Neuropilin-1 (NRP1), a receptor for vascular endothelial growth factor (VEGF) family members, has three distinct extracellular domains, a1a2, b1b2, and c. To determine the VEGF<sub>165</sub> and placenta growth factor 2 (PIGF-2)-binding sites of NRP1, recombinant NRP1 domains were expressed in mammalian cells as Myc-tagged, soluble proteins, and used in co-experimentation with <sup>125</sup>I-VEGF<sub>165</sub> and <sup>125</sup>I-PIGF-2. Anti-Myc antibodies immunoprecipitated <sup>125</sup>I-VEGF<sub>165</sub> and <sup>125</sup>I-PIGF-2 in the presence of the b1b2 but not of the a1a2 and c domains. Neither b1 nor b2 alone was capable of binding <sup>125</sup>I-VEGF<sub>165</sub>. In competition experiments, VEGF<sub>165</sub> competed PIGF-2 binding to the NRP1 b1b2 domain, suggesting that the binding sites of VEGF<sub>165</sub> and PIGF-2 overlap. The presence of the a1a2 domain greatly enhanced VEGF<sub>165</sub> but not PIGF-2 binding to b1b2. Heparin enhanced the binding of both <sup>125</sup>I-VEGF<sub>165</sub> and <sup>125</sup>I-PIGF-2 to the b1b2 domain by 20- and 4-fold, respectively. A heparin chain of at least 20–24 monosaccharides was necessary for binding. In addition, the b1b2 domain of NRP1 could bind heparin directly, requiring heparin oligomers of at least 8 monosaccharide units. It was concluded that an intact b1b2 domain serves as the VEGF<sub>165</sub>, PIGF-2-, and heparin-binding sites in NRP1, and that heparin is a critical component for regulating VEGF<sub>165</sub> and PIGF-2 interactions with NRP1 by physically interacting with both receptor and ligands.

Neuropilins (NRP)s are 130–140-kDa cell surface glycoproteins that mediate neuronal guidance and angiogenesis (1). There are two NRP genes, NRP1 and NRP2 (2, 3). NRP1 is essential for normal development of the nervous and cardiovascular systems. Overexpression of NRP1 in mouse embryos results in ectopic sprouting and defasciculations of nerve fibers along with excess capillary growth and malformed hearts (4).

NRP1-deficient mice show severe neuronal abnormalities as well as deficiencies in neuronal vascularization, aortic arch malformations, and diminished and disorganized yolk sac vascularization (5, 6). NRP1 also contributes to tumor angiogenesis. Induction of NRP1 expression in tumor cells in vivo results in larger and more vascular tumors (7).

In the nervous system, NRP1 is expressed on axons of dorsal root ganglia, as well as sympathetic and motor neurons as a receptor for class 3 semaphorins (2, 8). Semaphorin 3A (Sema3A) is a chemorepellent for axons and collapses their growth cones (9). NRP1 is the binding receptor for Sema3A however, Sema3A signaling is transduced by plexins. Plexins are transmembrane kinases that do not bind Sema3A directly but form a complex with NRP1 that enhances the binding of Sema3A to NRP1 (10, 11).

In the vascular system, NRPs are the second class of vascular endothelial growth factor (VEGF) receptors to be described (12, 13), the first being the receptor tyrosine kinases VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3 (Flt-4). NRP1 acts as a co-receptor for VEGF<sub>165</sub> activation of VEGFR2 (13). NRP1 also binds placenta growth factor 2 (PIGF-2), VEGF-B, and VEGF-E (14–16).

Heparin and heparan sulfate (HS) play important roles in mediating growth factor-receptor interactions, for example, the binding of basic fibroblast growth factor, heparin-binding EGF-like growth factor, and hepatocyte growth factor (17–19). The heparin binding properties of VEGF have been well established. All the VEGF isoforms are heparin-binding with the exception of VEGF<sub>121</sub>, which lacks exons 6 and 7 that encode the VEGF heparin-binding domains (20). VEGF<sub>165</sub> interactions with endothelial cells are mediated by heparin. In cross-linking analysis, heparin potentiated the binding of VEGF<sub>165</sub> to VEGFR2 (21). Heparin was also shown to enhance the binding of VEGF<sub>165</sub> to a 130-kDa receptor (21, 22) that we subsequently showed to be NRP1 (13). In a binding study using surface plasmon resonance technology (BIAcore system), heparin increased the affinity of VEGF<sub>165</sub> for the immobilized NRP1 extracellular domain (23).

