Structural studies of neuropilin/antibody complexes provide insights into semaphorin and VEGF binding

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Neuropilins (Nrp)s are co-receptors for class 3 semaphorins and vascular endothelial growth factors and important for the development of the nervous system and the vasculature. The extracellular portion of Nrp is composed of two domains that are essential for semaphorin binding (a1a2), two domains necessary for VEGF binding (b1b2), and one domain critical for receptor dimerization (c). We report several crystal structures of Nrp1 and Nrp2 fragments alone and in complex with antibodies that selectively block either semaphorin or vascular endothelial growth factor (VEGF) binding. In these structures, Nrps adopt an unexpected domain arrangement in which the a2, b1, and b2 domains form a tightly packed core that is only loosely connected to the a1 domain. The locations of the antibody epitopes together with *in vitro* experiments indicate that VEGF and semaphorin do not directly compete for Nrp binding. Based upon our structural and functional data, we propose possible models for ligand binding to neuropilins.

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Introduction

Neuropilin-1 and -2 (Nrp1 and Nrp2), non-tyrosine kinase receptors with a molecular weight of approximately 120 kDa,

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were initially described as axonally expressed proteins and later recognized as binding partners for class 3 semaphorins (Chen *et al*, 1997; He and Tessier-Lavigne, 1997; Kolodkin *et al*, 1997). They further serve as isoform-specific VEGF (vascular endothelial growth factor) co-receptors on endothelial cells and are involved in vascular development and tumorigenesis (Soker *et al*, 1998; Gluzman-Poltorak *et al*, 2000; Ellis, 2006). Genetic ablation of *Nrp1* or *Nrp2* indicate that both homologs play critical, but nonoverlapping roles during neuronal and vascular development (Kawasaki *et al*, 1999; Chen *et al*, 2000; Giger *et al*, 2000; Takashima *et al*, 2002; Yuan *et al*, 2002). Upregulation of Nrp expression has been proposed to contribute to tumor formation in a number of diseases such as prostate, breast, and colon cancer (reviewed in Ellis, 2006), and has been implicated in increasing mortality in acute myeloid leukemia (Kreuter *et al*, 2006). The linkage between neuronal and vascular wiring along with the upregulation of neuropilin in tumors has prompted considerable efforts to understand the molecular roles of Nrps in the processes of angiogenesis, neuronal development, and tumor biology, and makes these receptors attractive targets for drug development.

Nrp1 and Nrp2 share a sequence identity of 44% (Chen *et al*, 1997; Kolodkin *et al*, 1997); they are composed of five extracellular domains followed by a single transmembrane segment and a short cytoplasmic tail. The extracellular region of Nrps contains two N-terminal CUB domains (C1r/C1s, uEGF, bone morphogenetic protein; also known as a1 and a2), two coagulation factor V/VIII homology domains (F5/8; also known as b1 and b2), and a single MAM domain (meprin, A5, and μ -phosphatase; also known as c) (Takagi *et al*, 1991; Beckmann and Bork, 1993). The cytoplasmic tail includes about 42–44 amino acids and does not display catalytic activity of its own, but presents a binding site for the PDZ domain of Nrp1-interacting protein (NIP or GIPC) (Cai and Reed, 1999; Wang *et al*, 2006). The association of Nrp and NIP may represent one pathway that is important for signaling. Additional signaling pathways are mediated by the recruitment of semaphorins and their respective co-receptors, the type A Plexins (Takahashi *et al*, 1999; Tamagnone *et al*, 1999; Yaron *et al*, 2005), or by binding specific isoforms of the five mammalian VEGF family members (VEGF-A, -B, -C, -D, and PlGF) and their co-receptor tyrosine kinases (VEGFR1–3) (Migdal *et al*, 1998; Soker *et al*, 1998; Makinen *et al*, 1999; Fuh *et al*, 2000; Gluzman-Poltorak *et al*, 2000, 2001; Karkkainen *et al*, 2001; Mamluk *et al*, 2002; Soker *et al*, 2002; Favier *et al*, 2006; Karpanen *et al*, 2006; Pan *et al*, 2007b).

Class 3 semaphorins are secreted members of the semaphorin family and interact with Nrps on two distinct sites. One binding site includes residues from the N-terminal β -propeller or 'Sema' domain of the semaphorins and the a1a2 domains of Nrp. This site dictates the specificity for Nrp1 to

bind *Sema3A* and *Sema3C* and for *Nrp2* to recognize *Sema3C* and *Sema3F* (Chen *et al*, 1998; Giger *et al*, 1998; Nakamura *et al*, 1998; Renzi *et al*, 1999; Gu *et al*, 2002; Antipenko *et al*, 2003). The second binding site, formed between the carboxyl tail of the semaphorins and the b1 domain of *Nrp*, increases the affinity of the complexes (Giger *et al*, 1998; Nakamura *et al*, 1998; Gu *et al*, 2002; Chen *et al*, 1998; Renzi *et al*, 1999) but does not influence selectivity between the binding partners. The key angiogenic factor *VEGF*₁₆₅, as well as *VEGF-B* and *PlGF-2*, binds the b1 domain and to a lesser extent the b2 domain of the *Nrps* via a C-terminal heparin-binding domain (Giger *et al*, 1998; Migdal *et al*, 1998; Soker *et al*, 1998; Makinen *et al*, 1999; Fuh *et al*, 2000; Gluzman-Poltorak *et al*, 2000; Gu *et al*, 2002; Mamluk *et al*, 2002; Vander Kooi *et al*, 2007). Shorter *VEGF* variants such as *VEGF*₁₀₉, which only contain the *VEGF* receptor-binding domain that is responsible for binding to *VEGFRs* (Wiesmann *et al*, 1997; Fuh *et al*, 2000), are unable to recognize *Nrps*. In addition to binding *VEGF*, the *Nrp* b1b2 domains interact with heparin (Migdal *et al*, 1998; Fuh *et al*, 2000; Gluzman-Poltorak *et al*, 2000; Mamluk *et al*, 2002; Vander Kooi *et al*, 2007). The presence of heparin improves binding of *VEGF*₁₆₅ to *Nrp* up to 100-fold (Fuh *et al*, 2000; Mamluk *et al*, 2002), suggesting that heparin or heparan sulfate containing proteoglycans can either bridge or stabilize the interface between *Nrps* and *VEGF*. *VEGF-C* and *-D* contain no known heparin-binding motif yet they are able to bind the b1b2 domain as well (Karpanen *et al*, 2006). Finally, the membrane-proximal c domain of *Nrps* appears to have no direct role in ligand binding; however, it is critical for dimerization or oligomerization (Chen *et al*, 1998; Giger *et al*, 1998; Nakamura *et al*, 1998; Takahashi *et al*, 1998; Renzi *et al*, 1999).

