Vascular Endothelial Growth Factor Receptor-1 and Neuropilin-2 Form Complexes

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The products of the neuropilin-1 (Np-1) and neuropilin-2 (Np-2) genes are receptors for factors belonging to the class 3 semaphorin family and participate in the guidance of growing axons to their targets. In the presence of heparin-like molecules, both receptors also function as receptors for the heparin-binding 165-amino acid isoform of vascular endothelial growth factor (VEGF165). Both receptors are unable to bind to the 121-amino acid isoform of vascular endothelial growth factor (VEGF121), which lacks a heparin-binding domain. Interestingly, complexes corresponding in size to 125I-VEGF121-neuropilin complexes are formed when 125I-VEGF121 is bound and cross-linked to porcine aortic endothelial cells co-expressing VEGFR-1 and either Np-1 or Np-2. These complexes do not seem to represent complexes of 125I-VEGF121 with a truncated form of VEGFR-1, presumably formed as a result of the presence of Np-1 or Np-2 in the cells, because such truncated forms could not be detected with anti-VEGFR-1 antibodies. Antibodies directed against VEGFR-1 co-immunoprecipitated the 125I-VEGF121-Np-2 sized cross-linked complex along with 125I-VEGF121-VEGFR-1 complexes from cells expressing both VEGFR-1 and Np-2 but not from control cells, indicating that VEGFR-1 and Np-2 associate with each other. To perform the reciprocal experiment we have expressed in porcine aortic endothelial cells a Np-2 receptor containing an in-frame myc epitope at the C terminus. Surprisingly, the myc-tagged Np-2 receptor lost most of its VEGF165 binding capacity but not its semaphorin-3F binding ability. Nevertheless, when Np-2myc was co-expressed in cells with VEGFR-1, it partially regained its VEGF165 binding ability. Antibodies directed against the myc epitope co-immunoprecipitated 125I-VEGF165-Np-2myc and 125I-VEGF165-VEGFR-1 complexes from cells co-expressing VEGFR-1 and Np-2myc, indicating again that VEGFR-1 associates with Np-2. Our experiments therefore indicate that Np-2, and possibly also Np-1, associate with VEGFR-1 and that such complexes may be part of a cell membrane-associated signaling complex.

The various forms of the growth factors belonging to the VEGF family (VEGF, PlGF, VEGF-B, VEGF-C, and VEGF-D)

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The abbreviations used are: VEGF, vascular endothelial growth factor; BS3, bis(sulfosuccinimidyl) suberate; VEGFR-1, vascular endothelial growth factor receptor-1; VEGFR-2, vascular endothelial growth factor receptor-2; PAGE, polyacrylamide gel electrophoresis; VEGF165, 165-amino acid form of vascular endothelial growth factor; VEGF121, 121-amino acid form of vascular endothelial growth factor; Np-1, neuropilin-1; Np-2, neuropilin-2; PAE, porcine aortic endothelial cells; sema, semaphorin; PlGF, placenta growth factor; PBS, phosphate-buffered saline; AP, alkaline phosphatase.

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form-specific receptors (31). The receptors were purified from such cells using affinity chromatography on VEGF<sub>165</sub> affinity matrices followed by partial protein sequencing and were found to be the products of the neuropilin-1 (Np-1) gene (32). It was subsequently observed that the heparin-binding form of placent growth factor (PIGF-2) and VEGF-B, two additional members of the VEGF family of growth factors, are also able to bind to Np-1 (33, 34). When the role of Np-1 as a VEGF receptor was discovered, it was already known that Np-1 functions in the nervous system as receptor for sema-3A. sema-3A is a Np-1 agonist that causes repulsion of growing tips of axons (35, 36). It was recently observed that sema-3A is also able to inhibit migration of endothelial cells (37). These results indicate that signaling via Np-1 affects angiogenesis and possibly the development of the cardiovascular system. Targeted disruption of the Np-1 gene resulted in severe cardiovascular defects, confirming these suspicions (38–40). Np-1 is part of a gene family that includes the closely related receptor Np-2. In the nervous system Np-2 is activated by another class 3 semaphorin, sema-3F, which also induces the repulsion of axons that express Np-2 (41). We have recently observed that Np-2 is also able to bind VEGF<sub>165</sub> and PIGF-2 but not VEGF<sub>121</sub>. However, unlike Np-1, Np-2 was also able to interact with the VEGF<sub>145</sub> form of VEGF. VEGF<sub>145</sub> lacks the peptide encoded by exon 7 of VEGF, which is included in VEGF<sub>165</sub>, but contains instead the heparin-binding domain encoded by exon 6 of the VEGF gene (9, 42).

