VEGF-A121a binding to Neuropilins – A concept revisited

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ABSTRACT
All known splice isoforms of vascular endothelial growth factor A (VEGF-A) can bind to the receptor tyrosine kinases VEGFR-1 and VEGFR-2. We focus here on VEGF-A121a and VEGF-A165a, two of the most abundant VEGF-A splice isoforms in human tissue, and their ability to bind the Neuropilin coreceptors NRP1 and NRP2. The Neuropilins are key vascular, immune, and nervous system receptors on endothelial cells, neuronal axons, and regulatory T cells respectively. They serve as co-receptors for the Plexins in Semaphorin binding on neuronal and vascular endothelial cells, and for the VEGFRs in VEGF binding on vascular and lymphatic endothelial cells, and thus regulate the initiation and coordination of cell signaling by Semaphorins and VEGFs. There is conflicting evidence in the literature as to whether only heparin-binding VEGF-A isoforms – that is, isoforms with domains encoded by exons 6 and/or 7 plus 8a – bind to Neuropilins on endothelial cells. While it is clear that VEGF-A165a binds to both NRP1 and NRP2, published studies do not all agree on the ability of VEGF-A121a to bind NRPs. Here, we review and attempt to reconcile evidence for and against VEGF-A121a binding to Neuropilins. This evidence suggests that, in vitro, VEGF-A121a can bind to both NRP1 and NRP2 via domains encoded by exons 5 and 8a; in the case of NRP1, VEGF-A121a binds with lower affinity than VEGF-A165a. In in vitro cell culture experiments, both NRP1 and NRP2 can enhance VEGF-A121a-induced phosphorylation of VEGFR2 and downstream signaling including proliferation. However, unlike VEGF-A165a, experiments have shown that VEGF-A121a does not ‘bridge’ VEGFR2 and NRP1, i.e. it does not bind both receptors simultaneously at their extracellular domain. Thus, the mechanism by which Neuropilins potentiate VEGF-A121a-mediated VEGFR2 signaling may be different from that for VEGF-A165a. We suggest such an alternate mechanism: interactions between NRP1 and VEGFR2 transmembrane (TM) and intracellular (IC) domains.

VEGF-A isoforms exhibit differential binding to VEGFRs and NRPs

VEGF-A is one of five VEGF genes in humans, the others being VEGF-B, -C, -D, and PIGF. VEGF-A encodes multiple splice isoforms, and there are additional proteolytic isoforms generated by post-translational enzymatic cleavage. All of the known isoforms share the same binding sites for receptor tyrosine kinases; as anti-parallel homodimers, VEGF-A ligands are bivalent and bind two monomers of their cognate receptors, VEGFR1 and/or VEGFR2. There are two additional functional binding domains, but these are present only in select isoforms. First, the longer splice isoforms can include heparin binding domains (encoded by exons 6 and/or 7) that confer on those isoforms an ability to bind to, and be sequestered by, proteoglycans of the extracellular matrix or cell surface (HSPGs). Second, the ‘xxx’ isoforms contain the short C-terminal exon 8a-encoded sequence, which can confer the ability to bind to the Neuropilin coreceptors (Fig. 1, 2), while the ‘xxxb’ isoforms, containing the exon 8b-encoded sequence, cannot. All pro-angiogenic VEGF-A isoforms retain the exon 8a-encoded sequence. However, while the exon 8a-encoded sequence appears to be necessary for Neuropilin binding, experiments had not directly clarified whether it is sufficient; VEGF-A121a, for example, has been shown in some studies to bind Neuropilins and in other studies not to bind. Here we review and compare that literature.

