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Effect of C-terminal Sequence on Competitive Semaphorin Binding to Neuropilin-1

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Abstract

Neuropilins (Nrp) are type I transmembrane proteins that function as receptors for Vascular Endothelial Growth Factor (VEGF) and class III Semaphorin (Sema3) ligand families. Sema3s function as potent endogenous angiogenesis inhibitors, but require proteolytically processing by furin to compete with VEGF for Nrp binding. This processing liberates a C-terminal arginine (C_R) that is necessary for binding to the b1 domain of Nrp, a common feature shared by Nrp ligands. The C_R is necessary but not sufficient for potent Nrp inhibition and the role of upstream residues is unknown. We demonstrate that the second-to-last residue (C-1), immediately upstream of the C_R, plays a significant role in controlling competitive ligand binding by orienting the C-terminus for productive Nrp binding. Utilizing a peptide library derived from Sema3F, C-1 residues that preferentially adopt an extended bound-like conformation, including proline and β -branched amino acids, were found to produce the most avid competitors. Consistent with this, analysis of the binding thermodynamics revealed that more favorable entropy is responsible for the observed binding enhancement of C-1 proline. We further tested the effect of the C-1 residue on Sema3F processing by furin and found an inverse relationship between processing and inhibitory potency. Analysis of all Sema3 family members reveals two non-equivalent furin processing sites differentiated by the presence of either a C-1 proline or C-1 arginine and resulting in up to a forty-fold difference in potency. These data reveal a novel regulatory mechanism of Sema3 activity and define a fundamental mechanism for preferential Nrp binding.

Keywords

VEGF; angiogenesis; furin; proteolysis; peptide library

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INTRODUCTION

The Neuropilin (Nrp) family of type I transmembrane receptors coordinate critical signaling events in the cardiovascular and nervous system where they are essential in development, homeostasis, and pathogenesis. The two families of canonical Nrp ligands are the Vascular Endothelial Growth Factor (VEGF) family of pro-angiogenic cytokines and the Semaphorin-3 (Sema3) family of axon guidance molecules (rev. in¹). VEGF signaling via Nrp and a VEGFR receptor tyrosine kinase family member is essential for physiological and pathological angiogenesis and plays a causative role in tumorigenesis (rev. in^{2; 3}) and wet macular degeneration^{4; 5}. Additionally, it has recently been shown that Nrp is critical for autocrine cancer stem cell activation and maintenance^{6; 7}. Sema3 signaling via Nrp and Plexin receptors mediates physiological axon guidance and contributes to pathological axon repulsion following CNS injury (rev. in^{8; 9}).

Because of its role in multiple pathological conditions, Nrp represents an attractive therapeutic target. Peptides^{10; 11; 12}, peptidomimetics¹³, soluble receptor fragments¹⁴, and monoclonal antibodies^{15; 16; 17} have all been explored as Nrp binding and blocking molecules. Peptides are the best-studied class of Nrp targeting and modulating molecules and have been developed not only for their competitive ligand binding activity but also for other diverse purposes including *in vivo* Nrp diagnostic imaging^{18; 19} and for cargo targeting to Nrp-expressing tumors^{20; 21}. Nrp ligand-blocking peptides include sequences derived from endogenous Nrp ligands^{12; 22}, the naturally occurring immunostimulatory peptide Tuftsin¹¹, and phage-display derived peptides^{10; 20}. Initial mechanistic and developmental work has provided critical insights into Nrp ligand binding, but additional insights are needed to produce peptides that are optimized for potency, selectivity, and stability.

Biochemical and structural approaches have demonstrated that all known Nrp ligands require a C-terminal arginine (C_R) for binding to a conserved pocket in the Nrp b1 domain^{12; 23; 24; 25}. Alternative splicing generates a C_R in many VEGF families, including VEGF-A (rev. in²⁶) and VEGF-B²⁷, but proteolytic maturation is required in the case of VEGF-C and VEGF-D²⁸. Similarly, Sema3 family members require proteolytic activation by furin-family proteases to liberate a C_R^{29; 30; 31} that then allows them to function as endogenous competitive inhibitors of Nrp^{12; 32; 33}. Indeed, relative levels of VEGF and Sema3 family members have been shown to critically contribute to tumorigenesis^{34; 35}. Furin family proteases cleave substrates following an arginine residue³⁶. There are between one and three canonical RXXR furin cleavage sites in the C-terminal basic domain of Sema3 family members, producing Sema3 ligands with alternative forms of the C-terminal domain²⁹.

