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>*

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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 VEGFR-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 VEGFR-2 does not bind to Npn-1 (18, 23) and because mice engineered to express only the VEGFR-2 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 vasculization and suffer from ischemic cardiomyopathy, implying that VEGFR-2 cannot substitute for VEGF in development of the cardiac vasculature during the period of postnatal cardiac development (37). These data suggest that the inability of VEGFR-2 to interact with Npn-1 may result in insufficient signaling to support the maturation of the cardiac vasculature.

The ability of VEGFR-2 to bind to VEGF-2 and Npn-1 through distinct regions of the VEGF-165 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 VEGF-165 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 potencies that are observed in cultured endothelial cells for VEGF-165 and VEGFR-2-mediated stimulation of VEGFR-2 activation.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free recombinant VEGF-165 was purchased from R & D Systems Inc. (Minneapolis, MN). Na125I was purchased from Amersham Pharmacia Biotech. Heparin sulfate (catalog number 9399) and fetal bovine serum (catalog number A3294) were purchased from Sigma. PD-10 columns and protein G-Sepharose beads were purchased from Amersham Pharmacia Biotech. Donor calf serum, LipofectAMINE-2000, and OptiMEM I were purchased from Life Technologies, Inc. Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). pJFEE14 vector and hVEGFR-2/pJEFE14 construct were obtained from Regeneron Pharmaceuticals (Tarrytown, NY). pLTRMCSires-GFP and pLTR2hFLK1(full)IRES-GFP retroviral constructs 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 PlGF152 Exon 6 I-Neo vector used to construct hNpn-1 retroviral construct was obtained from Regeneron Pharmaceuticals. The pBMN-Z-I-Neo vector was used to construct an hNpn-1 retroviral construct that was obtained from Dr. Gary P. Nolan (Stanford University). The PIGF152 Exon 6 peptide (amino acids 142–157) was synthesized by SynPeptide (Dublin, CA). All of the other reagents were obtained from commercial sources.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) 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% antimitotics, 1% nonessential amino acids). COS-1 cells obtained from American Type Culture Collection were cultured in COS-1 cell growth medium (Dulbecco’s modified Eagle’s medium supplemented with 1% l-glutamine, 1% nonessential amino acids, 1% antimitotics, 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 pJFEE14 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 pLTHFlk1(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 pBMNZ-I-Neo vector.

Transient Expression of Receptors—COS-1 cells were transiently transfected with pJFEE14 (7.0 μg), hVEGFR-2 (5.0 μg of hVEGFR-2 + 2.0 μg of pJEFE14), hVEGFR-2 (5.0 μg) + hNpn-1 (2.0 μg), or hNpn-1 (2.0 μg of hNpn-1 + 5.0 μg of pJEFE14) constructs using LipofectAMINE-2000 in OptiMEM I according to the manufacturer’s instructions. The cells were plated 20 h after transfection.
Analysis of VEGF-2-Npn-1 Co-receptor Complex