Evidence against binding of VEGF-A121a to Neuropilins

The work of Hela Gitay-Goren, Shay Soker, Gera Neufeld, Michael Klagsbrun, and colleagues in the late 1990s elucidated the role of Neuropilins as VEGF coreceptors, and in particular showed that...
VEGF-A165a formed a 'bridge' between VEGFR2 and NRP1 by binding both receptors simultaneously.\(^{11-14}\) The authors used cross-linking to fix \(^{125}\)I-VEGF-A121a and \(^{125}\)I-VEGF-A165a to human umbilical endothelial (HUE) cells that express VEGFR1, VEGFR2, NRP1 and NRP2 at densities detectable by western blotting.\(^{13}\) They used western blotting to assess the appearance of \(^{125}\)I-VEGF-A121a-bound or \(^{125}\)I-VEGF-A165a-bound NRP and VEGFR complexes by bands corresponding to their combined molecular weight.\(^{13}\) In Fig. 2 of their paper\(^{13}\), bands corresponding to the molecular weight of NRP1+\(^{125}\)I-VEGF-A121a did not appear with excess VEGF-A121a (10–20 ng.mL\(^{-1}\) ~ 0.25–0.52 nM). Furthermore, treatment with an excess of VEGF-A121a did not inhibit formation of NRP1+\(^{125}\)I-VEGF-A165a complexes\(^{13}\) or does so to a small extent.\(^{10}\) The authors concluded that either VEGF-A121a does not bind to NRPs, or that the affinity of VEGF-A121a to these receptors is lower;\(^{13}\) competition experiments are not always sufficiently sensitive to detect low affinity ligand binding.\(^{15}\)

In another 1996 paper, the same authors used cross-linking to test whether VEGF-A165a or VEGF-A121a were capable of binding to MDA-MB-231 cells (expressing NRP1 but not VEGFR2) and HUVECs (expressing both NRP1 and VEGFR2)\(^{11}\). As shown in figure 5 of their paper, the authors observed 5–10 ng.mL\(^{-1}\) of VEGF-A121a cross-linking to HUVECs but not the 231 cells, and did not observe VEGF-A121a competition with \(^{125}\)I-VEGF-A165a for binding to NRP1 on MDA-MB-231 cells.

In 1998, the same authors assessed whether VEGF-A121a and VEGF-A165a bound to porcine aortic endothelial (PAE) cells engineered to express VEGFR2, or NRP1, or both VEGFR2 and NRP1.\(^{12}\) As shown in figure 5A of that paper, VEGF-A121a did not cross-link to PAE cells or PAE/NRP1 cells, but did cross-link to PAE/VEGFR2 or PAE/VEGFR2/NRP1 cells.\(^{12}\) The difficulty in cross-linking VEGF-A121a to PAE cells in the absence of VEGFR2 could be an issue with the specific reagent used (DSS: disuccinimidyl suberate). The DSS cross-linker used here has an spacer arm length of ~11.4 A and cross-linking may depend on both the molecules (due to availability of Lysines) and the cell type (due to expression of different extracellular matrix (ECM) species).\(^{16}\)
Evidence that the exon 8a domain, which VEGF-A121a has, is responsible for Neuropilin binding

Crystal structures of NRP1 b1 domain demonstrate distinct contacts with VEGF-A exon 7 and 8a encoded domains

A VEGF-A-bound NRP1 crystal structure solved by Vander Kooi, Leahy and colleagues in 2007 demonstrated that exon 8a-encoded residues of VEGF-A165a are the direct site of interaction between NRP1 domains b1b2 and VEGF-A165a (Fig. 2 of[17]). Thus, NRP1 binding specificity towards VEGF-A isoforms is regulated by VEGF-A exon 8a-encoded sequence, not exon 7 (supplementary figure 8 of[17]). In 2012, Parker et al reported a crystal structure of mouse VEGF-A164a exons 7+8a-encoded sequence bound to NRP1.18 The intermolecular interface, and experimental mutagenesis in the exon 8a-encoded sequence and NRP1 b1 domain residues, showed that exon 8a-encoded amino acids are critical for high affinity binding of any VEGF-A isoform to NRP1.18

All NRP1-binding proteins and peptides are found to possess a C-terminal arginine.17,19-21 The authors further showed that NRP1 has a C-terminal arginine-binding pocket in the b1 domain. Mutating VEGF’s C-terminal exon 8a-encoded arginine (R164) resulted in up to 97% loss in retention of mouse VEGF-A164a by NRP1, thus this residue plays a critical role in VEGF-A/NRP1 interactions (Fig. 3 of[18]). Additional support for the key role of this arginine comes from a different VEGF ligand encoded by a different gene, VEGF-C, which can bind NRP2. Processed peptides corresponding to the VEGF-C C-terminus (219-SIIRR-223) can bind to NRP2 b1 domain21, and the R223E mutation results in loss of VEGF-C binding to NRP2.21