PIGF is a member of the VEGF family. Three PIGF isoforms were generated by mRNA alternative splicing (24, 25). PIGF-2 is the only heparin-binding isoform (26). Interestingly, the PIGF-2 isoform binds NRP1, whereas PIGF-1 binds neither heparin nor NRP1. Moreover, PIGF-2 binding to NRP1 is heparin/HS-dependent (14). PIGF binds and activates VEGFR1 but not VEGFR2. The role of VEGFR1 activation by PIGF in endothelial cells is not yet established. Similarly, the significance of NRP1 as a PIGF-2 receptor is unknown. NRP1 was shown to bind directly to VEGFR1, and this interaction was competed by heparin (23). This is in...
contrast to the potentiation of VEGF<sub>165</sub>/VEGFR2/NRP1 interactions by heparin.

The structure of NRP1 may explain in part how NRP1 can interact with multiple structurally unrelated ligands. NRP1 contains a relatively large extracellular domain of 860 amino acids and a short cytoplasmic domain of 40 amino acids. The extracellular part has 3 subdomains, designated a, b, and c. The a and b domains consist of 2 tandem repeats, a1a2 and b1b2. The c domain contains a MAM domain shown to be involved in dimerization of proteins, such as the zinc-metalloprotease meprins (27, 28). Precise identification of VEGF<sub>165</sub> and PIgF-2-binding sites on NRP1 and characterization of their specific interactions with NRP1 could be of interest in understanding structure-function relationships. In this report, we have used soluble NRP1 extracellular domain fragments to demonstrate that the NRP1 b1b2 domain is the binding site for VEGF<sub>165</sub> and PIgF-2; nevertheless, the two ligands differ in their NRP1 b1b2 domain binding characteristics. Furthermore, we demonstrate for the first time that the b1b2 domain is the NRP1 heparin-binding domain and that heparin potentiates VEGF/b1b2 interactions. We conclude that the b1b2 domain serves as PIgF-2-, VEGF<sub>165</sub>- , and heparin-binding sites, that this domain is central to NRP1/ligand interactions, and that heparin plays an important role in regulating these interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**

VEGF<sub>165</sub> was a gift from Dr. Judith Abraham Chiron (Emeryville, CA). Materials were purchased from the following sources. Recombinant mouse PlGF-2 was from R&D Systems (Minneapolis, MN); fetal bovine serum, Dulbecco's modified Eagle's medium, F-12 medium, and bovine serum, Dulbecco's medium containing 10% fetal bovine serum were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% glutamine/penicillin/streptomycin mixture were from Invitrogen; anti-Myc antibody clone 9E10 was from Santa Cruz Biotechnology (Santa Cruz, CA); FuGENE 6 transfection reagent was from Roche Molecular. Materials were purchased from the following sources. Recombinant NRP1 heparin-binding domain and that heparin potentiates VEGF/b1b2 interactions. We conclude that the b1b2 domain serves as PIgF-2-, VEGF<sub>165</sub>- , and heparin-binding sites, that this domain is central to NRP1/ligand interactions, and that heparin plays an important role in regulating these interactions.

**Cell Culture**

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics/BioWhittaker (Walkersville, MD) and grown according to the manufacturer's protocol in EGM<sup>TM</sup> medium. MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% glutamine/penicillin/streptomycin. Porcine aortic endothelial cells (PAEC) (13) were cultured in F-12 medium with 10% fetal bovine serum and 1% glutamine/penicillin/streptomycin.

**Cloning and Expression of Recombinant NRP1 Extracellular Domains**

Human NRP1 cDNA was used as a template for PCR amplification of the domains described below. For in-frame cloning, an XhoI site was added to the 5' end of all cDNAs (at the second and third codons of the forward primers). The following primer pairs were used for PCR amplification of NRP1 domains: a1a2 domain (Phe<sup>23</sup>-Phe<sup>773</sup>, 5'-CTCATGAGGATCCACGCAACGATAAATGTGG and 3'-CTCTAGAAGCTTATGTAAAGCGTCTG-3' for 50 cycles: 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 1–4 min). The following primer pairs were used for PCR amplification of NRP1 domains: a1a2 domain (Phe<sup>23</sup>-Phe<sup>773</sup>, 5'-CTCATGAGGATCCACGCAACGATAAATGTGG and 3'-CTCTAGAAGCTTATGTAAAGCGTCTG-3').