### RESULTS

**VEGF<sub>165</sub> and VEGF<sub>121</sub> Exhibit Differential Potency in Stimulating VEGF-2 Phosphorylation Despite Similar Affinity at VEGF-2**—Although VEGF<sub>165</sub> and VEGF<sub>121</sub> have the same affinity for the isolated purified VEGF-2 receptor (15), these two ligands exhibit a differential ability to activate the VEGF-2 receptor in vitro (15–17). In native HUVEC, this differential activity manifests as an increased potency of VEGF<sub>165</sub> relative to VEGF<sub>121</sub>. This difference in potency is characterized by an EC<sub>50</sub> (concentration of agonist that provokes a response halfway between the baseline and maximal responses) for VEGF<sub>165</sub> of 7.76 × 10<sup>-11</sup> M (log EC<sub>50</sub> = −10.11 ± 0.113, n = 14) and for VEGF<sub>121</sub> of 2.45 × 10<sup>−9</sup> M (log EC<sub>50</sub> = −8.61 ± 0.091, n = 14) in stimulating phosphorylation of VEGF-2, with a representative experiment illustrated in Fig. 1. The reported similarity in affinity between VEGF<sub>165</sub> and VEGF<sub>121</sub> at the isolated VEGF-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, VEGF-2 is overexpressed in COS-1 cells, and the ability of these two ligands to compete for [<sup>125</sup>I]VEGF<sub>165</sub> binding is measured. In the COS-1 cells overexpressing the full-length VEGF-2, the IC<sub>50</sub> values for VEGF<sub>165</sub> (IC<sub>50</sub> = 6.34 × 10<sup>−10</sup> M) and VEGF<sub>121</sub> (IC<sub>50</sub> = 3.12 × 10<sup>−10</sup> M) binding are nearly identical, indicating similar affinity to VEGF-2 when it is expressed in isolation. Surprisingly, VEGF<sub>121</sub> has only limited ability to compete for [<sup>125</sup>I]VEGF<sub>165</sub> binding in HUVEC (Fig. 2B), despite the presence of functional VEGF-2 in these cells (Fig. 1). Because the HUVECs 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, VEGF<sub>121</sub> 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 VEGF-2 (Fig. 2, B and D). The data from the COS-1 cells expressing defined receptor populations suggest that the relative inability of VEGF<sub>121</sub> to compete for binding in HUVEC may be attributed to excess Npn-1 expression relative to VEGF-2. We therefore explored the possibility that VEGF-2 and Npn-1 form a co-receptor complex and that the preferential ability of VEGF<sub>165</sub> to signal through this complex is responsible for the increased potency of VEGF<sub>165</sub> relative to VEGF<sub>121</sub> in the VEGF-2 autophosphorylation assay.

**VEGF-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 [<sup>125</sup>I]VEGF<sub>165</sub> chemically cross-linked, and immunoprecipitated with antibodies

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**Table I**

Saturation analysis results from multiple cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>Receptors</th>
<th>Clone</th>
<th>Log K&lt;sub&gt;d&lt;/sub&gt; ± S.E.</th>
<th>K&lt;sub&gt;d&lt;/sub&gt;</th>
<th>B&lt;sub&gt;max&lt;/sub&gt; ± S.E.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS-1</td>
<td>Transient</td>
<td>VEGF-2</td>
<td></td>
<td>−9.46 ± 0.15</td>
<td>3.39 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>13,670 ± 2886</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEGF-2-Npn-1</td>
<td></td>
<td>−8.84 ± 0.00</td>
<td>1.43 × 10&lt;sup&gt;−9&lt;/sup&gt;</td>
<td>245,700 ± 6,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Npn-1</td>
<td></td>
<td>−8.68 ± 0.17</td>
<td>2.09 × 10&lt;sup&gt;−9&lt;/sup&gt;</td>
<td>151,950 ± 55,957</td>
<td>3</td>
</tr>
<tr>
<td>Balb/c</td>
<td>Stable</td>
<td>VEGF-2</td>
<td>D7R2</td>
<td>−8.95 ± 0.31</td>
<td>1.11 × 10&lt;sup&gt;−9&lt;/sup&gt;</td>
<td>8,032 ± 2,080</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEGF-2-Npn-1</td>
<td>D7R2-Npn-1 (4)</td>
<td>−9.48 ± 0.33</td>
<td>3.31 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>228,467 ± 159,993</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Npn-1</td>
<td>C-19</td>
<td>−9.35 ± 0.13</td>
<td>4.17 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>143,506 ± 37,410</td>
<td>5</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Native</td>
<td>VEGF-2</td>
<td>PGBMGH</td>
<td>−9.77 ± 0.12</td>
<td>1.69 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>49,450 ± 3,600</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Balb/c cells contain readily detectable levels of endogenous Npn-1 as measured in Western blot analysis (Fig. 7B).

