEGF121, VEGF165 can cluster VEGFR-2 through its ability to bind VEGFR-2 in a manner similar to VEGF121 as well as through the preformed Npn-1-VEGFR-2 complex that binds via Exon 7.

The model incorporates the experimental data we have described but also incorporates data from a variety of other receptor systems. It has been demonstrated that the concentration of receptors at the cell surface can affect the position of the dose-response curve for G-protein-coupled receptors (53–55), as well as for RTK (56, 57) ligands, with detection of the ligand-mediated response occurring at progressively lower ligand concentrations as receptor expression increases. By analogy, the ability of VEGF165 to bind to both VEGF2 and Npn-1 may serve to increase the local concentration of VEGFR-2 upon binding VEGF165 relative to that achieved with VEGF121 (Fig. 9) because Npn-1 has the potential to form a ligand-independent complex with VEGFR-2 (Fig. 5). It is also possible that Npn-1 could function to increase the local ligand concentration in the vicinity of VEGFR-2 (58) and that the multivalency of VEGF165 relative to VEGF121 could serve to increase the avidity of the ligand to VEGFR-2, resulting in increased activity at lower ligand concentrations for VEGF165 relative to VEGF121. The latter provides an explanation for the limited ability of VEGF121 to compete for the binding of [125I]VEGF165 in cells co-expressing VEGFR-2 with Npn-1 (Fig. 2).

There is precedence for the preclustering of different members of multi-component receptors (59–61), wherein ligand binding to different receptor clusters can have different signaling consequences (61, 62). Such precedence is also available for the receptor tyrosine kinases. In the case of the Ephrin receptors, the oligomeric state of the ligand has been demonstrated to confer signaling specificity through the EphB1 and EphB2 receptors in endogenous endothelial cell systems, with cellular attachment and recruitment of the low molecular mass protein-tyrosine phosphatase to the receptor cytoplasmic domains only occurring in higher order ligand-receptor oligomers (63). Both platelet-derived growth factor receptor β and VEGF2 have been demonstrated to exist in a ligand-independent complex with the αβ integrin through an interaction of the RTK with the β3 extracellular domain, although association of VEGFR-2 with αβ requires the presence of the αα integral receptor subunit (64). This finding indicates that oligomerization of receptor subunits need not occur through enzyme-substrate interactions of the cytoplasmic domains. The attachment of endothelial cells to vitronectin potentiates signaling through VEGFR-2, and an antibody to the integrin β3 subunit that does not antagonize cellular adhesion is capable of inhibiting the

FIG. 8. The PlGF152 peptide antagonizes VEGF165 signaling without affecting VEGF121 signaling in HUVEC cells. The experimental design is the same as for Fig. 1, with the exception that the PlGF152 peptide was added to the indicated samples at a final concentration of 100 μM. If the PlGF152 peptide was acting solely as an antagonist of VEGFR-2, it would shift both the VEGF165 and VEGF121 dose-response curves to the right. In contrast, only the VEGF165 response curve is affected. This implies that the PlGF152 Exon 6 peptide is specifically affecting signaling through the VEGF2-Npn-1 complex. The nonlinear regression analysis (Prism) of the phosphorytyrosine (pY/R2 signal ratio reveals the following: VEGF165 EC50 = 283 pm; VEGF165 + PlGF152 Exon 6 peptide EC50 = 1.06 nm; VEGF121 EC50 = 1.84 nm; VEGF121 + PlGF152 Exon 6 peptide EC50 = 2.38 nm. This experiment has been repeated once with similar results.
specifically through the VEGFR-2-Npn-1 complex, without an effect on signaling of VEGF_{121} through VEGFR-2. Because a concurrent up-regulation of VEGF_{165} with VEGFR-2 and Npn-1 correlates with increased vascular density in certain pathologies (66, 67), it is tempting to speculate that antagonism of signaling through the VEGFR-2-Npn-1 receptor complex may attenuate pathological angiogenesis without affecting the function of VEGFR-2 in the quiescent vasculature (68, 69).

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