**Purification of Recombinant Domain-specific Proteins**

To produce pure recombinant proteins, pSecTag plasmids encoding the various NRP1 domains were transfected into CHO-K1 cells using the FuGENE 6 transfection reagent. Cells were selected with Zeocin (0.5 mg/ml) for 12 days. Resistant clones were chosen and expanded, and their CM were assayed for protein expression by Western blotting using an anti-Myc antibody. Positive clones expressing the recombinant protein were expanded further. One stable clone representing each NRP1 domain was used to condition serum-free media. Protein purification was carried out as previously described (29). Briefly, CM was concentrated, adjusted to 20 mM Hepes, pH 7.2, 0.5 mM NaCl (concanavalin A binding buffer), and incubated with concanavalin A beads overnight at 4 °C. Concanavalin A-binding proteins were eluted with 0.2 M methyl-a-D-mannopyranoside and applied onto a 1-ml Hi-Trap chelating column (Amersham Biosciences) loaded with cobalt and attached to a Pharmacia FPLC system. Bound proteins were eluted by a linear gradient of 5–150 mM imidazole. Fractions were analyzed for the presence of recombinant domain protein by Western blot analysis and Coomassie Blue staining following SDS-PAGE. Pooled fractions were concentrated and adjusted to 20 mM Hepes, pH 7.2, 50 mM NaCl. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad).

**Binding and Cross-linking of <sup>125</sup>I-VEGF<sub>165</sub> and <sup>125</sup>I-PIgF-2 to NRP1 Domains and Cells**

**Binding of VEGF to NRP1 Domains in Solution—** VEGF<sub>165</sub> and PIgF-2 were iodinated as described (12). Five nanograms of VEGF<sub>165</sub>, or PIgF-2 were incubated with pure NRP1 b1b2 or a1a2/ b1b2 domains or CM containing recombinant NRP1 domains (5–20 µg/ml) with 20 mM Hepes, pH 7.4, 0.15 mM NaCl, 0.1% Tween 20, 0.1% bovine serum albumin, with or without 1 µM unfractrated heparin or heparin oligosaccharides for 2 h at 25 °C. Complexes were incubated overnight with anti-Myc antibodies (1 µg/tube), followed by incubation for 1 h with protein G-Sepharose beads. Immunocomplexes were washed three times with 20 mM Hepes, pH 7.4, 0.15 mM NaCl, 0.1% Tween 20, and bound <sup>125</sup>I-VEGF<sub>165</sub> was eluted by boiling the beads in Laemmli’s sample buffer for 5 min. The samples were analyzed by 10% SDS-PAGE followed by autoradiography. Aliquots of each sample of CM were analyzed by Western blot with an anti-Myc antibody to verify that equal amounts of NRP1 domains were being included in the immunoprecipitation experiments. For quantification of immunoprecipitated <sup>125</sup>I-VEGF<sub>165</sub> or PIgF-2, 3-µl aliquots of each sample were measured in a γ-counter.

**Cross-linking of VEGF<sub>165</sub> or PIgF-2 to NRP1 Domains—** Binding of VEGF<sub>165</sub> or PIgF-2 to NRP1 recombinant domains was carried out as described above. Complexes were cross-linked in 0.2 mM disuccinimidyl carbonate for 15 min, and the reaction was stopped by the addition of 10 mM Tris, pH 7.4, 250 mM glycine, 2 mM EDTA. Complexes were separated by 7.5% SDS-PAGE, and gels were autoradiographed.

**Competition of VEGF<sub>165</sub> Binding to Cells by NRP1 Domains—** Binding of VEGF<sub>165</sub> to HUVEC and MDA-MB-231 cells was performed as described (12). In competition experiments, 1–25 ng/ml VEGF<sub>165</sub> was incubated with either CM containing recombinant NRP1 domains or pure recombinant domains. Samples were pre-incubated at 25 °C for 30 min prior to cell binding. Cell binding experiments were carried out in 24-well plates in triplicate for 2.5 h on ice with gentle agitation followed by 3 washes with ice-cold phosphate-buffered saline. Cells were lysed with 0.2 M NaOH, and associated radioactivity was measured with a γ-counter.
NRP1 b1b2 Domain Binds PlGF-2, VEGF<sub>165</sub> and Heparin

**RESULTS**

**Identification of the NRPI Domain That Binds VEGF<sub>165</sub>**

VEGF<sub>165</sub> binds sNRP1 (29), suggesting that the VEGF<sub>165</sub>-binding site is located within the a1a2/b1b2 domains of NRPI. To identify the binding domain more precisely, individual PCR-amplified NRPI extracellular domains were cloned into a mammalian expression system containing the IgG signal peptide and a Myc tag (Fig. 1A). Recombinant soluble proteins were expressed transiently in PAEC. The various domain products were released into CM with the expected sizes (Fig. 1B, lower panel). The CM containing the various Myc-tagged NRPI domains (about 10 μg/ml) were incubated in solution with <sup>125</sup>I-VEGF<sub>165</sub> and putative VEGF-NRP1 domain complexes were immunoprecipitated with anti-Myc antibodies in "pull-down" experiments. <sup>125</sup>I-VEGF<sub>165</sub> was immunoprecipitated by the anti-Myc antibodies only when incubated with b1b2 or a1a2/b1b2 but not with a1a2 or c domains (Fig. 1B, upper panel, lanes 2, 4, 6, and 8), demonstrating that the VEGF<sub>165</sub> binding site in NRPI is located within the b1b2 domain. The binding of <sup>125</sup>I-VEGF<sub>165</sub> to NRPI b1b2 and a1a2/b1b2 domains was greatly enhanced in the presence of 1 μg/ml heparin (Fig. 1B, lanes 6 versus 2 and 8 versus 4). Similar potentiation of VEGF<sub>165</sub>/NRPI interactions by heparin has been demonstrated before in cellular and solid-phase systems (12, 23). Therefore, the binding studies reported here are routinely carried out in the presence of 1 μg/ml heparin unless indicated otherwise.