Based on the domain architecture and functional properties, the extracellular domains of *Nrps* are typically divided into three functional units: a1a2, b1b2, and c that represent the primary semaphorin-binding, *VEGF*-binding, and dimerization regions, respectively (Takagi *et al*, 1991; Chen *et al*, 1998; Giger *et al*, 1998; Nakamura *et al*, 1998; Renzi *et al*, 1999; Gu *et al*, 2002; Mamluk *et al*, 2002). Although structure-function studies define the overall domain requirements for neuropilin function, the molecular details of these receptor/ligand complexes remain poorly understood. To date, structural information on *Nrps* is limited to the b1b2 domain of *Nrp1* (Lee *et al*, 2003; Vander Kooi *et al*, 2007). The crystal structure of *Nrp1* b1b2 in complex with Tuftsin, a tetrapeptide homologous to the C terminus of *VEGF*₁₆₅, provided the first structural information on the interaction of *Nrp* with its binding partner *VEGF* (von Wronski *et al*, 2006; Vander Kooi *et al*, 2007).

Here, we present the crystal structure of *Nrp2* a1a2b1b2 in complex with a Fab fragment (anti-panNrp^A) of an antibody that blocks *Sema3* binding and function mediated through both *Nrp1* and *Nrp2* *in vitro*. We also compare and contrast structures of the a2b1b2 and b1b2 fragments of both *Nrp1* and *Nrp2*. Finally, we illustrate the structure of the b1 domain of *Nrp1* combined with a Fab fragment (anti-Nrp1^B) of a recently described phage-derived antibody (Liang *et al*, 2007; Pan *et al*, 2007a) that specifically inhibits *VEGF*₁₆₅ binding to *Nrp1*. *In vivo*, this antibody not only reduces vascular remodeling in the mouse retina, but also works as a single agent and in combination with anti-*VEGF* to slow tumor growth (Liang *et al*, 2007; Pan *et al*, 2007a). Together, these structures

present a detailed picture of a large portion of the *Nrp* extracellular domain and suggest models for *VEGF* and semaphorin binding.

Results and discussion

Construction and function of anti-Nrp1^B and anti-panNrp^A

We recently described a strategy to develop phage-derived antibodies that selectively block binding of either semaphorin or *VEGF* to *Nrp1* (Liang *et al*, 2007; Pan *et al*, 2007a). These monoclonal antibodies were designed as research tools to discriminate between *Nrp1*-mediated responses to either ligand and to evaluate their potential as therapeutics in murine tumor models. Anti-Nrp1^B is a *VEGF*-blocking antibody that binds *Nrp1* with an affinity of 0.2 nM. Although this antibody blocks the interaction between *VEGF* and *Nrp1*, it does not antagonize *Sema3A* function (Liang *et al*, 2007; Pan *et al*, 2007a).

Here, we introduce an antibody (anti-panNrp^A) that cross-reacts with both *Nrp1* and *Nrp2* with affinities of 0.21 and 0.15 nM, respectively (Supplementary Figure S1). A thorough description of the phage-library construction and antibody screening will be detailed elsewhere (manuscript in preparation). In contrast to anti-Nrp1^B, anti-panNrp^A does not affect the binding of *VEGF*₁₆₅ or *VEGF-C* to *Nrp1* and *Nrp2* (data not shown). We also evaluated the capacity of both antibodies to inhibit the *Sema3A*-mediated collapse of axon growth cones from murine dorsal root ganglia (DRG) (Figure 1). The addition of *Sema3A* results in the retraction of the actin processes within the DRG growth cones; this function of *Sema3A* is completely antagonized by anti-panNrp^A, but not by anti-Nrp1^B (Figure 1).

Crystallization of Nrp1, Nrp2, and Nrp/Fab complexes

For structural studies, the smaller *Nrp* fragments that include the type b domains were expressed in *E. coli* while the larger *Nrp* constructs required production as secreted proteins from baculovirus-infected insect cells. An overview of the seven structures is presented in Figure 2. Crystals of the *VEGF*-binding portion (b1b2) of *Nrp1* and *Nrp2* diffracted to a maximum resolution of 1.8 and 1.95 Å, respectively. Crystals of the a2b1b2 domains of *Nrp1* and *Nrp2* were refined to 2.0 and 2.3 Å resolution, respectively, while the b1 domain of *Nrp1* in complex with the *VEGF*-blocking Fab, anti-Nrp1^B, diffracted to 2.2 Å resolution. Finally, the crystal structure of the *Nrp2* a1a2b1b2 domains was solved in complex with the semaphorin-blocking Fab, anti-panNrp^A; monoclinic (space group C2) and trigonal (space group P3₂21) crystal forms of this complex were identified that diffracted to 2.75 and 3.1 Å, respectively. All structures were solved by molecular replacement and are reported with final *R*_{work}/*R*_{free} values below 19/25% and good stereochemistry (Table I). The *Nrp1* a2b1b2 structure has two N-linked glycosylation sites on opposite sides of the a2 domain (Figure 3B). The monoclinic form of the *Nrp2*/Fab complex also shows two glycosylation sites within the a2 domain (Figure 3A); however, these sugar moieties are not well defined in the electron density of the *Nrp2* a2b1b2 structure and therefore are not modeled (Figure 3B).