All known peptide inhibitors of Nrp also contain a C_R and target the conserved Nrp-b1 pocket, binding in a mode analogous to that of Nrp ligands^{20; 22; 37}. Recently, the structural basis for C_R dependent Nrp binding has been described. Crystal structures of the VEGF-A heparin binding domain (HBD)²⁴ and Tuftsin²³ in complex with Nrp1 revealed a shared mode of receptor engagement and have provided critical insight into the physical basis for Nrp binding. Two residues of the ligand contribute to Nrp-b1 binding. The C_R is critical for Nrp binding and mediates the majority of the interface via divalent engagement of both the

C_R side chain and carboxylate at the C-terminus²⁴. The third-to-last residue (denoted as residue-“C-2” hereafter) mediates the other interaction, with the C-2 backbone carbonyl forming a hydrogen bond with the aromatic hydroxyl of Nrp1-Y297. This interaction is also critical since mutation of Y297 results in loss of ligand binding³⁸. That this interaction critically depends on a backbone hydrogen bond is supported by the observation that for ATWLPPR, Nrp binding is C-2 sequence-independent but truncation smaller than a tetrapeptide eliminated activity³⁷. While a C_R residue is critical for all peptide inhibitors of Nrp, no upstream consensus has been identified. This led us to investigate the sequence-dependence for Nrp-ligand binding and inhibition.

To determine the role of residues upstream of the C_R, we studied the sequence dependence of Nrp binding and inhibition of Sema3F derived peptides. We found that the C-1 residue serves the critical role of positioning the C_R and C-2 residues to allow concurrent Nrp binding. A peptide library with substitution of all 20 natural amino acids at the C-1 position revealed that residues that naturally adopt an extended conformation enhance inhibitory potency by six-fold. A C-1 proline produced the most potent Nrp binding peptide, which we demonstrate is due to a specific reduction in the entropic cost of binding. We further demonstrate that there is an inverse relationship between furin processing of Sema3 and inhibitory potency across the Sema3 family. These data provide critical insight into the mechanism of Nrp ligand binding and potent inhibition, and describe a novel mechanism for regulated Sema3 furin processing and Nrp receptor engagement.

RESULTS AND DISCUSSION

C-1 sequence variation critically affects peptide potency

To determine the contribution of the C-1 residue to Nrp binding and inhibitory potency, a peptide library derived from the C-terminal domain of Sema3F (WDQKKPRNRR) was synthesized with all twenty natural amino acids substituted at the C-1 position. The library was tested for the ability to inhibit alkaline phosphatase (AP)-tagged VEGF-A binding to Nrp1 utilizing an *in vitro* plate-binding assay. Retained AP-VEGF-A was measured as a function of peptide concentration. For all peptides, full AP-VEGF-A inhibition was achieved and the concentration of peptide resulting in half AP-VEGF-A inhibition (IC₅₀) was determined. Significant differences in inhibitory potency were observed, with wild type Sema3F (S3F-RR, black line, IC₅₀ = 11 μM) showing intermediate potency between the peptides with maximum and minimum potency, C-1 proline (S3F-PR, green line, IC₅₀ = 4.7 μM) and C-1 aspartate (S3F-DR, red line, IC₅₀ = 28 μM) peptides, respectively (Fig. 1A).

Sequence alteration at the C-1 residue resulted in a significant variation in peptide potency (Fig. 1B), indicating that this position plays a significant role in modulating Nrp binding. The most potent inhibitor was the C-1 proline peptide, which was six times more potent than the aspartate variant. Analysis of previously published peptide inhibitors reveals a preference for proline at the C-1 position. Modified Tuftsin (TKPPR) and the phage display derived peptide ATWLPPR possess a C-1 proline. The prototypic CendR peptide identified by phage display, RPARPAR, does not have this sequence element. However, an analysis of the other CendR peptides that were initially identified reveals that 30% of all peptides contained a C-1 proline, in striking contrast to the 5% theoretical representation if there were

no selective pressure at the C-1 position³⁹. Collectively these data reveal a significant role for the C-1 position in determining competitive binding to Nrp.