The b1 and b2 domains are a tandem repeat of two similar domains sharing a sequence identity of 38%. To determine whether the VEGF<sub>165</sub>-binding site was contained within the b1 or b2 domains alone or whether both were required, the individual Myc-tagged b domains were expressed in PAEC as above (Fig. 1A). The b1 recombinant protein was readily secreted at ~10–20 μg/ml (Fig. 1C, lower panel, lane 1), whereas no secreted b2 recombinant protein could be detected (data not shown). However, the b2 protein, which was associated with PAEC lysates (Fig. 1C, lower panel, lane 1), could be readily solubilized with 1% Triton X-100, an anionic detergent. Binding analysis indicated that neither secreted b1 nor solubilized b2 could bind <sup>125</sup>I-VEGF<sub>165</sub> (Fig. 1C, upper panel, lanes 1 and 2). The lack of b2 domain binding was not because of the presence of Triton X-100 since the detergent did not interfere with VEGF<sub>165</sub>b1b2 binding (Fig. 1C, upper panel, lane 3). An a1a2/b1 fragment was generated and tested for binding with the rationale that the presence of a1a2 might allow more proper folding of b1 (Fig. 1C, lane 4). However, a1a2/b1 did not bind <sup>125</sup>I-VEGF<sub>165</sub> (Fig. 1C, upper panel, lane 4). These results suggest that elements of both b1 and b2 are required for VEGF<sub>165</sub> binding.

The b1b2 Domain Inhibits VEGF<sub>165</sub> Binding to Cells Expressing NRPI—Further evidence for the b1b2 domain being the VEGF<sub>165</sub>-binding site was obtained by testing the ability of the various domain proteins to compete with <sup>125</sup>I-VEGF<sub>165</sub> for binding to cells expressing NRPI. Two cell types were analyzed, the breast carcinoma cell line MDA-MB-231 in which NRPI and NR2P are the only VEGF<sub>165</sub> receptors and HUVEC which express NRPI, NR2P, VEGFR1, and VEGFR2. Cells were incubated with <sup>125</sup>I-VEGF<sub>165</sub> and a 1000-fold excess of the various NRPI domains (Fig. 2). The b1/b2 domain inhibited <sup>125</sup>I-VEGF<sub>165</sub> binding to MDA-MB-231 cells and HUVEC by 65 and 42% (p < 0.05), respectively. The a1a2 domain did not compete for <sup>125</sup>I-VEGF<sub>165</sub> binding to either cell type. The c domain did not inhibit <sup>125</sup>I-VEGF<sub>165</sub> binding to HUVEC but reduced <sup>125</sup>I-VEGF<sub>165</sub> binding to MDA-MB-231 cells by 35% (p < 0.05), even though it does not directly bind VEGF<sub>165</sub> as was shown in Fig. 1B.

The a1a2/b1b2 Domain Has Higher VEGF<sub>165</sub> Binding Capacity Than the b1b2 Domain—The a1a2/b1b2 domain is similar in structure to the naturally occurring sNRP1, previously demonstrated to bind VEGF<sub>165</sub> and to compete with VEGF<sub>165</sub> for binding to cells expressing NRPI (29). The a1a2/b1b2 and b1b2 domains were compared for their ability to inhibit <sup>125</sup>I-VEGF<sub>165</sub> binding to MDA-MB-231 cells and HUVEC in a dose-
The b1b2 domain inhibits VEGF<sub>165</sub> binding to cells. 