<sup>b</sup> Statistically greater than PGBMGH (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 [125I]VEGF165 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 [125I]VEGF165 (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 [125I]VEGF165 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-
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...
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 \[^{125}I\]VEGF\(_{165}\) (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, lane 1), 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 VEGF\(_{165}\), 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 VEGF\(_{165}\) 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 VEGF\(_{165}\). The simplest explanation would be that Npn-1 acts as an affinity converter and that the affinity of VEGF\(_{165}\) 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 cell lines 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 (\(P = 0.050, B_{\text{max}} = 143,306 \pm 37,419, n = 5, \text{in } 1-2)\) versus \(48,825 \pm 16,118, n = 4 \text{ in PGBMGH})\) 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 \(B_{\text{max}}\) 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 VEGF\(_{165}\) 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 \(B_{\text{max}}\) 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^{-2} M) is similar to that observed in the D7R2 cells (2.91 \times 10^{-2} 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^{-2} M) is similar to that at VEGFR-2 expressed in the COS-1 cells (Table I). This indicates that the affinity of VEGF165 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 Co-receptor Complex Can Explain the Increased Potency of VEGF165 versus VEGF121 for Activation of VEGFR-2—To demonstrate that the enhanced potency of VEGF165 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 VEGF165 to Npn-1 to see whether the potency of VEGF165 in the anti-phosphotyrosine assay would be reduced to match that of VEGF121. 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]VEGF165 in HUVEC cells. These authors concluded that a peptide consisting of the first 16 amino acid residues from Exon 6 of PlGF152 was sufficient to block the binding of [125I]VEGF165 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]VEGF165 binding in HUVEC cells. With no competitor (lane 1) [125I]VEGF165 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 \([125\text{I}]\)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. 6B where the peptide is able to completely compete for \([125\text{I}]\)VEGF165 binding in COS-1 cells expressing Npn-1 but does not compete effectively for binding to VEGFR-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 various endothelial cell signaling assays (18). However, in the presence of 100 \(\mu\text{M}\) PlGF152 Exon 6 peptide, the signaling potency of VEGF165 is dramatically reduced (VEGF165 + PlGF152 Exon 6 peptide EC\(_{50}\) = 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 EC\(_{50}\) = 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 VEGF orfNZ2 (20), PlGF152 (21), 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). However, this conclusion was based solely on affinity labeling data generated in cells.
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\textsubscript{165} 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 \textsuperscript{125}I\textsubscript{VEGF}\textsubscript{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 VEGFR-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 \(\alpha\) subunit and the Ret receptor tyrosine kinase (RTK) co-receptor complex, where ligand binding is also observed to the non-receptor tyrosine kinase \(\alpha\) subunit in the absence of the RTK, and co-expression of the \(\alpha\) 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\textsubscript{165} 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-Plexin A1 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 VEGFR-2 in response to VEGF\textsubscript{165} versus VEGF\textsubscript{121}. Previous attempts at blocking Npn-1 involvement in VEGF\textsubscript{165} 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 \textsuperscript{125}I\textsubscript{VEGF}_{165} binding to both VEGFR-2 and Npn-1, (18) and reduces signaling in response to both VEGF\textsubscript{165} and VEGF\textsubscript{121} (16). Because VEGF\textsubscript{21} 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
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 VEGFR2-Npn-1 complex. The nonlinear regression analysis (Prism) of the phosphorylsine (pY/R2 signal ratio reveals the following: VEGF165 EC_{50} = 283 pM; VEGF165 + PlGF152 Exon 6 peptide EC_{50} = 1.06 nM; VEGF121 EC_{50} = 1.84 nM; VEGF121 + PlGF152 Exon 6 peptide EC_{50} = 2.38 nM. This experiment has been repeated once with similar results.

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 [^{125}I]VEGF165 to Npn-1, with a proportional competition for [^{125}I]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 VEGFR2-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 VEGFR-2-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 VEGF165 binding epitope similar to that observed with VEGF121, and the Npn-1 binding epitope occurring symmetrically in the Exon-7–8 regions of the VEGF165 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 VEGFR-2 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 [^{125}I]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 domain only occurring in higher order ligand-receptor oligomers (63). Both platelet-derived growth factor receptor β and VEGFR-2 have been demonstrated to exist in a ligand-independent complex with the αβ subunit through an interaction of the RTK with the β subunit extracellular domain, although association of VEGFR-2 with αβ requires the presence of the α 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 β subunit that does not antagonize cellular adhesion is capable of inhibiting the
specifically through the VEGFR-2-Npn-1 complex, without an effect on signaling of VEGF121 through VEGFR-2. Because a concurrent up-regulation of VEGF165 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 VEGF-2-Npn-1 receptor complex may attenuate pathological angiogenesis without affecting the function of VEGF-2 in the quiescent vasculature (68, 69).

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Analysis of the VEGFR-2-Npn-1 Co-receptor Complex

Vascular Endothelial Growth Factor Receptor-2 and Neuropilin-1 Form a Receptor Complex That Is Responsible for the Differential Signaling Potency of VEGF\textsubscript{165} and VEGF\textsubscript{121}

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