Figure 1 VEGF does not block Semaphorin 3A-induced growth cone collapse of DRG neurons. **(A)** Images of axon growth cones. Untreated dorsal root ganglia (DRG) have large actin-rich growth cones (arrowheads) that are significantly reduced in number upon addition of Semaphorin 3A. Anti-Nrp antibodies were added at 50 $\mu\text{g}/\text{ml}$; Semaphorin 3A was added at 2.2 nM. Only anti-panNrp^A blocks Semaphorin 3A-mediated collapse. **(B)** Quantification of Semaphorin 3A-induced growth cone collapse. The percentage of collapsed growth cones were calculated by counting collapsed and uncollapsed growth cones ($N = 4$ explants per condition). Error bars represent standard error of the mean.

The overall domain architecture of the Nrp ectodomain

The Nrp extracellular region is often divided into three units that describe the primary semaphorin-binding (a1a2), VEGF-binding (b1b2), and dimerization (c) domains. The crystal structure of rat Nrp1 b1b2 identified a large interface between the b1 and b2 domains and, as the residues buried between domains are conserved, this b1b2 domain arrangement was recognized as a general feature of the Nrp family (Vander Kooi *et al*, 2007). We have solved four crystal structures of Nrp that include the a2, b1, and b2 domains (Figures 2 and 3) and were crystallized from a variety of conditions (Supplementary Table S1). Two models include the a1 domains bound to Fab fragments, while two others include calcium ions in the a2 domains. Despite these differences, the three domains (a2, b1, and b2) share the same arrangement in all crystal structures and pack tightly around a pseudo-three-fold axis (Figure 3). The Nrp1 and Nrp2 a2b1b2 structures are very similar and superimpose with a root mean square deviation (r.m.s.d.) of 1.2 Å over 317 C α atoms. As predicted (Vander Kooi *et al*, 2007), the interface between b1 and b2 is conserved between both neuropilins, burying

about 1200–1500 Å² of solvent accessible surface. Notably, the interface between the a2 domain and the b1b2 is conserved in all structures as well and buries more than 2000 Å² of solvent accessible surface, indicating that all three domains form a rigid structural unit (Figure 3).

In contrast, the domain arrangement between a1 and a2b1b2 is not preserved between the two crystal forms of the Nrp2/anti-panNrp^A-Fab complex (Figure 3C). When superimposing the a2b1b2 cores of the receptors, the a1 domains are displaced by about 7 Å with respect to each other. The interface between a1 and a2b1b2 is small (approximately 800 Å² buried surface area) and not conserved. The lack of strong interactions identifies flexibility of the a1 domain, suggesting that it can undergo conformational changes with respect to the remainder of Nrp in solution or upon receptor binding.

Nrp CUB domains include calcium- and semaphorin-binding sites

The a1 and a2 domains of Nrp1 and Nrp2 are CUB domains (Takagi *et al*, 1991). In the CUB domain prototype, spermad-

Figure 2 Summary of the neuropilin crystal structures. (A) The Nrp ectodomain is comprised of tandem CUB (a1a2), tandem coagulation factor V/VIII (b1b2), and one MAM (c) domain. Cartoon representation of the seven crystal structures presented in this report with the resolution limits listed below. Magenta spheres indicate a bound calcium ion in the a2 domain. (B) Sequence alignment of the a1a2b1b2 domains of human Nrp1 and Nrp2. Secondary structure elements refer to the Nrp2 a1a2b1b2 structure (blue, a1; green, a2; yellow, b1; red, b2) and are named according to conventions adopted for the spermathecin CUB domain (Romero *et al*, 1997) and the coagulation factor V C2 domain (Macedo-Ribeiro *et al*, 1999). Residues boxed in blue and yellow delineate the antibody epitopes for anti-panNrp^A and anti-Nrp1^B, respectively. Amino acids highlighted in orange indicate the Ca²⁺-binding site in a2, while residues in red represent a putative Ca²⁺-binding site in the a1 domain. Residues shaded in green highlight the positions of amino acid that when substituted disrupt interactions between Sema3A and Nrp1 (Gu *et al*, 2002). This alignment was produced with EsPript (Gouet *et al*, 2003).

hesin (Romero *et al*, 1997), the fold comprises two five-stranded β -sheets that form a β -sandwich. Strands β 2, β 4, β 9, β 6, and β 7 form one β -sheet with the other sheet containing strands β 1, β 3, β 10, β 5, and β 8; however, strands β 1 and β 2 are frequently absent in CUB domains from several complement family proteins (Feinberg *et al*, 2003; Gregory *et al*, 2003, 2004). Similar to these proteins, the a1 and a2 domains of Nrps lack the β 1 strand (Figure 4). Overall, CUB domains display a high degree of similarity. For example, the Nrp2 a2 domain is structurally similar to the Nrp1 a2 domain (r.m.s.d. of 0.9 Å; 108 C α atoms), the Nrp2 a1 domain (r.m.s.d. of 0.9 Å; 96 C α atoms), and the CUB domain from spermathecin (r.m.s.d. of 1.4 Å; 86 C α atoms).