**Fig. 2.** The b1b2 domain inhibits VEGF<sub>165</sub> binding to cells. 

**Fig. 3.** The a1a2b1b2 domain enhances VEGF<sub>165</sub> binding to the b1b2 domain. 

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**Characterization of NRP1/PIGF-2 Interactions—PIGF-2 is a VEGF<sub>165</sub> homologue that specifically binds VEGF<sub>R1</sub> and NRP1 and stimulates angiogenesis by a yet unknown mechanism.**

Because NRP1 is a receptor shared by PIGF-2 and VEGF<sub>165</sub>, the PIGF-2-binding domain of NRP1 was determined and compared with that of VEGF<sub>165</sub>. 125<sup>I</sup>-PIGF-2 was incubated in solution with heparin and the various NRP1 domains, and putative PIGF-2/NRP1 domain complexes were immunoprecipitated with anti-Myc antibodies (as in Fig. 1). 125<sup>I</sup>-PIGF-2 was immunoprecipitated only when incubated with b1b2 (Fig. 4A, lane 2) or with a1a2/b1b2 (Fig. 4A, lane 3) but not with a1a2 or c domains (data not shown). As with VEGF<sub>165</sub>, heparin greatly enhances PIGF-2 binding to the NRP1 b1b2 domain (Fig. 4B, lane 3 versus 2). Although both ligands bind to the same NRP1 b1b2 domain, the binding capacity of PIGF-2 for NRP1 appears to be relatively much less than that of VEGF<sub>165</sub> (Fig. 4B). Over 10-fold more 125<sup>I</sup>-VEGF<sub>165</sub> was pulled down in solution by anti-Myc antibody in the presence of equal concentrations of b1b2 domain and equal specific activities of 125<sup>I</sup>-PIGF-2 and 125<sup>I</sup>-VEGF<sub>165</sub> (Fig. 4B, lane 5 versus 2, lane 6 versus 3). Because of the relatively low affinity of PIGF-2 for b1b2, it has not been possible to determine a reliable K<sub>D</sub> for this interaction.

To address whether VEGF<sub>165</sub> and PIGF-2 bind to similar or distinct sites, VEGF<sub>165</sub> and PIGF-2 competitive binding to the NRP1 b1b2 domain in solution was analyzed (Fig. 4C). Pure b1b2 domain (300 ng) was incubated with 5 ng of 125<sup>I</sup>-PIGF-2, and binding was competed with excess unlabeled VEGF<sub>165</sub>. The binding of PIGF-2 to the b1b2 domain was inhibited in a dose-dependent manner by 26, 45, and 80% with 5 (1-fold excess), 50 (10-fold excess), and 500 ng (100-fold excess) of VEGF<sub>165</sub>, respectively. On the other hand, PIGF-2 could not compete for the binding of 125<sup>I</sup>-VEGF<sub>165</sub> to the NRP1 b1b2 domain even at 200-fold excess (data not shown). However, lack of competition by PIGF-2 might be technical because of the low binding capacity of PIGF-2 for the NRP1 b1b2 domain in solution. These results suggest that VEGF<sub>165</sub> and PIGF-2 have overlapping binding sites within the NRP1 b1b2 domain.

**The NRP1 a1a2 Domain Enhances the Binding of VEGF<sub>165</sub> but Not PIGF-2, to the b1b2 Domain—The interactions of a1a2/b1b2 and b1b2 with VEGF<sub>165</sub> and PIGF-2 were compared by cross-linking analysis (Fig. 5). Two major complexes were formed between VEGF<sub>165</sub> and b1b2 with molecular masses of about 65 and 80 kDa (Fig. 5A, lane 2, open arrows). The sizes of the 65- and 80-kDa complexes are consistent with binding of 45-kDa b1b2 to the VEGF<sub>165</sub> monomer (20 kDa) and dimer (40 kDa), respectively. The binding of VEGF<sub>165</sub> to the a1a2/b1b2 generated two complexes, one of 110–130 kDa and another greater than 200 kDa (Fig. 5A, lane 3, solid arrows). The 110–130-kDa complexes are consistent in size with the molecular mass of the VEGF<sub>165</sub> monomer or dimer bound to the 85-kDa a1a2/b1b2. The high molecular mass complexes greater than 200 kDa may correspond to multimers of VEGF<sub>165</sub> and a1a2/b1b2. The intensity of the autoradiography bands demonstrate clearly that 125<sup>I</sup>-VEGF<sub>165</sub> binds to a1a2/b1b2 much more efficiently than to b1b2, about 14–20-fold greater.

On the other hand, the cross-linking of 125<sup>I</sup>-PIGF-2 to a1a2/b1b2 and b1b2 domains gave very different results (Fig. 5B). One specific complex was formed between 125<sup>I</sup>-PIGF-2 and b1b2, of about 65 kDa, consistent with binding of 45-kDa b1b2 to 20-kDa PIGF-2 monomer (Fig. 5B, lane 2, open arrow). One complex of about 120–125 kDa was formed between the 85-kDa a1a2/b1b2 and 125<sup>I</sup>-PIGF-2 (Fig. 5B, lane 3, solid arrow). The cross-linking profiles of 125<sup>I</sup>-VEGF<sub>165</sub> and 125<sup>I</sup>-PIGF-2 were strikingly different. First of all, 125<sup>I</sup>-PIGF-2 was bound much more efficiently to the b1b2 domain than to a1a2/b1b2, by about 5-fold (Fig. 5B, lane 2 versus lane 3), whereas the opposite was true for 125<sup>I</sup>-VEGF<sub>165</sub> (Fig. 5A, lane 2 versus lane 3). In addition, 125<sup>I</sup>-PIGF-2 binding to NRP1 domains (Fig. 5B) did not result in the formation of the very large molecular mass complexes that were formed in the presence of 125<sup>I</sup>-VEGF<sub>165</sub> (Fig. 5B). These results suggest that structural elements within the a1a2 domain specifically contribute to a more efficient binding of VEGF<sub>165</sub> to the b1b2 domain, but that these elements have no effect on PIGF-2 binding to the NRP1 b1b2 domain.