The neuropilins have a short intracellular domain and are unlikely to function as independent receptors. Indeed, no responses to VEGF<sub>165</sub> were observed when cells expressing either Np-1 or Np-2 but no other VEGF receptors were stimulated with VEGF<sub>165</sub> (32, 42). It was recently found that plexins form complexes with neuropilins and that these complexes mediate signal transduction by sema-3A (43, 44). It is possible that neuropilins associate with additional cell surface molecules to form complexes that transduce VEGF signaling. We present evidence indicating that Np-2 and possibly also Np-1 form complexes with the VEGF-1 receptor and that formation of these complexes changes the binding characteristics of neuropilins so that they are now able to bind VEGF<sub>121</sub>, a splice form that is not recognized by neuropilins in cells that do not express VEGF<sub>1</sub>.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies directed against the intracellular tyrosine-kinase domain of VEGF-R1 were obtained from Santa Cruz Biotechnology. Immunoprecipitating antibodies that bind to the extracellular part of VEGF-R1 were generated as previously described (45). Antibodies directed against the myc epitope were purchased from Santa Cruz Biotechnology. Np-2 and Np-1 expressing PAE cells were generated as previously described (42). A sema-3F expression plasmid was kindly given to Dr. David Ginty by permission from Dr. Marc Tessier-Lavigne. This construct contains the semaphorin-3F cDNA fused in-frame to alkaline phosphatase at the N terminus of sema-3F (41). LipofectAMINE was purchased from Life Technologies, Inc.

**Generation of Anti-Np-2 Antibodies—** A 780-base pair Np-2 Sph-1/Pst-1 cDNA fragment was ligated into the bacterial expression vector pQE-30 (Qiagen). This plasmid was used for the production of the recombinant, His<sub>6</sub>-tagged 30-kDa peptide according to the manufacturer’s instructions. The peptide was cleaved from bacterial cell extracts using nickel affinity chromatography according to the instructions of the vendor and purified again using SDS/PAGE. The gel was electroblotted onto nitrocellulose, and the band containing the peptide was cut out, solubilized in Me<sub>2</sub>SO, and used to immunize rabbits. The antibody was purified on a protein A column to which the recombinant peptide was coupled using a previously described method (46). The antibody was eluted from the column using 0.1 M glycine at pH 3. The antibody generated in this manner recognized Np-2 specifically in Western blots and did not recognize other proteins even when these proteins contained a His<sub>6</sub> tag but did not immunoprecipitate Np-2 (data not shown).

**Cell Lines and cDNAs—** PAE cells (20) were kindly provided by Dr. Carl Hedin. The PAE-VEGFR-1 cell lines were generated by transfecting PAE cells with the VEGF-R1 expression vector BCMGNSneo-hu-flt-1 (23), and selection of VEGF-R1 expressing cell lines was done using 0.5 mg/ml G418. The cells were continuously maintained in medium containing 0.25 mg/ml G418. Cell lines expressing VEGF-R1 and Np-2 were generated by co-transfection of VEGF-R1 expressing plasmid with the PEC/Np-2 expression vector and with the pBabe-puro vector (47). Stable cell lines were isolated by double selection with 0.5 μg/ml puromycin and 0.25 mg/ml G418. For the generation of PAE cells expressing VEGF-R1 and Np-1, the VEGF-R1 expression vector BCMGNSneo-hu-flt-1 was transfected into the previously described PAE/Np-1 cells (32), and stable cell lines expressing cells were selected using 0.5 mg/ml G418. Transfections were carried out using LipofectAMINE according to the instructions of the vendor. Human umbilical vein-derived endothelial cells were cultured as previously described (30).

**Construction and Expression of Np-2myc in PAE Cells**—The primer 5′-GCTCTAGAGGGCCCTCATGATCTTCGATGATTTTT-GTCAGCTCGAGACGACATTTTGG-3′ containing the myc epitope (underlined) and the primer 5′-CAACTCTAGGCTCTGCGC-3′ were used to introduce a myc epitope after the last amino acid of Np-2α22 (41). The primers were used to amplify the modified C terminus using the Np-2 cDNA (42) as a template. The myc epitope-containing fragment was ligated back into the Np-2 cDNA using a unique NarI site and an XbaI site donated by the plasmid (pCDNA3.1 hygro). Following sequencing, the correct expression vector (pCDNA3.1 hygro/myc/myc) was transfected into PAE or PAE-VEGFR-1 cells using LipofectAMINE. Stable cell lines were selected using hygromycin (0.3 mg/ml). In the case of the PAE-VEGFR-1-Np-2myc cells the selective medium also contained G418 (0.5 mg/ml).