Potency is correlated with the propensity of the C-1 residue to adopt an extended conformation

Intriguingly, the identity and chemical properties of the C-1 residue, including charge and hydrophobicity, seemed to have little systematic impact on potency. Structural analysis indicates that the C-1 side-chain is dynamic and shows little if any direct interaction with Nrp. Instead, we hypothesized that the C-1 residue critically tethers the C-2/Nrp1-Y297 hydrogen bond with the C_R mediated salt-bridge and hydrogen bond network. Using structures of Nrp1 complexes, we measured the backbone torsion angles of the C-1 Tuftsin (PDB=2ORZ) and VEGF-A (PDB=4DEQ) residue to determine whether a specific orientation is required to tether the adjacent C_R and C-2 interaction interfaces. Although VEGF-A contains a C-1 arginine and Tuftsin a C-1 proline, residues with markedly different physiochemical properties, their backbone conformations were very similar (Fig. 2A). Analysis of the backbone dihedral angles demonstrate that they lie within the extended region of the Ramachandran plot with $\phi, \psi = -89^\circ, 108^\circ$ and $-85^\circ, 158^\circ$, respectively (Fig. 2B).

The extended conformation seen for both proline and arginine indicates that residues with an inherent propensity to adopt an extended conformation could, when present at the C-1 position, enhance Nrp binding. Upon analysis of all variants, it was striking to observe that proline and β -branched amino acids (isoleucine, valine and threonine) produced the most potent C-1 variants (Fig. 1B). We examined the relationship between the IC₅₀ for each C-1 variant and the Chou-Fasman β -sheet propensity scale for each amino acid⁴⁰ to determine whether these properties are correlated (Fig. 2C). Indeed, potency and β -sheet propensity showed a highly significant correlation (correlation coefficient (r) = -0.74 , $p = 0.0003$). These data support a correlation between competitive potency and preferred C-1 backbone conformation.

Preferential backbone conformation minimizes the entropic cost associated with binding

Thus, we hypothesized that peptides with a C-1 residue that inherently favors an extended conformation would have increased potency due to decreased entropic cost associated with Nrp1 binding. Therefore, we measured the thermodynamic parameters of S3F-RR (WDQKKPRNRR) and S3F-PR (WDQKKPRNPR) binding to Nrp1 using isothermal titration calorimetry (ITC).

With Nrp1 in the sample cell, peptides were titrated to saturation and the resulting binding isotherms were fit with a one-site binding model, allowing determination of thermodynamic binding parameters. S3F-RR (Fig. 3A) and S3F-PR (Fig. 3B) fit to a 1:1 stoichiometry, consistent with a single C_R ligand binding to monomeric Nrp1. S3F-RR bound to Nrp1 with a $K_a = 3.0 \times 10^4 \text{ M}^{-1}$ ($K_d = 33 \text{ }\mu\text{M}$) and S3F-PR bound with significantly higher affinity, with a $K_a = 10.1 \times 10^4 \text{ M}^{-1}$ ($K_d = 10 \text{ }\mu\text{M}$). The binding enhancement for S3F-PR relative to S3F-RR is consistent between direct binding and inhibitor potency (3.0-fold and 2.3-fold, respectively). Binding of the less potent wild-type S3F-RR was actually more favorable

enthalpically ($\Delta H = -18.5$ and -17.0 kcal/mole, for S3F-RR and S3F-PR, respectively, $p = 0.04$). However, there was a significantly larger loss of entropy upon S3F-RR binding as compared to S3F-PR binding ($\Delta S = -12.2$ and -10.0 kcal/mole for S3F-RR and S3F-PR, respectively, $p = 0.009$). Thus, the difference in entropy accounts for the preferential binding potency of S3F-PR.

The measured thermodynamic parameters of binding represent the combined changes for both Nrp1 and the peptide upon binding. No significant differences in the orientation of the bound product in the Nrp b1 binding site (Fig. 2A), conformation of the Nrp1 binding pocket itself (R.M.S.D. = $0.6\text{\AA}$ for Ca over residue range 274–429), or protonation state have been indicated. Thus, the difference in entropy between S3F-RR and S3F-PR binding can be best interpreted in terms of conformational bias in the free ligand. Indeed, it was recently suggested that peptide backbone rigidity is able to enhance Nrp binding⁴¹. These data directly support the hypothesis that proline substitution at the C-1 position enhances Nrp binding by preferentially positioning the free peptide backbone in a ligand bound-like extended conformation that allows engagement of the C-2 and C_R interactions. With proline at the C-1 position the backbone is pre-organized for optimal receptor engagement.