The crystal structures of CUB domains from the complement family proteins C1s and MASP-2 (Gregory *et al*, 2003, 2004) contain a calcium-binding site at one end of the sandwich. The a2 domain from the Nrp1 and Nrp2 a2b1b2 structures also contain a bound ion at an identical location (Figures 3B and 4A). In the Nrp1 structure, the ion is clearly observed in the electron density (Supplementary Figure S2) even though no divalent cations were included during crystallization (Supplementary Table S1). In Nrp1, the calcium ion

is coordinated by three negatively charged side chains (Glu¹⁹⁵, Asp²⁰⁹, and Asp²⁵⁰), two carbonyl oxygens (Ala²⁵² and Ile²⁵³), and a water molecule (Figure 4B). Similarly, calcium coordination involves three negatively charged side chains in Nrp2 (Supplementary Figure S2), and these three amino acids are absolutely conserved in the a2 domain from 12 distantly related species (Supplementary Figure S3).

A sequence alignment of the a1 and a2 domains of Nrps (Supplementary Figures S2 and S3) shows that this triad of charged residues is also strictly conserved within the a1 domain of the Nrps. However, in the Nrp2/anti-panNrp^A-Fab complex, there is no indication of a bound ion in the a1 domain. The loops with the residues that define the putative a1 Ca²⁺-binding site are poorly ordered, suggesting that Ca²⁺ plays a role in stabilizing the fold of the domain. Based on the high degree of sequence conservation, it is likely that calcium binding represents a shared feature of Nrp CUB domains.

Neuropilins function as co-receptors for select members of the class 3 family of semaphorins. The semaphorin N terminus contains the signature 'Sema' domain, a seven-bladed β -propeller (Antipenko *et al*, 2003), which is required for

Table 1 Data collection and refinement statistics

	Nrp1 b1b2	Nrp1 a2b1b2	Nrp1 b1/Fab	Nrp2 b1b2	Nrp2 a2b1b2	Nrp2 a1a2b1b2/Fab	Nrp2 a1a2b1b2/Fab
<i>Data collection</i>							
PDB code	2QQI	2QQM	2QQN	2QQJ	2QQO	2QQK	2QQQL
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁	H3	P2 ₁ 2 ₁ 2 ₁	P2 ₁	C2	P3 ₂ 21
Cell dimensions							
<i>a</i> , <i>b</i> , <i>c</i> (Å)	65.9, 67.0, 74.7	53.2, 68.2, 66.6	214, 214, 45.5	36.5, 70.5, 122	50.1, 193, 66.2	148, 106, 92.4	121, 121, 203
α , β , γ (deg)	90, 90, 90	90, 102, 90	90, 90, 120	90, 90, 90	90, 90.1, 90	90, 98.8, 90	90, 90, 120
Resolution (Å)	50–1.8	50–2.0	50–2.2	50–1.95	50–2.3	50–2.75	50–3.1
No. of reflections	30 653	30 901	39 195	23 691	52 659	36 321	31 929
Completeness (%)	97.6 (99.3)	97.5 (88.7)	99.0 (98.0)	99.8 (99.9)	94.5 (85.6)	99.6 (100)	99.6 (98.9)
Redundancy	4.6 (4.6)	3.5 (3.0)	4.4 (3.6)	5.3 (5.2)	4.2 (4.1)	4.2 (4.2)	3.8 (3.6)
<i>R</i> _{sym}	5.0 (51.5)	8.6 (32.2)	9.6 (48.5)	8.9 (53.0)	5.6 (34.4)	10.4 (52.7)	6.1 (51.5)
<i>I</i> / σ <i>I</i>	26.9 (3.2)	13.8 (2.4)	14.3 (2.2)	16.8 (3.1)	20.7 (3.7)	10.9 (3.3)	18.3 (2.6)
<i>Refinement</i>							
Resolution (Å)	20–1.8	20–2.0	20–2.2	20–1.95	20–2.3	30–2.75	30–3.1
No. of reflections	29 018	29 213	37 085	22 404	49 667	34 487	30 265
<i>R</i> _{work} / <i>R</i> _{free}	0.159/0.201	0.183/0.243	0.159/0.207	0.172/0.235	0.185/0.239	0.186/0.243	0.188/0.232
No. of atoms							
Protein	2549	3459	4540	2509	6813	7593	7740
Ligand/ion	6	25	12	6	34	0	0
Sugar	0	76	0	0	0	28	0
Water	282	194	249	239	365	0	0
B-factors (Å ²)	26.6	36.3	33.2	22.5	31.8	74.9	97.1
R.m.s. deviations							
Bond lengths (Å)	0.014	0.012	0.013	0.013	0.013	0.011	0.010
Bond angles (deg)	1.5	1.5	1.4	1.5	1.4	1.3	1.3

One crystal was used for data collection for each structure. Values in parentheses refer to the highest-resolution shell.

Figure 3 Overall domain architecture of neuropilins. (A) Domain organization of Nrp2 (blue, a1; green, a2; yellow, b1; red, b2) in complex with the Fab fragment of anti-panNrp^A (tan, heavy chain; gray, light chain). N-glycosylated residues are indicated in magenta. (B) Ribbon representation of the Nrp a2b1b2 structures; the orange spheres highlight a bound calcium ion. (C) Superposition of the Nrp2/Fab complex from two different crystal forms based on the a2b1b2 domains. Note the poor superposition of the a1 domains (yellow arrows) in comparison to the a2b1b2 region (black arrows). Structure figures were produced with PyMol (<http://www.pymol.org>).