**Binding and Cross-linking—** VEGF<sub>121</sub> and VEGF<sub>165</sub> were produced using the baculovirus expression system and iodinated as described (30, 48). Binding of <sup>125</sup>I-VEGF<sub>121</sub> or <sup>125</sup>I-VEGF<sub>165</sub> to cells was carried out essentially as previously described (42). The water-soluble cross-linker BS<sub>3</sub> was used to cross-link bound VEGFs to receptors. BS<sub>3</sub> was dissolved in PBS to a final concentration of 0.2 mM and applied to cells. The cross-linking procedure was done essentially as previously described (30). All experiments were performed at least twice with similar results.

**Immunoprecipitation of Complexes Cross-linked to <sup>125</sup>I-VEGF<sub>121</sub> or <sup>125</sup>I-VEGF<sub>165</sub>**—Using Anti-VEGFR-1 or Anti-myc Epitope Antibodies—Anti-VEGFR-1 antibodies directed against the extracellular domain of VEGF-R1 (45) or commercial anti-myc epitope antibodies were used in immunoprecipitation experiments. <sup>125</sup>I-VEGF<sub>121</sub> or <sup>125</sup>I-VEGF<sub>165</sub> was bound and cross-linked to cells expressing various VEGF receptors. Following cross-linking, the cells were lysed using lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM dithiothreitol, and protease inhibitors (2 μg/ml of leupeptin and aprotinin and 1 mM phenylmethylsulfonyl fluoride)) for 10 min. at 4°C. The lysate was cleared by centrifugation, and protein content was measured using the Bio-Rad protein assay according to the instructions of the vendor. Equal amounts of protein from the different lysates were taken for immunoprecipitation. The lysates were incubated overnight at 4°C with anti-myc or anti-VEGFR-1 antibodies. Protein A-Sepharose was added the next day and incubated with the antibody for 2 h at 4°C, and the beads were subsequently washed three times with cold PBS. SDS/PAGE sample buffer was then added to the beads, and the beads were boiled for 3 min. The supernatant was then separated using SDS/PAGE followed by autoradiography and phosphorimaging analysis. All experiments were performed at least twice with similar results.

**Western Blot Analysis**—Detection of VEGF-R1 by Western blot analysis was performed using a commercial anti-VEGFR-1 antibody (Santa Cruz Biotechnology) directed against the intracellular tyrosine-kinase domain of the receptor using the ECL system (Amersham Pharmacia Biotech) as previously described (9). Detection of Np-2 in Western blots was performed similarly using our affinity purified anti-Np-2 rabbit derived polyclonal antibodies. All experiments were performed at least twice with similar results.

**Production and Binding of sema-3F**—The production of sema-3F was done essentially as described (41). The Np-2 expressing PAE cells or parental PAE cells were grown in gelatin-coated 48-well dishes to confluence. The cells were washed once with PBS and incubated with 50 μg/ml sema-3F to which the radiolabeled COS-7 cells expressing alkaline phosphatase (AP)-tagged sema-3F. The conditioned medium was supplemented with 1 mg/ml gelatin, 10 ng/ml HEPES buffer, pH 7.3, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride prior to the binding experiment. The cells were incubated with the conditioned medium at room temperature for 2 h, washed three times with PBS, and then fixed with 4% paraformalde
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**RESULTS**