Arginine is conserved at the C-1 position in Sema3F to maintain efficient proteolytic maturation

Although a C-1 proline residue confers an advantage for Nrp1 binding, the majority of furin consensus sequences present in Sema3 family members possess a C-1 arginine residue. This opens the question of why arginine, as opposed to proline, is preferred at the C-1 position of Sema3 family members. The Sema3 family of ligands require processing by furin family proteases for biological function²⁹. This processing event liberates a C_R and is required for Nrp1 binding¹². We hypothesized that the C-1 residue may directly affect furin-dependent proteolysis.

To test this hypothesis, we expressed the Sema3F Ig-basic domains with a C-terminal human growth hormone (Hgh) fusion as either the wild type sequence (S3F-Hgh) or with the C-1 residue mutated to proline (R778P-Hgh) (Fig. 4A). S3F-Hgh and R778P-Hgh were secreted at similar levels, but processing by furin was significantly different (Fig. 4B). When expressed in wild type CHO cells, over 40% of S3F-Hgh protein was cleaved but only 10% of R778P-Hgh could be processed. No processing was observed for either S3F-Hgh or R778PHgh when expressed in CHO cells deficient in furin activity (FD11)⁴², demonstrating that the observed processing is due to furin activity. In contrast, in CHO cells stably overexpressing furin⁴², the majority (70%) of secreted S3F-Hgh was furin processed, while 30% of R778P-Hgh was processed. These data demonstrate that a C-terminal di-arginine motif allows optimal proteolysis by furin family proteases and that variation at the C-1 position alters proteolytic efficiency. It is notable that R778P-Hgh is partially processed and that this processing increases in cells with higher levels of furin activity. Thus, tissue specific differences in furin activity or differential expression of different proprotein convertase (PPC) family members may serve as a regulatory mechanism underlying activating proteolysis of the different furin sites.

Cumulatively, these data demonstrate that there is an inverse relationship between furin processing and Nrp binding. While a C-1 arginine is best suited for furin processing, a C-1 proline is best suited for Nrp1 engagement. Intriguingly, while the majority of furin sites in the different Sema3 family members have a C-1 arginine, those without an arginine exclusively possess a proline residue at this position, suggesting a possible tissue-specific regulatory mechanism of Sema3 activity.

C-1 variation in the Sema3 family alters Nrp1 binding

Sema3F contains a single furin proteolysis site in its C-terminus. This site has a strong furin consensus (KXRXR) and is conserved in five of the seven Sema3 family members (Fig. 5A, site 2). While it is clear that this extended basic sequence serves as an ideal furin substrate, different Sema3 family members possess up to two other furin sites that have a conserved dibasic RXXR motif^{43; 44} and have been demonstrated to be processed^{29; 30; 31}. For all family members, cleavage at the first and second furin site results in a C-1 arginine, while processing at the third site results in a C-1 P residue (Fig. 5B). By aligning the terminal four amino acids of all Sema3 family members, the differential usage of arginine and proline at the C-1 position is clearly illustrated (Fig. 5C) indicating two distinct classes of furin cleavage sites.

We tested the VEGF inhibitory potency of peptides representing all forms of each member of the Sema3 family and found very significant differences. Greater than a 40-fold difference was observed between the different Sema3 family members and an 18-fold difference between different sites in the same Sema3 family member (Sema3A) (Fig. 6A). Sema3E.3 was the most potent inhibitor with an $IC_{50} = 0.76 \mu M$ and Sema3A.2 was the weakest inhibitor with an $IC_{50} = 34 \mu M$. Notably, all peptides with a C-1 proline were more potent than all peptides with a C-1 arginine (Fig. 6A). Indeed, when grouped by their C-1 residue (Fig. 6B), peptides with a C-1 arginine had a mean potency of $19 \pm 11 \mu M$, as opposed to peptides with a C-1 proline that had a mean potency of $2.4 \pm 1.4 \mu M$ ($p = 0.02$). Otherwise, no correlation between potency and sequence, equivalent to that for the C-1 residue, was observed at other upstream positions. This does not rule out additional secondary effects, since a range of IC_{50} values are observed within the two C-1 groups. For instance, it is interesting to note that furin processing at site 2, the most conserved in the Sema3 family, always produces a C-2 asparagine (Fig. 5B), and these peptides showed the weakest VEGF inhibitory potency (Fig. 6A). A strongly negative preference for aspartate/asparagine is observed in the C-1 position (Fig. 2C), and it may be that the sidechain of an asparagine residue in either the C-1 or C-2 position competes with Nrp for mainchain interactions, thereby lowering affinity.