Figure 4 Molecular details of the neuropilin CUB domains. (A) In the a2b1b2 structures, the a2 domain includes a bound calcium ion (orange). The Nrp a1 (panel C) and a2 domains lack the β 1 strand found in spermadhesin. (B) The calcium ion of the Nrp1 a2 domain is coordinated by three negatively charged amino acids, two main-chain carbonyl oxygens, and a water molecule. These interactions are highly conserved among Nrps (Supplementary Figures S2 and S3). (C) (left panel) Amino acids are colored according to the percentage of solvent-accessible surface that is buried at the Nrp2/Fab interface (red, 75–100%; orange, 50–74%; yellow, 25–49%). Anti-panNrp^A is crossreactive for Nrp1 and Nrp2, and 11 of the 14 residues in the structural epitope are identical (black text, identical; white text, nonconserved; asterisks indicate residues in which the side chain points toward the a1 protein core). Residues outlined in green indicate the position of amino acids that when replaced were shown to abrogate binding between Nrp1 and Sema3A (Gu *et al*, 2002). (right panel) The C α atoms of these amino-acid substitutions are indicated as green spheres. C α atoms shaded in purple represent a putative calcium-binding site. (D) The anti-panNrp^A/Nrp2 interface. Nrp2 is shown as a molecular surface with local concentrations of charged residues colored blue (basic) or red (acidic) as calculated by PyMol. The antibody contact residues are dominated by aromatic residues from CDRs H2, H3, and L3.

binding the a1a2 domains of neuropilins. Anti-panNrp^A blocks Sema3A-induced growth cone collapse mediated through Nrp1 (Figure 1) and Sema3F-mediated growth cone collapse mediated through Nrp2, but does not affect VEGF binding (data not shown). In the structure of the Nrp2/anti-panNrp^A-Fab complex (Figure 3A), the Fab fragment contacts only the a1 domain of Nrp2 on the β 8-5-10-3 face of the sandwich, burying approximately 1400 Å² of the solvent accessible surface (Figure 4C). The interface recognized by this antibody is well conserved between Nrp1 and Nrp2. Eleven of the 14 Nrp2 residues that have more than 25% of their accessible surface buried in the interface are identical between the two receptors (Figure 4C). This recognition of an epitope that is conserved between Nrp1 and Nrp2 explains the ability of the antibody to bind both receptors with affinities in the sub-nanomolar range (Supplementary Figure S1).

On the antibody side, 11 side chains contact Nrp2 from complementary-determining regions (CDRs) L3, H2, and H3, seven of which are aromatic in character (Figure 4D). Previous site-directed mutagenesis studies (Gu *et al*, 2002) outlined the semaphorin-binding site of Nrp1. Based on a model of spermadhesin, putatively solvent-exposed residues

on the surface of the a1 domain were selected for amino-acid substitutions. Using this approach (Gu *et al*, 2002), a number of mutants were identified that disrupt the interactions between Sema3A and Nrp1 and when mapped onto Nrp1 a1, they are located on the loops at one pole of the a1 β -sandwich (Figure 4C). Substitutions at the other end of the domain had no effect on Sema3A binding (Gu *et al*, 2002). The mutations disrupting Sema3A binding are adjacent to the epitope recognized by the Sema3A-blocking Fab (Figure 4C), strongly suggesting that the sema domain binds the loops and the β 8-5-10-3 face of the sandwich within the Nrp2 a1 domain. The location of the semaphorin-binding site is also adjacent to the putative calcium-binding site of the a1 domain (Figure 4C), suggesting that calcium binding may play a role in the interactions between the ligand and receptor.

To test this hypothesis, we examined the effects of the Ca²⁺ chelator EGTA *in vitro*. While Sema3A results in the collapse of actin-rich growth cones in DRG, pre-incubation with EGTA blocks this activity of Sema3A (Supplementary Figure S4). These observations coupled with the high conservation of the amino acids that coordinate the calcium ion suggest that calcium may play an important role for Sema3/Nrp interactions.

Nrp type b domains contain the heparin- and VEGF-binding sites

The b domains from the human neuropilin b1b2 structures (Supplementary Figure S5) share significant homology with the phospholipid-binding (type C2) modules from coagulation factors V and VIII (Takagi *et al*, 1991; Macedo-Ribeiro *et al*, 1999; Pratt *et al*, 1999; Lee *et al*, 2003; Vander Kooi *et al*, 2007), and to the galactose-binding domain of bacterial sialidase (Gaskell *et al*, 1995). Collectively, these domains define the discoidin fold that is topologically classified as a distorted jelly-roll β -barrel composed of eight core β -strands. One pole of the domain contains three extended 'spikes' or loops (Supplementary Figure S5) that typically constitute the ligand-binding site for discoidin family members (Gaskell *et al*, 1995; Macedo-Ribeiro *et al*, 1999; Pratt *et al*, 1999; Vander Kooi *et al*, 2007). The b1b2 fragment of Nrp1 and Nrp2 share 50% sequence identity and superimpose with an r.m.s.d. of 2.3 Å over 307 C α atoms. While the b1 domains of Nrp1 and Nrp2 are nearly indistinguishable (r.m.s.d. = 0.6 Å), the b2 domains superimpose less well (r.m.s.d. = 2.7 Å) with the differences largely in the conformations of the 'spikes' (Supplementary Figure S5). As these spikes frequently define binding sites within the discoidin family (Gaskell *et al*, 1995; Macedo-Ribeiro *et al*, 1999; Pratt *et al*, 1999; Vander Kooi *et al*, 2007), the dissimilarity between Nrp1 and Nrp2 may represent flexibility in this region, and thus a way to recognize multiple-binding partners.