**VEGF<sub>165</sub> and PIGF-2 Binding to the b1b2 Domain of NRP1 in the Presence of Heparin Fragments—As shown previously in Figs. 1 and 4, heparin greatly enhanced the binding of 125<sup>I</sup>-VEGF<sub>165</sub> (Fig. 6A) and 125<sup>I</sup>-PIGF-2 (Fig. 6B) to the NRP1 b1b2
domain, by about 20- and 10-fold, respectively, compared with the lack of heparin (Fig. 6, A and B, last lanes). To test the effect of heparin chain length on VEGF₁₆₅/b₁b₂ and PlGF-2/ b₁b₂ interactions, heparin fragments from 4 to 24 monosaccharide units were tested for the ability to facilitate binding in solution (Fig. 6). A chain length of 18 monosaccharide units failed to promote VEGF₁₆₅/b₁b₂ interactions, heparin-agarose precipitates were formed with the b₁b₂ domain (open arrow), lane 1, no NRP1 domain; lane 2, a complex of 65 and 80 kDa was formed with the b₁b₂ domain (open arrow), lane 3, a complex of 120 kDa was formed with a1a1/b₁b₂ (solid arrow). Moreover, a chain length of over 20 monosaccharides is needed to facilitate NRP1/VEGF₁₆₅ and NRP1/PlGF-2 interactions.

The b₁b₂ Domain Is Heparin Binding—To test the possibility that NRP1 by itself binds heparin, recombinant NRP1 domain proteins were incubated with heparin-agarose beads. Heparin-agarose precipitated the NRP1 b₁b₂ and a₁a₂/b₁b₂ but not the a₁a₂ or c domains (Fig. 7A). Furthermore, b₁b₂ and a₁a₂/b₁b₂ proteins bound to the TSK-heparin columns and were eluted with ~0.65 M NaCl (Fig. 7B). The entire b₁b₂ but neither b₁ nor b₂ alone could bind heparin (data not shown). The binding of b₁b₂ to heparin was dependent on the length of the heparin chain (Fig. 7C). Soluble heparin oligosaccharides of increasing length were analyzed for competition of heparin-b₁b₂ binding. Binding was reduced significantly by 26% upon addition of a chain as short as 8 monosaccharide units. A heparin chain length of 16 saccharide units reduced b₁b₂ binding by 75% and binding was totally inhibited by 20 saccharide units. These results demonstrate that NRP1 binds directly to heparin through its b₁b₂ domain, and that this interaction can occur between the b₁b₂ domain and monosaccharides as short as 8 units.

**DISCUSSION**

NRP1 plays a critical role in the development of the neuronal and vascular systems as a receptor for multiple disparate ligands. It binds secreted factors such as the semaphorins, which are regulators of axonal guidance, and members of the VEGF family, which are regulators of vasculogenesis and angiogenesis. VEGF₁₆₅ and PlGF-2 are both heparin-binding members of the VEGF family that regulate angiogenesis and bind NRP1. We were interested in determining which structural elements of NRP1 are responsible for the binding of VEGF₁₆₅ and PlGF-2, and whether these structural elements overlap or are distinct. We have demonstrated that the b₁b₂ domain of NRP1 binds VEGF₁₆₅ and PlGF-2 and that their binding sites appear distinct. We have demonstrated that the b₁b₂ domain of NRP1 binds heparin directly and heparin facilitates NRP1/VEGF₁₆₅ and NRP1/PlGF-2 interactions.