While furin processing is critical, a differential physiological role and how processing at the distinct sites is regulated is currently unknown. These data indicate that there are two physically distinct classes of non-equivalent furin sites in the C-terminal domain of Sema3 family members that are differentiated by their C-1 residue. Indeed, it has been demonstrated within the context of full-length protein that Sema3A cleaved solely at site 3 (Sema3A.3) shows maximal potency and that when processing at this site is abolished, function is markedly reduced *in situ*²⁹. Additionally, conformational flexibility may be

important for productive assembly of the Sema3 signaling complex, as suggested by the recent structural characterization of the Nrp1/Sema3A/PlexinA2 complex⁴⁵. Differential furin processing could modulate the interdomain distance between the Sema3 sema domain and basic domain C_R, which engage Nrp a1 and b1 domains, respectively^{1; 46}. It is interesting to note that Sema3A.3 is the most potent sequence as well as the most C-terminal furin site opening the possibility for coupling between binding site sequence and spacing. Thus, in physiological context, processing at the different sites can produce different amplitude of Sema3 signaling, thereby providing a mechanism for control of Sema3 signaling by regulation of both expression and selective processing.

Intriguingly, these data also have direct implications for the *in vivo* function of differential furin processing in Kallmann's syndrome, a genetic disease characterized by incomplete development of olfactory nerve fibers and defective migration of neuroendocrine cells. A recent analysis of patients revealed mutations in the Sema3A gene underlying the disease, including R773H⁴⁷. R773 is the C-1 residue of the Sema3A.1 furin site²⁹. Although R773H was efficiently expressed and secreted, it exhibited a marked and significant reduction in its ability to signal⁴⁷. Thus, differential processing and Nrp engagement of the different furin sites has important function in both normal physiology and pathology. Our data provide a mechanistic basis for understanding the effect of mutations on both furin processing and Nrp binding.

CONCLUSION

These data demonstrate that the C-1 position of Nrp binding peptides and ligands is critical for regulating both potency and processing. This provides insight into the mechanism of potent Nrp-binding and inhibition and lays the groundwork for continued improvements to the potency and specificity of peptide inhibitors of Nrp. Further, by studying the C-1 residue in the context of the C-terminus of Sema3 we have gained an understanding of physiological Sema3 ligand function. Interestingly, the Sema3 basic domain contains three non-equivalent furin processing sites defined by possessing either a C-1 Arg or Pro. These data define an inverse relationship between processing and potency, providing an important new mechanism for post-translational regulation of Sema3 activity. It was previously thought that furin processing simply functioned as a binary activation mechanism. However, our data indicates that the coupled relationship between differential furin processing and Nrp engagement allows production of Sema3 forms with a range of biological activities (Fig. 6C). This provides insight into the mechanistic basis for functional differences for Sema3 both *in situ* and *in vivo*. Recently reported disease-associated mutations in Sema3A underscore the biological importance of Sema3 processing and Nrp engagement. Indeed, mutation in the furin processing sites of individual Sema3 family members that differentially effect processing and Nrp engagement represents an important avenue for future study in both neuronal and cardiovascular diseases.

MATERIALS AND METHODS

Protein expression and purification

The core ligand binding domains of Nrp1, domains b1b2, were produced in *Escherichia coli* as a 6xHis-tag fusion from pET28 and purified by immobilized metal ion affinity chromatography (IMAC) using HIS-Select resin (Sigma-Aldrich, St. Louis, MO) followed by heparin-affinity chromatography (GE Healthcare Life Sciences, Piscataway, NJ)²³. Alkaline-phosphatase tagged VEGF-A₁₆₄ (AP-VEGF-A) was expressed in Chinese Hamster Ovary (CHO) cells via large-scale transient transfection from pAPtag-5 vector (GenHunter, Nashville, TN)¹².

In vitro plate binding assay

Nrp1 affinity plates were produced by diluting Nrp1-b1b2 to 25 ng/μl in 50 mM Na₂CO₃ pH 10.4 and 100 μl was added to 96 well protein high-binding plates (Costar, 9018), incubated for 1 hr at 37 °C, washed 5× 100 μl with PBS-T (PBS, 0.1% Tween20), and stored at 4 °C. Two peptide libraries, one in which the C-1 residue of the S3F C-terminus (WDQKKPRNRR) was substituted for all twenty amino acids and another that corresponded to the last nine residues of all Sema3 family members, were synthesized and purified by HPLC, with an average purity of 90% (NEO Group, Cambridge, MA). An N-terminal tryptophan was added to aid in quantitation.