Despite the differences between the b2 domains of the Nrp1 and Nrp2, the b domains are tightly packed and form a rigid scaffold (Figure 5). The interdomain junction forms a

deep cleft that runs approximately perpendicular to the long axis of the two b domains. This cleft, created by the β 4: β 5 loop of b1 and the β 5: β 6 loop of b2, is surrounded by a number of positively charged residues, which based on mutagenesis experiments on rat Nrp1, represents the heparin-binding site (Vander Kooi *et al*, 2007). The electropositive patch is present in the structure of both human Nrps as well (Figure 5A). The C-terminal domain of VEGF₁₆₅ (also known as VEGF₅₅) also contains a heparin-binding site (Fairbrother *et al*, 1998). As heparin increases the affinity of b1b2 for VEGF₁₆₅ up to 100-fold (Fuh *et al*, 2000; Mamluk *et al*, 2002), it is likely that Nrps use heparin to recruit VEGF₁₆₅ to this region.

In addition to this heparin-mediated interaction between exon 7 of VEGF₁₆₅ and Nrps, the C-terminal tail of VEGF (CDKPRR_{COOH}) encoded by exon 8 may bind directly to Nrps. In the crystal structure of rat Nrp1 b1b2 in complex with Tuftsin (Vander Kooi *et al*, 2007), a tetrapeptide mimetic (TKPR_{COOH}) of the VEGF tail (von Wronski *et al*, 2006), the C-terminal arginine is tucked tightly into an acidic groove created by residues from the conserved 'spikes' of the b1 domain. In several of our structures, including all three Fab complexes, the C-terminal residue (a histidine) from a symmetry-related molecule occupies the same acidic pocket as the Tuftsin peptide (Supplementary Figure S6).

To further detail potential residues involved in VEGF binding, we examined the conservation of Nrp residues on the b1b2 surface (Figure 5B). Two contiguous patches are conserved among 12 Nrp1 and Nrp2 proteins (Supplementary Figure S3). One of these sites maps directly to the Tuftsin-

Figure 5 Features of the Nrp VEGF- and heparin-binding domains. **(A)** The molecular surface of the rat (PDB code 2ORZ) (Vander Kooi *et al*, 2007) and human b1b2 crystal structures are colored as described in Figure 4D. Green arrows indicate an acidic groove that is formed by the 'spikes' in the b1 domain (Supplementary Figure S5); this feature forms the Tuftsin-binding site of rat Nrp1. Yellow arrows indicate the approximate location of the heparin-binding patch. **(B)** The sequence conservation (green, 100%; yellow, $\geq 75\%$) of the b1b2 domains among 12 Nrps (Supplementary Figure S3) was mapped onto the surface of the human Nrp1 b1b2 structure. Two highly conserved patches are delineated in red. Residues outlined in cyan indicate those residues that contact the Fab in the anti-Nrp1^B-Fab/b1 complex. The a2 domain (tan) is shown by using a superposition of the b1b2 and a2b1b2 structures from Nrp1. **(C)** Ribbon representation of the anti-Nrp1^B-Fab/b1 complex (yellow, b1; orange, heavy chain; gray, light chain). **(D)** The anti-Nrp1^B/b1 interface. The b1 domain is depicted as a molecular surface with a green arrow indicating the Tuftsin/VEGF tail-binding groove. Only CDRs H3 and L1 contact b1.

binding site, while the second site includes residues on the edge of the heparin-binding patch, suggesting an additional area for interactions between Nrp and VEGF.

In the crystal structure of the Nrp1 b1/anti-Nrp1^B-Fab complex (Figure 5), the Fab contacts an epitope located between these two putative VEGF-binding sites and partially overlaps with the Tuftsin-binding cleft. The epitope of this VEGF-blocking antibody is unusual, involving only residues from CDRs L1 and H3. In general, Fab/antigen interfaces bury an average of approximately 1700 Å² of solvent-exposed surface (Lo Conte *et al*, 1999); however, only 900 Å² are shielded in the Nrp1 b1/Fab interface. Despite the small interface, anti-Nrp1^B binds tightly to Nrp1 with an affinity of 0.2 nM (Liang *et al*, 2007; Pan *et al*, 2007a).

VEGF and Sema3A do not compete for Nrp binding

Several studies have reported that VEGFs and semaphorins compete for binding on the cell surface and that this competition involves a partially overlapping binding site on the b1 domain (Miao *et al*, 1999; Gu *et al*, 2002; Narazaki and Tosato, 2006).