To determine the VEGF-binding sites, Myc-tagged NRP1 extracellular domains were expressed in mammalian cells as secreted proteins. Using two assays, binding in solution of...
125I-VEGF165 to recombinant NRP1 subdomains and competition of 125I-VEGF165 binding to HUVEC and MDA-MB-231 cells, it was concluded that the VEGF 165-binding site was localized solely to the b1b2 domain. The a1a2 and c domains did not bind VEGF165. In addition, they did not compete VEGF 165 cell surface binding with the exception that the c domain inhibited 125I-VEGF165 binding to MDA-MB-231 cells by about 30%. The c domain has been implicated in NRP1 dimerization (31). Because MDA-MB-231 cells express only NRPs and in abundance, it may be that the c domain fragment dimerizes with the MDA-MB-231 transmembrane NRP1, thus altering the binding site and partially inhibiting VEGF165 binding. However, in HUVEC where the higher affinity receptors VEGFR1 and VEGFR2 are expressed, this effect may not be detected. An attempt was made to narrow the VEGF165-binding domain further. The NRP1 b domain has 316 amino acids and contains two subdomains as a tandem repeat, b1 and b2. When recombinant b1 and b2 were expressed separately, neither protein alone could bind VEGF165. In addition, they did not compete VEGF165 cell surface binding with the exception that the c domain inhibited 125I-VEGF165 binding to MDA-MB-231 cells by about 30%. The c domain has been implicated in NRP1 dimerization (31). Because MDA-MB-231 cells express only NRPs and in abundance, it may be that the c domain fragment dimerizes with the MDA-MB-231 transmembrane NRP1, thus altering the binding site and partially inhibiting VEGF165 binding. However, in HUVEC where the higher affinity receptors VEGFR1 and VEGFR2 are expressed, this effect may not be detected. An attempt was made to narrow the VEGF165-binding domain further. The NRP1 b domain has 316 amino acids and contains two subdomains as a tandem repeat, b1 and b2. When recombinant b1 and b2 were expressed separately, neither protein alone could bind VEGF165. Truncated b1b2 lacking 30 amino acids from its C terminus also did not bind VEGF165 (data not shown). These results suggest that elements of both b1 and b2 are required for VEGF165 binding to NRP1. Interestingly, it has been shown that the b1 and b2 subdomains each contain a stretch of 18 amino acids that is responsible and sufficient for the cell-cell adhesion activity of NRP1 (32). Whether the NRP1 b1b2 domain contains a single stretch of amino acids or whether non-contiguous motifs are responsible for VEGF165 binding is currently under investigation.

The NRP1 b1b2 domain was also identified as the binding site for PlGF-2. However, PlGF-2 did not bind to b1b2 as well as did VEGF165. Under similar experimental conditions of b1b2 concentration and radiiodinated ligand specific activity, 10
times less $^{125}$I-PlGF-2 was immunoprecipitated with the NRP1 b1b2 domain than was $^{125}$I-VEGF$_{165}$. Whereas, a $K_D$ of 30–40 $\times 10^{-9}$ M could be determined for VEGF$_{165}$/b1b2 interactions, we were not able to determine reliably a $K_D$ for PlGF-2/b1b2 interactions, presumably because the low amounts of $^{125}$I-PIGF-2 that bind NRP1 domains in our system impaired the sensitivity of the assay. Although the binding efficiency between the NRP1 b1b2 domain and the two ligands differ substantially, their binding sites appear to overlap. VEGF$_{165}$ could very efficiently compete with PlGF-2 for b1b2 binding, 30% inhibition was detected by a 1:1 $^{125}$I-PIGF-2 to VEGF$_{165}$ concentration ratio and an excess of 200-fold VEGF$_{165}$ totally abolished $^{125}$I-PIGF-2 binding. The poor binding efficiency of PlGF-2 compared with VEGF$_{165}$ precluded the reverse analysis of PlGF-2 competing with VEGF$_{165}$-binding sites.

Although a1a2 by itself does not bind VEGF$_{165}$, this domain appears to mediate binding to b1b2. The a1a2/b1b2 recombinant protein was 50 times more potent than the b1b2 domain at inhibiting VEGF$_{165}$ binding to cell surface receptors. The affinity of VEGF$_{165}$/b1b2 interactions in solution as measured by Scatchard analysis was 4–8 times lower than that previously determined for the a1a2/b1b2 domain (29). Finally, the a1a2/b1b2 domain cross-linked $^{125}$I-VEGF$_{165}$ more strongly than did b1b2 alone. It may be that the presence of a1a2 provides structural elements that optimize VEGF$_{165}$ binding to b1b2. Unlike the b1b2 domain, the a1a2/b1b2 domain forms mostly high molecular complexes in solution that appear to contain VEGF$_{165}$/a1a2b1b2 multimers. These multimers might stabilize VEGF$_{165}$/b1b2 interactions. High molecular weight complexes were also observed when VEGF$_{165}$ was cross-linked to endothelial cells (21, 22). Another possibility is that the a1a2 domain enables proper folding that optimizes the three-dimensional structure of the VEGF$_{165}$/binding site within the b1b2 domain.