Peptides were resuspended in binding buffer and combined with AP-VEGF-A at peptide concentrations ranging from 500 μM to 230 nM and a final AP-VEGF-A activity of 1 μmol of pNPP hydrolyzed/min per μl. The pre-mixed AP-VEGF-A/peptide solution was then added to Nrp1 affinity plates and incubated for 1 hr at 25 °C. Wells were washed with 100 μl of PBS-T three times using an EL404 plate washer (BioTek, Winooski, VT) and an additional 100 μl of PBS-T was added to the plate and incubated for 5 min. The wash solution was then removed and 100 μl of 1X alkaline phosphatase substrate was added⁴⁸. After 25 minutes the reaction was quenched by adding 100 μl of 0.5N NaOH and the plate was read at 405 nm on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Retained AP-VEGF-A binding was plotted against peptide concentration to generate an inhibition curve that was fit with a four-parameter sigmoidal dose response curve to determine the half-maximal inhibitory concentration (IC₅₀). Results are reported for each peptide as the mean IC₅₀ ± standard deviation of two independent experiments.

Isothermal titration calorimetry

Peptides with sequence WDQKKPRNRR (S3F-RR) and WDQKKPRNPR (S3F-PR) were synthesized by solid-phase synthesis and purified to >95% purity (LifeTein, South Plainfield, NJ). Purified Nrp1-b1b2 and the S3F peptides were exhaustively dialyzed against ITC buffer (10 mM phosphate, 238 mM NaCl, 2.7 mM KCl, pH 7.4). Following dialysis, Nrp1 was concentrated to 40 μM and S3F peptides were diluted to 390 μM as measured by OD₂₈₀ (NanoDrop 1000, Thermo Scientific).

Binding between Nrp1 and the S3F peptides was measured using a VP-ITC Microcalorimeter (MicroCal, GE Healthcare) and data was processed with Origin software

(OriginLab, Northampton, MA). Nrp1-b1b2 was transferred into the sample cell and the syringe was loaded with S3F. Sample measurements were made at 30 °C and the system was set to provide a reference power of 10 µcal/sec. Thirty 10 µl injections were made, spaced 250 sec. apart. To determine the heat of ligand dilution, S3F-RR and S3F-PR were separately injected into ITC buffer utilizing the same parameters as the experimental runs. The heat of ligand dilution was subtracted from the heat of binding to generate a binding isotherm that was fit with a one-site binding model in Origin, allowing determination of the association constant (K_a), enthalpy (ΔH), and entropy (ΔS) of the interaction. K_a was used to calculate K_d ($K_d=1/K_a$). ITC data was collected in quadruplicate for each peptide with independent preparations of Nrp1-b1b2.

Western blot analysis of Sema3F furin processing

The Ig-basic domains of human Sema3F (residues 605–785, S3F-Hgh), as well as the single point mutant, R778P (R778P-Hgh), were cloned into the pLexM vector⁴⁹ for expression with an N-terminal PTP- α signal sequence and C-terminal Hgh fusion⁵⁰. Protein was expressed by transient transfection from CHO, furin deficient FD11, and furin overexpressing cells⁴². Western blot analysis was performed on conditioned medium from all cell types to detect the Ig-basic-Hgh fusion protein (unprocessed) or free Hgh (processed) as previously described¹². An anti-Hgh polyclonal primary antibody (1:10000 dilution, RD1-HGHabrX1, Fitzgerald Industries, Acton, MA) and anti-rabbit HRP secondary antibody (1:20000 dilution, sC-2301, Santa Cruz Biotechnology, Santa Cruz, CA) were utilized and the blot was developed using SuperSignal West Pico (Pierce Biotechnology, Rockford, IL). Image Lab software (Bio-Rad Laboratories, Hercules, CA) was used to calculate the percent of processed Ig-basic-Hgh relative to total protein.

Prism (Graphpad Software, La Jolla, CA) was used for analyzing and graphing data and for calculating the statistical significance between sets of data as determined by a student's t-test. Molecular graphics were generated using Pymol (The PyMOL Molecular Graphics System, Version 1.5.0.2 Schrödinger, LLC).