As the carboxyl tail of VEGF₁₆₅ and class 3 semaphorins are both rich in basic residues, it was suggested that these tails might compete for the electronegative groove created by the 'spikes' in the b1 domain (Lee *et al*, 2003; Vander Kooi *et al*, 2007). The recent b1b2/Tuftsin crystal structure indicated that the VEGF₁₆₅ tail occupies this binding site. However, the C terminus of Sema3A does not end with the highly conserved arginine of VEGF₁₆₅ or Tuftsin that is tucked in the VEGF-binding groove on the b1b2 structure (Vander Kooi *et al*, 2007); instead, it contains a hydrophobic valine, rendering it unlikely that the C terminus of semaphorins occupy the same binding pocket. We have previously shown that anti-Nrp1^B does not antagonize Sema3A-mediated collapse of axons from DRG (Figure 1) (Liang *et al*, 2007; Pan *et al*, 2007a). As the VEGF-blocking antibody does not affect Sema3 function, we examined whether VEGF₁₆₅ might antagonize Sema3A-mediated collapse of axonal growth cones from DRGs. In this assay, even at concentrations up to 100 nM, VEGF₁₆₅ did not reduce the ability of Sema3A (at 2.2 nM) to cause the retraction of actin processes (Figure 1). In addition, saturating concentrations of Sema3A do not alter VEGF-induced endothelial cell migration (Pan *et al*, 2007a). To test whether VEGF and Sema3A exclude each other from binding to Nrps, we studied the interaction between immobilized Nrp1 and its ligands VEGF₁₆₅ and Sema3A using surface plasmon resonance. Both Nrp-binding partners show specific binding to Nrp1-Fc, although they display different binding modes as illustrated in the differences between their association and dissociation phases (Supplementary Figure S7). When VEGF₁₆₅ and Sema3A are coinjected at equimolar amounts, the resulting binding sensorgram is almost identical to the sum of the two single binding sensorgrams, as would be expected for simultaneous binding (Supplementary Figure S7). Antibodies that have been shown to block binding of Sema3A and VEGF₁₆₅ to Nrp1 (Liang *et al*, 2007; Pan *et al*, 2007a) also completely block binding in this *in vitro* assay (Supplementary Figure S7). Furthermore, in our Nrp/Fab crystal structures, the binding epitopes blocking VEGF and Sema3 binding are separated by 65 Å and located on opposite sites of the Nrp (Supplementary Figure S8). The large distance between the

interfaces for VEGF- and Sema3-blocking Fabs supports the notion that both ligands, semaphorin and VEGF, can bind Nrps simultaneously and do not compete for each other.

Previous competition experiments (Miao *et al*, 1999; Gu *et al*, 2002; Narazaki and Tosato, 2006), indicating that VEGF and semaphorins compete for Nrp binding, employed VEGFs and semaphorins with heterologous tags (such as alkaline phosphatase) at the C termini of these proteins. It might be possible that the observed competition is a result of steric clashes with the tags rather than the result of direct competition between the VEGF and the Sema3 tail.

Models for neuropilin dimerization

Nrp1 and Nrp2 form homo- or hetero-multimers even in the absence of ligand (Chen *et al*, 1998; Nakamura *et al*, 1998; Takahashi *et al*, 1998). Data have demonstrated that the c domain is important for multimerization; however, since truncation mutants lacking this domain can self-associate, the c domain might not be the only domain involved in Nrp oligomerization (Chen *et al*, 1998; Giger *et al*, 1998; Nakamura *et al*, 1998; Renzi *et al*, 1999). Nrp constructs that include the a1a2 and b1b2 domains have higher affinity for VEGFs than constructs that span only the b1b2 domains (Gu *et al*, 2002; Mamluk *et al*, 2002; Karpanen *et al*, 2006) even though the a1a2 domains do not bind VEGF. It is therefore possible that the a1a2 domains contribute directly to Nrp dimerization and strengthen interactions between the Nrp dimer and the VEGF dimer by stabilizing the 2:2 complex.

Interestingly, the crystal structures of a1a2b1b2 domains from two Nrp2 crystal forms (Table I) and contain a conserved, crystallographic interface that is mediated by a1. In this dimer, the a1 domains align in an approximately anti-parallel arrangement along the β7 and β8 strands. The interface buries approximately 1200 Å² of solvent accessible surface area and is dominated by hydrophobic interactions (Supplementary Figure S8). Notably, similar dimers have been observed for other CUB domain family members (Romero *et al*, 1997). Other features of the crystallographic dimer are less supportive that the observed a1 interface is biologically relevant. For one, the amino acids along the interface are only moderately conserved (Supplementary Figure S8); in addition, the shape complementarity of the interface is poor with an Sc value of only 0.4 (Lawrence and Colman, 1993). Therefore, we examined the molecular mass of three Nrp2 constructs (a2b1b2, a1a2b1b2, and a1a2b1b2c) by multiangle light scattering, but in this experiment none of the constructs formed dimers even when the c domain was present. Although immunoprecipitation experiments have demonstrated that Nrp self-associates (Chen *et al*, 1998; Giger *et al*, 1998; Nakamura *et al*, 1998; Takahashi *et al*, 1998, 1999), there is no data that rigorously show the formation of Nrp dimers in solution. This underscores the difficulty in designing experiments that directly measure dimerization of wild-type or mutant Nrps that might disrupt the a1 interface.

Although Nrp2 a1a2b1b2 is monomeric by dynamic light scattering, the orientation of crystallographic Nrp2 dimer suggests a model for VEGF binding. The a1 interface creates a saddle-shaped dimer with a width of approximately 70 Å (Figure 6), large enough to accommodate a VEGF₁₀₉-dimer with dimensions of about 35 by 60 Å. Importantly, the

Figure 6 A Nrp2 crystallographic dimer suggests models for VEGF and Sema3 binding. (A) Nrp2 forms a saddle-shaped dimer in both crystal forms of the Nrp2/Fab complex. This figure highlights the Nrp2 a1a2b1b2 domains from the monoclinic crystal form of the Fab complex. The putative VEGF tail-, heparin-, and semaphorin-binding sites are indicated. (B) Potential models of VEGF/Nrp and semaphorin/Nrp complexes based on the Nrp a1-mediated dimer in the crystal structures.

Tufts binding sites and the heparin-binding patches are found on the inner surface of the saddle. Heparin could stabilize interactions between the heparin-binding sites of Nrp and VEGF and further facilitate the association of the VEGF tail with the 'spikes' of b1. This arrangement would also be able to accommodate VEGFR binding via the VEGF receptor-binding domain for downstream signaling. Therefore, although the a1 domains do not directly engage VEGF, they could enhance VEGF binding of Nrps by facilitating Nrp dimerization. Alternatively, it has been suggested that heparin binding induces dimerization of Nrp1 b1b2 (Vander Kooi *et al*, 2007) and that this activity could explain how Nrps dimerize in the absence of the MAM domain.