Unlike VEGF$_{165}$, the binding of PlGF-2 to b1b2 was not enhanced by the presence of the a1a2 domain. Moreover, PlGF-2 was not associated with high molecular weight complexes when cross-linked to the a1a2/b1b2 domain. These results suggest that whereas the a1a2 domain contributes structural elements that facilitate VEGF$_{165}$ binding to the b1b2 domain, this domain is already optimal for PlGF-2 binding.

The domains found in naturally occurring soluble NRP1 isoforms (sNRP1) are a1a2 and b1b2 (29, 33). sNRP1s are 60–90-kDa secreted proteins that bind VEGF$_{165}$ in solution, and inhibit VEGF$_{165}$-induced VEGFR2 phosphorylation and VEGF binding in cells overexpressing VEGFR2, NRP1, or both. When overexpressed in tumor cells, sNRP1 adversely affects the tumor vasculature and induces tumor cell apoptosis, possibly by a VEGF withdrawal mechanism (29). Our studies have elucidated the structural elements of sNRP1 responsible for its activity.

The binding of VEGF$_{165}$ to b1b2 is consistent with previous reports that came to a similar conclusion (34, 35). In these experiments individual NRP1 domains were deleted, and the various NRP1 deletion mutants were expressed as membrane-anchored proteins (34). It was observed that VEGF$_{165}$ bound equally well to wild type NRP1 as to the b1b2 transmembrane domain. In contrast, our results indicated that although the intact b1b2 is required and sufficient to bind VEGF$_{165}$, additional elements from the a1a2 domain contribute to efficient binding. In light of the multiple interactions of NRP1 with various cell surface and secreted proteins, our in vitro approach for studying VEGF$_{165}$/NRP1 interactions provides a more controlled system and complements the studies that used NRP1 membrane-anchored deletion mutants expressed on cells.

Heparin plays an active role in the interaction between NRP1 domains and VEGF$_{165}$ or PlGF-2. Initially, it was found that VEGF$_{165}$ binding to its cell surface receptor tyrosine kinases was augmented by heparin and HS (21, 22). Subsequently, formation of an additional complex, now known to have contained VEGF$_{165}$ and NRP1, was shown in particular to be heparin-dependent (12). We have now demonstrated that heparin is essential for promoting the binding of VEGF$_{165}$ and PIGF-2 to its NRP1 b1b2-binding site. Besides facilitating VEGF$_{165}$ and PIGF-2 binding to b1b2, heparin also binds to the b1b2 domain directly as can be shown by heparin affinity chromatography. Thus, b1b2 is a VEGF$_{165}$, a PlGF-2, and a binding-domain in common. A possible mechanism is that VEGF$_{165}$ or PlGF-2, b1b2, and heparin form ternary complexes that facilitate ligand NRP1 interactions by bringing these proteins in direct association.

VEGF$_{165}$ and PlGF-2 binding to the NRP1 b1b2 domain are dependent on the heparin chain length with a minimum length being 20 and 24 monosaccharide units, respectively. Similar heparin size requirements were demonstrated for facilitating these two ligands binding to cell surface NRP (12, 14). On the other hand, heparin/b1b2 binding requires a shorter heparin oligomer of only 8 monosaccharides. The natural cell surface polysaccharide in vivo is heparan sulfate, not heparin. These polysaccharides are chemically related, being composed of the same basic repeating units. Heparin is highly sulfated throughout the entire chain, whereas in HS the sulfated sugar residues tend to occur in clusters of variable length (S-domains) separated by regions of low sulfation (36). The majority of S-domains contain 6–10 sugars and thus fall within the minimum range for heparin binding to the b1b2 domain, 8 monosaccharides. We speculate that several NRP1 molecules could bind to a single HS chain leading to clustering of multiple NRPs. In support, a recently published study has shown that in the presence of heparin, the apparent molecular weight of NRP1 was increased by 3–4-fold, suggesting multimerization of NRP1 (23). However, a single S-domain could not promote the binding of VEGF$_{165}$ or PlGF-2 to NRP1, which requires at least a 20-sugar residue heparin sequence. Thus HS may act as a template for accelerating the ligand/NRP interactions in a similar manner to the way heparin catalyzes the binding of thrombin and antithrombin (37). It will be important to examine the sugar sequence and sulfation requirements for HS recognition of NRP1, and to determine whether HS induces a conformational change in NRP1 that is necessary for its binding to VEGF$_{165}$ and PIGF-2.

In summary, we have identified the NRP1 b1b2 domain as the binding site for VEGF$_{165}$ and PIGF-2, and that the a1a2 domain augments the VEGF$_{165}$ but not PIGF-2 binding to the b1b2 domain. In addition, we show here for the first time that the b1b2 domain of NRP1 interacts directly with heparin. Future studies will determine the specific binding sites within the b1b2 domain.

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J. Biol. Chem. 2002, 277:24818-24825,
doi: 10.1074/jbc.M200730200 originally published online May 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200730200

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