**Np-2 Appears to Gain a VEGF121 Binding Ability When Co-expressed with VEGFR-1**—We have previously found that recombinant Np-2 is unable to bind 125I-VEGF121 when it is expressed on its own in PAE cells (Fig. 1A, lane 1) (42). When 125I-VEGF121 was bound and cross-linked to PAE cells expressing either VEGFR-1 (PAE-VEGFR-1 cells) or VEGFR-1 and Np-2 (PAE-VEGFR-1-Np-2 cells), two diffuse complexes of ~190 and ~210 kDa were formed, corresponding to the expected mass of 125I-VEGF121-VEGFR-1 complexes (Fig. 1A, lanes 2 and 3). Surprisingly, we have found that two additional labeled complexes corresponding in mass to the expected masses of 125I-VEGF121-Np-2 complexes (~140 and ~160 kDa) were formed in PAE-VEGFR-1-Np-2 cells (Fig. 1A, lane 3, arrow). This observation suggested that the presence of VEGFR-1 affects the ligand binding specificity of Np-2, enabling 125I-VEGF121 binding, and that VEGFR-1 and Np-2 may perhaps form complexes. This effect on 125I-VEGF121 binding seems to be specific to VEGFR-1 because we could not detect binding of 125I-VEGF121 to Np-2 (not shown).

**Western Blot Analysis Does Not Reveal Shorter Forms of VEGFR-1 in Cells Expressing Both VEGFR-1 and Np-2**—Although the ~140- and ~160-kDa complexes observed in the previous experiment correspond in size to 125I-VEGF121 complexes, it was still possible that these cross-linked complexes do not represent complexes of 125I-VEGF121 and Np-2 but rather complexes formed as the result of the binding of 125I-VEGF121 to truncated forms of VEGFR-1. Such truncated VEGFR-1 forms could perhaps be generated as a result of the presence of Np-2 in the cells. To exclude this possibility, extracts from parental PAE cells, PAE-VEGFR-1-Np-2 cells, and PAE-VEGFR-1-Np-2 cells were analyzed for the presence of shorter forms of VEGFR-1 using commercial antibodies directed against the tyrosine-kinase domain of VEGFR-1. The cells were solubilized and subjected to SDS/PAGE chromatography followed by Western blot analysis. Two VEGFR-1 high molecular weight forms of ~170 and ~190 kDa were easily detected in PAE-VEGFR-1 and in PAE-VEGFR-1-Np-2 cells but not in Np-2-expressing PAE cells (Fig. 2). A band of ~140 kDa was observed in all of the PAE-derived cells including the
Fig. 3. Antibodies directed against VEGFR-1 co-immunoprecipitate a labeled complex corresponding in mass to a 125I-VEGF165-Np-2 complex. A, 125I-VEGF165 (10 ng/ml) (lane 1) or 125I-VEGF121 (30 ng/ml) (lanes 2 and 3) were bound to PAE cells expressing Np-2 (lane 1), VEGFR-1 (lane 2), or Np-2 and VEGFR-1 (lane 3) in the presence of 1 μg/ml heparin. Binding was carried out 2 h at 4 °C. Bound 125I-VEGF was cross-linked (CL) to the cells, which were subsequently lysed as described. Equal amounts of protein representing about 10% of the lysates were chromatographed on a 6% SDS/PAGE gel, and autoradiographed. Lane 4, immunoprecipitate from a PAE-Np-2 cell lysate to which 125I-VEGF was bound and cross-linked. Lane 5, immunoprecipitate from a PAE-VEGFR-1 cell lysate to which 125I-VEGF121 was bound and cross-linked. Lane 6, immunoprecipitate from a PAE-VEGFR-1-Np-2 cell lysate to which 125I-VEGF121 was bound and cross-linked. Parental nontransfected cells (not shown). This band probably represents nonspecific binding of the antibody to an unknown antigen and has a mass that is higher than that of Np-2 (Fig. 2). These experiments therefore indicate that it is unlikely that PAE-VEGFR-1-Np-2 cells express smaller forms of VEGFR-1 as a result of the presence of Np-2 in these cells.