This dimer could further accommodate Sema3 binding (Figure 6). In the Sema3A crystal structure, two 'sema' domains pack tightly together at an interface. Upon Nrp binding, the sema domains of Sema3A dissociate to allow interactions with the primary binding site on a1a2 (Antipenko *et al*, 2003). In our model of the a1-mediated Nrp dimer, the two putative Sema3A-binding sites are located on opposite sides, and distant enough to accommodate the large β -propellers of two bound Sema3 molecules (Figures 6 and Supplementary S5).

Our structural analysis provides the first detailed picture of both the VEGF-binding (b1b2) and semaphorin-binding (a1a2) portions of the Nrp ECD. The antibody complexes along with previous mutagenesis studies (Gu *et al*, 2002; Vander Kooi *et al*, 2007) delineate the Nrp-binding sites for the Sema domain of semaphorins, and the heparin-binding domain of VEGF. These structures provide a basis for future experiments and provide strategies to elucidate the structures of Nrps in complex with their ligands (VEGFs and semaphorins) and signaling receptors (VEGFRs and plexins).

Materials and methods

Functional assays and antibody-binding affinities

Collapse assays and determination of anti-Nrp antibody-binding affinities were performed as previously described (He and Tessier-Lavigne, 1997; Liang *et al*, 2007; Pan *et al*, 2007a).

Protein expression and purification

Nrp1 b1, Nrp1 b1b2, and Nrp2 b1b2 (Supplementary Table S1) were cloned into pET15b (Novagen) and expressed in *E. coli* following induction at 37°C (Nrp1 b1 and b1b2) or 16°C (Nrp2 b1b2). All neuropilin type b fragments are expressed as soluble proteins without the need for a refolding protocol. Following cell lysis, proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) resin in 50 mM Tris (pH 8.0), 300–500 mM NaCl, and 20 mM imidazole, and eluted in the same buffer plus 250 mM imidazole. The his₆-tags were removed with thrombin, and samples were further purified using a Superdex-75 column equilibrated in 25 mM Tris (pH 8.0) and 150 mM NaCl.

Recombinant baculoviruses were generated to facilitate the secretion of Nrp a2b1b2, a1a2b1b2, and full-length ECD constructs (Supplementary Table S1). Nrp2 a1a2b1b2 and the full-length Nrp2-ECD were subcloned with the Nrp2 native secretion signal and a C-terminal His₆-tag into pENTR/D-TOPO (Invitrogen) and recombined into pDEST8 (Invitrogen) to generate a viral bacmid. Nrp1 and Nrp2 a2b1b2 were cloned into pAcGP67B (Clontech). Following infection, the culture media were collected and supplemented with 50 mM Tris (pH 8.0), 5 mM CaCl₂, and 1 mM NiCl₂; proteins were purified with Ni-NTA and gel filtration chromatography as described for bacterial-expressed constructs.

The Fab fragments for anti-Nrp1^B (YW107.4.87) (Liang *et al*, 2007; Pan *et al*, 2007a) and anti-panNrp^A (YW68.11.26) were expressed in *E. coli*, captured on a Protein G column equilibrated in PBS, and eluted with 0.58% acetic acid. Protein fractions were further purified by ion exchange chromatography (SP-sepharose) in 20 mM MES (pH 5.5) and eluted with a gradient from 0 to 250 mM NaCl. Fab/Nrp complexes were typically mixed at 1:1 molar ratio and further purified using a Superdex-200 column equilibrated in 25 mM Tris-HCl (pH 7.5) and 200 mM NaCl. For crystallization, all

unbound neuropilin and Nrp/Fab complex samples were concentrated as detailed in Supplementary Table S1.

Crystallization, structure determination, and refinement

All crystals were obtained by vapor diffusion at 19°C by mixing equal volumes of protein plus well solution (Supplementary Table S1). Crystals were cryoprotected as described in Supplementary Table S1. Data sets were collected at the Advanced Light Source (beam lines 5.0.1 or 5.0.2) or the Stanford Synchrotron Radiation Laboratory (beam lines 9-2 or 11-1) and processed with the HKL package (Otwinowski and Minor, 1997).

All crystal structures were solved by molecular replacement using Phaser (McCoy *et al*, 2005). The Nrp1 b1b2 structure was solved using the Nrp1 b1 crystal structure (pdb code 1KEX) (Lee *et al*, 2003), and the refined coordinates of the Nrp1 b1b2 structure served as the search probe for the Nrp2 b1b2 structure. The monoclinic form of the Nrp2 a1a2b1b2/anti-panNrp^A-Fab complex was solved using the Nrp2 b1b2 structure and Fab fragments containing either the variable domains (V_H/V_L) or the constant domains (C_{H1}/C_L) from the B20-4/VEGF complex (pdb code 2FJH) (Fuh *et al*, 2006). The a2 domain was identified by molecular replacement using the N-terminal CUB domain from MASP-2 (pdb code 1NT0) (Feinberg *et al*, 2003); the a1 domain was placed manually within the electron density. The Nrp1 b1/Fab complex was solved using the Nrp1 b1 crystal structure and the B20-4 Fab fragments described above. All remaining structures were solved

using the refined coordinates of the b1b2 structures and the a2 CUB domain from the Nrp2 a1a2b1b2/Fab complex. Atomic models were built using Coot (Emsley and Cowtan, 2004) and refined with Refmac (Murshudov *et al*, 1997).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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