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Abbreviations

Nrp	Neuropilin
Sema3	class III Semaphorin family
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
C_R	C-terminal arginine

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Highlights

- Nrp ligands utilize a C-terminal domain for receptor engagement
- The C-1 position critically modulates Nrp binding and VEGF inhibitory potency
- Sema3 sequence variability balances proteolytic processing and Nrp engagement
- A C-terminal proline-arginine motif allows optimal Nrp engagement by its ligands

Figure 1. Inhibitory potency of C-1 peptide variants

(A) Peptides were titrated to determine their inhibitory potency versus AP-VEGF-A. Shown are representative inhibition curves for wild type peptide (S3F-RR, black line) and the variants with maximum and minimum potency, S3FPR (green line) and S3F-DR (red line), respectively. (B) Table of all C-1 variants with their respective IC₅₀. Values are reported as the mean ± standard deviation.

Figure 2. The C-1 residue of Nrp1 ligands adopts an extended backbone conformation
 (A) Superimposition of the product bound structures of VEGF-A (green) (PDB=4DEQ) and Tuftsin (gold) (PDB=2ORZ) in complex with Nrp1 (PDB=2ORZ). There are two distinct interaction interfaces, one of which is mediated by the C_R and the other by the C-2 carbonyl of the ligand. The C-1 residue serves the critical role of tethering the adjacent interacting residues and correctly orienting them with respect to one another within the binding pocket.
 (B) Ramachandran plot of the ϕ and ψ angles of the C-1 VEGF-A (C-1 R) and Tuftsin (C-1 P) residue reveals that they adopt an extended conformation.
 (C) Plotting the IC_{50} for each C-1 variant against the β -sheet propensity of each amino acid reveals a correlation between potency and the inherent preference of amino acids to adopt an extended conformation. Proline was excluded from the fit due to its inability to conform to the backbone hydrogen bonding pattern present in β -sheets and, therefore, its low β -sheet propensity.

Figure 3. Analysis of peptide binding by ITC

Using ITC the thermodynamic parameters of Nrp1 binding were measured for wild type peptide (S3F-RR, A) and the C-1 proline variant (S3F-PR, B). A representative binding isotherm is shown for each peptide. Values are reported as the mean $\pm$ standard deviation of four independent experiments.

Figure 4. Mutation of the Sema3F C-1 residue alters processing efficiency

(A) The Igbasic domains of Sema3F were expressed as a C-terminal Hgh fusion. In addition to wild type protein (S3F-Hgh), a mutant with the C-1 arginine residue of the furin consensus site mutated to proline (R778P-Hgh) was expressed. The arrow indicates the site of furin proteolysis. (B) S3F-Hgh and R778P-Hgh were expressed in CHO, furin deficient (FD11), and furin over expressing (Furin) cells and the efficiency of processing was measured by blotting for Hgh. S3F-Hgh was processed in CHO and Furin cells, as detected by the presence of the processed form, but significantly less processing was seen for R778P-Hgh in these cell types. Neither construct was processed in FD11 cells.

Figure 5. Furin proteolysis of Sema3 generates divergent C-terminal sequences

(A) The Sema3 family of ligands are furin processed at multiple sites within their C-terminus that result in either a C-terminal arginine-arginine motif (site 1 and 2) or a proline-arginine motif (site 3). (B) Peptides corresponding to all furin cleavage sites (black arrow) within the basic domain of Sema3 family members were synthesized with a leading tryptophan (grey). Sema3 variants are labeled according to family member (A–G) and cleavage site number (1–3). (C) An alignment of the four C-terminal residues of all Sema3 peptides illustrating the either-or preference for proline and arginine at the C-1 position. The height of the amino acids at each position represents their relative conservation within the alignment.

Figure 6. Sema3 family variation in potency is explained by the C-1 amino acid

(A) The ability of all Sema3 ligand variants to inhibit VEGF-A binding to Nrp1 was measured. The data are reported as the mean IC₅₀ ± standard deviation. (B) The IC₅₀ for each peptide was plotted against the amino acid present at the C-1 position. Peptides with a C-1 proline (average IC₅₀ = 2.4 ± 1.4 μM) were significantly more potent than those with a C-1 arginine (average IC₅₀ = 19 ± 11 μM). (C) The C-1 residue inversely effects furin consensus strength and Nrp affinity.