Antibodies Directed against VEGFR-1 Co-immunoprecipitate a Labeled Complex Corresponding in Mass to a 125I-VEGF121-Np-2 Complex—The experiments described above suggested that VEGFR-1 may be able to form complexes with Np-1 and Np-2. To test this hypothesis directly, we used antibodies directed against VEGFR-1 to co-immunoprecipitate Np-2 from PAE cells co-expressing VEGFR-1 and Np-2 receptors. 125I-VEGF121 was bound and cross-linked to PAE-Np-2, PAE-VEGFR-1, or PAE-VEGFR-1-Np-2 cells. The cells were then lysed, and radiolabeled complexes were immunoprecipitated using anti-VEGFR-1 antibodies. This method takes advantage of the high sensitivity afforded by the use of 125I-labeled VEGF but cannot be used to determine whether complex formation is VEGF-dependent or not (50). This experiment revealed that the ~140- and ~160-kDa 125I-VEGF121-containing complexes seen in the experiment shown in Fig. 1 could be immunoprecipitated along with the 125I-VEGF121-VEGFR-1 complex from PAE-VEGFR-1-Np-2 cells but not from PAE-VEGFR-1 cells (Fig. 3B, lane 5 versus lane 6). The anti-VEGFR-1 antibodies did not immunoprecipitate a similar 125I-VEGF165-Np-2 complex from cells expressing Np-2 but no VEGFR-1, indicating that the anti-VEGFR-1 antibodies do not cross-react with Np-2 (Fig. 3B, lane 4). It should be noted that the PAE-Np-2 cells used in this control experiment contain large amounts of Np-2 as revealed by 125I-VEGF165 binding/cross-linking (Fig. 3A, lane 1). These results therefore indicate that VEGFR-1 forms complexes with Np-2. Np-2 Tagged with a myc Epitope at the C Terminus Loses its VEGFR-1 Binding Ability—To obtain further independent experimental evidence for the formation of complexes between VEGFR-1 and Np-2, we have attempted to immunoprecipitate VEGFR-1 from cells expressing both VEGFR-1 and Np-2 using our anti-Np-2 affinity purified antibodies. However, our antibodies turned out to be poor precipitating antibodies. We have therefore tagged Np-2 by expressing a myc epitope in-frame at the end of the intracellular C-terminal domain of Np-2. When we expressed the Np-2myc construct in PAE cells the cDNA directed the expression of tagged Np-2 as revealed in Western blots employing anti-Np-2 antibodies (Fig. 4A, lane 2). The amount of Np-2myc in the transfected cells was about 5-fold lower than the amount of Np-2 in our Np-2 expressing PAE cells (Fig. 4A, lane 1). However, in contrast to our expectations, we could not detect specific 125I-VEGF165 binding to the Np-2myc expressing PAE cells (Fig. 4B). Only at very high concentrations was there some residual specific binding. The maximal specific binding observed was at least 40-fold less than the specific binding of 125I-VEGF165 to PAE-Np-2 cells (Fig. 4B). Surprisingly, the PAE-Np-2myc cells were still able to bind sema-3F. The amount of sema-3F bound per cell was about 5-fold lower than the binding of sema-3F to PAE-Np-2 cells (Fig. 4C) and therefore in good agreement with the experiment shown in Fig. 4A. These experiments therefore indicate that the attachment of the myc epitope selectively inhibits the binding of 125I-VEGF165 to Np-2 but does not affect significantly the binding of sema-3F to Np-2myc. This experiment indicates indirectly that sema-3F and VEGF165 may bind to different domains on the extracellular part of Np-2. This conclusion was further supported by an experiment showing that the binding of sema-3F to PAE-Np-2 cells could not be inhibited by the inclusion of 2 μg/ml of VEGF165 in the binding reaction (Fig. 5).