Though VEGF-A121a (human) and VEGF-A120a (mouse) both contain the exon 8a-encoded domain, a crystal structure for either isoform binding to NRPs is yet to be elucidated. Binding affinities of mouse VEGF-A120a and 164a to NRP1 (Fig. 2 of[18]) were measured to be 3 nM and 22 nM respectively. VEGF-A120a, which lacks exons 6 and 7 (the ‘heparin-binding domain’22), binds with higher affinity to NRP2 than to NRP1; mouse VEGF-A164a, which has exon 7, binds to NRP1 with higher affinity than to NRP2 (Fig. 2 and 5 of[18]). Thus, the exon 8a-encoded arginine is essential for the VEGF-A-NRP interaction, and the exon 7-encoded domain provides additional contacts (a larger intermolecular interface) that may provide specificity towards NRP1 but not NRP2.18

The crystal structure of VEGF-A165a has been determined, but as two separate fragments: a 110-amino acid fragment that includes the VEGFR-binding domains (Fig. 1B, exons 2–5), and a 55-amino acid fragment (corresponding to exons 6–8a) that includes the NRP-binding domain. The inability to crystallize both together suggests that the two domains are not rigid with respect to each other. Thus, the NRP1-binding site likely has some range of motion (distance and orientation) from the main VEGFR-binding domain in VEGF-A165a (Fig. 1B), due to the additional amino acids encoded by exon 7; this may make NRP binding less restricted in longer VEGF isoforms.

Mutations, peptidomimetics, and antagonists: The exon 8a-encoded tertiary fold is key to NRP1 binding

In a 2006 paper, Cébe Suarez et al showed that VEGF-A splice variants which either lack the exon 8-encoded domain entirely (i.e. VEGF-A159), or have their exon 8a replaced by exon 8b (8a: CDKPRR; 8b: SLTRKD; i.e. VEGF-A121b and VEGF-A165b), do not bind NRP1 at all (figure 4 of[23]). They conclude that exon 8a-encoded sequence is required for NRP1 binding, that the heparin-binding domains (exons 6 and/or 7) are not sufficient for NRP1 binding, and that exon 8a is necessary for proper
folding of the heparin-binding domains, as VEGF-A165b has reduced HS binding, no NRP1 binding and altered (delayed and attenuated) signaling characteristics via VEGFR2 and ERK kinases.

Only VEGF-A isoforms containing the exon 8a-encoded sequence (VEGF-Axxx; Fig. 1) compete with VEGF-A165a for binding to NRP1.23 In 2006, von Wronski et al demonstrated that Tuftsin, a naturally occurring short peptide antagonist mimicking the C-terminal sequence of VEGF-A (Tuftsin: TKPR; exon 8a: CDKPRR), blocks VEGF-A binding to NRP1 without blocking VEGF-A binding to VEGFR2.24 The peptide competed with VEGF-A165a on NRP1 and NRP2, and displaced VEGF-A165a from endothelial cells. The authors further showed that the peptide inhibits VEGF-A165a-induced phosphorylation of VEGF2 without directly inhibiting VEGF-A binding to VEGFR2. These results and homology between Tuftsin and the exon 8a-encoded sequence of VEGF-A suggests a specific role for exon 8a’s C-terminal residues in NRP1 binding.

In another 2006 article, Jia et al identified the NRP1 binding region of VEGF-A165a to be the C-terminal region of the peptide encoded by exons 7+8a, and showed that EG3287a, a VEGF-A165a mimetic bicyclic peptide with the three-dimensional conformation of VEGF-A exon 8a-encoded fold, binds to NRP1. This peptide significantly inhibited VEGF-A165a binding to NRP1 while...
lacking most of the exon 7-encoded domain and comprising largely of the exon 8a-encoded sequence (Fig. 3 of 26). The peptide inhibited VEGF-A165a-induced VEGFR2, PLC-γ and ERK activation in HUVECs. The authors further found that peptides lacking VEGF-A exon 7-encoded residues and comprised largely of the exon 8a-encoded residues retained most of their inhibitory activity with reduced potency compared to EG3287a. Mutagenesis on exon 8a residues, Lysine (K), Proline (P) and Arginine (R), demonstrated that all these residues are essential for VEGF-A binding to NRP1. These results demonstrate the significance of exon 8a-encoded sequence in NRP1 binding to VEGF-A isofoms; the presence of these exon 8a-encoded residues in VEGF-A121a, therefore, suggests that that isoform can also bind NRP1.