Np-2myc Partially Regains Its 125I-VEGF165 Binding Properties in the Presence of VEGFR-1, and Antibodies Directed against the myc Epitope Tag of Np-2 Co-precipitate VEGFR-1—The previous experiments have indicated that the addition of the myc tag inhibits the VEGF binding ability of Np-2. Nevertheless, when 125I-VEGF165 was bound and cross-linked to PAE cells co-expressing Np-2myc and VEGFR-1, we noted that 125I-VEGF165 formed complexes corresponding in size to VEGF165-Np-2myc complexes (Fig. 6A, lane 2). It therefore seems that the presence of VEGFR-1 enables Np-2myc to regain at least part of the 125I-VEGF165 binding ability of untagged Np-2. We have therefore attempted to co-immunoprecipitate VEGFR-1 from cells co-expressing Np-2myc and VEGFR-1 using an antibody directed against the myc epitope. 125I-VEGF165 was bound and cross-linked to the cells, and the anti-myc antibody was then used in the immunoprecipitation. As expected from the previous experiments, the anti-myc antibody was able to co-precipitate 125I-VEGF165-Np-2myc and 125I-VEGF165-VEGFR-1 complexes from the cells (Fig. 6B, lane 3). Because 125I-VEGF165 did not bind to Np-2myc in cells lacking VEGFR-1, no complex could be precipitated from such cells, nor did the anti-myc antibody precipitate any cross-linked complexes from cells co-expressing VEGFR-1 and native Np-2 following the cross-linking of 125I-VEGF165 to such cells (Fig. 6B, lanes 2 and 4). These observations therefore support the results obtained using the anti-VEGFR-1 antibodies (Fig. 3B, lane 6) indicating that VEGFR-1 is able to form complexes with Np-2.
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**Fig. 4.** myc epitope-tagged Np-2 looses its VEGF<sub>165</sub> binding ability but not its sema-3F binding properties. A, PAE cells expressing recombinant Np-2 (lane 1), Np-2<sup>myc</sup> (lane 2), VEGFR-1 and Np-2 (lane 3), VEGFR-1 and Np-2<sup>myc</sup> (lane 4), or VEGFR-1 alone (lane 5) were grown to confluence in 5-cm dishes (~3 x 10<sup>6</sup> cells/dish) and lysed. Lysates were chromatographed on a 6% SDS/PAGE gel. The amount of lysate loaded in each lane was equivalent to 2 x 10<sup>6</sup> cells. The proteins were transferred to nitrocellulose by electroblotting and probed with an affinity purified anti-Np-2 antibody. A secondary antibody coupled to peroxidase was used to detect bound primary antibody. Bound secondary antibody was detected using the ECL detection method. B, PAE cells expressing Np-1, Np-2, or Np-2<sup>myc</sup> were grown in gelatin-coated 24-well dishes to confluence. Increasing concentrations of <sup>125</sup>I-VEGF<sub>165</sub> were bound in duplicates to the cells in the presence of 1 mg/ml bovine serum albumin and lysed by the addition of 0.5 ml of 0.5 N NaOH. Aliquots of 0.4 ml were then counted in a ß counter. Nonspecific binding was subtracted from total binding to calculate specific binding values. The nonspecific binding did not exceed 15% of the total binding values. The experiment was repeated twice, and the error bars represent the standard deviations from the mean. C, control PAE cells transfected with the pBabe-Puro plasmid (42) as well as PAE cells expressing recombinant Np-2, Np-2<sup>myc</sup>, VEGFR-1 and Np-2, or VEGFR-1 and Np-2<sup>myc</sup> were grown to confluence in gelatin-coated 48-well dishes. The cells were incubated with conditioned medium containing AP-tagged sema-3F as described. Following washing, heat-resistant alkaline phosphatase activity of cell bound sema-3F was measured as described. Binding to pBabe-puro vector transfected PAE cells was 0.08 optical density units (O.D.) and was subtracted from the total binding values to calculate the specific binding values shown. The experiment was repeated twice in triplicate with similar results, and the error bars represent the standard deviations from the mean.

**DISCUSSION**

Np-1 and Np-2 were originally found to function as receptors for several class 3 semaphorins that repel growing tips of axons during the development of the nervous system (35, 36, 41). These discoveries were followed by experiments that have demonstrated that Np-1 and Np-2 can function in addition as receptors for VEGF<sub>165</sub>, one of the heparin-binding forms of the angiogenic factor VEGF (32, 42). These experiments indicated that the neuropilins may play a role in cardiovascular biology, in addition to their role in the nervous system. In the case of Np-1 these expectations were verified when it was shown that targeted disruption of the Np-1 gene results in severe cardiovascular defects (38). In agreement with this observation it was found that the Np-1 agonist sema-3A inhibits migration of endothelial cells (37), but the consequences of the binding of VEGF to Np-1 are not completely clear as yet. In contrast, mice lacking functional Np-2 receptors are viable, and no vascular defects were reported so far (51, 52). Nevertheless, the absence of vascular defects in these gene targeted mice does not necessarily preclude a physiological role for these receptors in vascular biology because the absence of a phenotype may be explained by redundancy with other signaling pathways.

We have not been able to demonstrate any biological responses to VEGF<sub>165</sub> in PAE cells expressing either recombinant Np-1 or recombinant Np-2 receptors and no other types of VEGF receptors (32, 42). These observations suggested that for the transduction of VEGF signals the neuropilins may perhaps have to associate with other membrane-bound proteins. Neuropilins possess short intracellular domains, and it was demonstrated that binding of sema-3A to Np-1 is not sufficient for induction of sema-3A mediated growth cone collapse (53). It was indeed found that neuropilins form complexes with plexin receptors to be able to transduce semaphorin signals (43, 44). Our binding/cross-linking experiments and co-immunoprecipitation experiments indicate that in addition, Np-2 can form complexes with VEGFR-1. Our experiments also suggest that Np-1 too can associate with VEGFR-1. This was recently verified in a manuscript that was published during the preparation of this manuscript in which complexes between Np-1 and VEGFR-1 were observed using completely different methods (49).

The mechanism by which VEGFR-1 enables the binding of VEGF<sub>121</sub> to Np-1 and Np-2 is unclear. The binding of VEGFR-1 to the neuropilins may induce a neuropilin conformation that binds VEGF<sub>121</sub> with increased affinity. It is possible that VEGF<sub>121</sub> binds initially to VEGFR-1, placing the bound neuropilins in close proximity to neuropilins in cells co-expressing both receptor types and effectively increasing the affinity of the neuropilins toward VEGF<sub>121</sub>. The effect of VEGFR-1 on VEGF<sub>121</sub> binding may therefore be similar to the potentiating effect that heparin-like molecules have on the binding of VEGF<sub>165</sub> to neuropilins (32, 33, 42).

VEGFR-1 and Np-2 may be able to form complexes prior to the addition of VEGF. Alternatively, it is possible that VEGFR-1 binds to Np-2 only subsequent to the binding of
VEGF to VEGFR-1. We have not been able to differentiate between these two possibilities. We have attempted to detect co-immunoprecipitated Np-2 or VEGFR-1 using Western blot analysis, but we have failed regardless of whether the cells were exposed or not to VEGF prior to the immunoprecipitation. It is possible that these experiments failed because the sensitivity of the assays was insufficient or because the VEGFR-1zNp-2 complexes are sensitive to the detergents used during the solubilization of the cells, making the detection of VEGFR-1zNp-2 complexes by less sensitive techniques than the technique we have used difficult. To circumvent these problems we have therefore used antibodies to immunoprecipitate recombinant receptors that have been covalently cross-linked to125I-VEGF prior to the immunoprecipitation using a previously described method (50). The method we used utilized the high sensitivity afforded by the use of125I but did not allow us to determine whether complex formation between Np-2 and VEGFR-1 was VEGF-dependent or not.

We have no data regarding the biological significance of VEGFR-1zNp-2 complexes at this stage. The formation of complexes between Np-2 and VEGFR-1 may contribute to VEGF-induced signal transduction by VEGFR-1. If that assumption is correct, then it may provide a clue to a puzzling observation. Mice deficient in VEGFR-1 expression die before birth because of severe cardiovascular defects (27). In contrast, mice that retain the extracellular and trans-membrane domains of VEGFR-1 but lack the signaling tyrosine-kinase domain develop normally (54). It is unclear how the extracellular domain of VEGFR-1 is able to restore the normal embryonic development of mice. It is possible that the extracellular domain is required for VEGF sequestration, so as to limit the activity of VEGF. Alternatively, the extracellular domain may associate with another membrane protein to form a signaling holo-receptor. It is possible that Np-2 and Np-1 may perhaps participate in the formation of such a putative VEGFR-1 containing holo-receptor.

In the course of our experiments we have found that when a myc epitope is inserted in-frame after the conserved SEA terminal tripeptide of Np-2a, the modified Np-2myc receptor loses most of its VEGF165 binding ability. It was shown that Np-1...
and Np-2 can form homodimers and heterodimers (55). Formation of such dimers may perhaps be required for high affinity binding of VEGF to neuropilins. The insertion of the myc epitope may perhaps interfere with dimer formation and consequently with VEGF binding. Interestingly, the VEGF
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myc binding ability of Np-2
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myc was restored to some extent in cells co-expressing VEGFR-1, perhaps because following complex formation a high affinity VEGF-binding conformation of Np-2
myc is favored. Interestingly, the sema-3F binding properties of Np-2 were not affected by the introduction of the myc epitope, perhaps because the sema-3F-binding domain of Np-2 seems to be distinct from the VEGF-binding domain as suggested by the results of the competition experiments.

To conclude, our experiments indicate that VEGFR-1 forms complexes with Np-2 and possibly also with Np-1. The presence of VEGFR-1 changes the specificity of VEGF binding, allowing VEGF
121 to bind to Np-2. However, the biological function of these VEGF-1-Np-2 complexes is still unclear. In addition our experiments indicate that changes in the intracellular domain of Np-2 can affect VEGF binding to Np-2 and provide evidence indicating that VEGF and semaphorins bind to nonoverlapping sites in the extracellular part of Np-2.

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