**In VEGF-A121a, domains encoded by both exons 5 and 8a are necessary for NRP binding**

Delcombel et al demonstrated that VEGF-A111a (a biologically active and proteolysis-resistant isoform 26), which contains the exon 8a-encoded sequence, does not bind to NRP1 (Fig. 2 of 26). Thus, the exon 8a-encoded sequence is not sufficient by itself to confer NRP1 binding. Unlike VEGF-A121a, VEGF-A111a has part of exon 5 missing, and thus Delcombel et al synthesized exon 5+8a encoded peptides. The results showed that exon 5 or 8a alone are not sufficient for NRP1 binding, and both domains must be present and intact (as is the case for VEGF-A121a), for binding to occur.9

**Evidence supporting direct VEGF-A121a binding to Neuropilins**

**Cell-free SPR demonstrates that VEGF-A121a can bind NRP1**

In 2007, Pan et al examined binding of VEGF-A165a, VEGF-A121a and VEGF-A109 to NRP1 using surface plasmon resonance (SPR) methodology.27 VEGF-A109 is a proteolytically-processed VEGF-A isoform which only contains exons 1–4 and most of exon 5; since it lacks the exon 8a-encoded sequence it should not and does not bind to Neuropilins. Figures 4 and 6 of that paper show that VEGF-A121a produced by a number of different sources binds to NRP1.27 This shows that well-characterized VEGF-A121a, possessing the correct sequence (including exon 8a), has a three dimensional structure that enables its binding to NRP1 with binding affinity comparable to VEGF-A165a (Kd of 220 nM compared to Kd of 110 nM). Note that SPR measurements represent the affinities for the interaction of a ligand with monomeric truncated NRP1, comprising only the a1/12-b1/b2 domains and lacking the MAM domain known to induce NRP oligomerization,28 the transmembrane domain known to homodimerize29 and the NRP1 GAG modification30, which may explain why these SPR-measured affinities are lower than those measured for on-cell VEGF-NRP interactions.31

Pan et al and Kawamura et al emphasized the importance of expression, purification or purchase of sequenced VEGF isoforms that are not degraded and have the correct exon 8a-encoded sequence and tertiary structure for proper receptor binding, and suggest that lack of VEGF-A121a/NRP1 binding in previous experiments could be due to inadvertently cleaved VEGF-A121a lacking the exon 8a-encoded sequence, as demonstrated in Fig. 2D and 4C of.27,32 Other technical considerations for any experiment probing ligand-receptor interactions in general include: 1) the presence of tags (e.g. myc, Flag) on purified or purchased VEGF-A121a that may sterically interfere with ligand-receptor binding; and 2) expression and purification in *E. coli* instead of mammalian cells, which may cause incorrect posttranslational modifications or VEGF-A disulfide bond formation, or low affinity of VEGF-A121a due to improper folding/structure.

In 2012, Parker et al also used SPR to demonstrate that VEGF-A121a binds to NRP1.18 In 2013 Delcombel et al also confirmed via SPR that VEGF-A121a bound to NRP1 with lower affinity compared to VEGF-A165a, and with distinct kinetics (Fig. 2 of 27). Going further, using multiple VEGF-Aexx,a and VEGF-Aexx,b isofoms, Delcombel et al demonstrated that: all VEGF-Aexx,a isofoms containing exon 8a encoded sequence except VEGF-A111a bind to NRP1. As noted above, this one exception (VEGF-A111a) is likely due to the lack of part of exon 5 encoded sequence on the VEGF-A111a isoform. The authors suggest that the lack of observed NRP1-binding by VEGF-A121a in earlier publications11-13 is due to degradation of exon 8a encoded sequence during expression and preparation of the ligand or during the experiments. They also showed that VEGF-Aexx,b isoform failing to bind NRP1 is due to lack of exon 8a encoded sequence/tertiary fold and is not due to the presence of exon 8b encoded sequence/tertiary fold.9

Parker et al also showed that VEGF-A120a (mouse) binds to both NRP1 and NRP2. However, VEGF-A120a binds to NRP2 with higher affinity than VEGF-A164a (mouse)33 (as summarized in Figure 6 of 28). VEGF-A164a (mouse) binds to NRP2 with close to 50-fold lower affinity as compared to its binding to NRP1.18

**VEGF-A121a and VEGF-A165a binding to NRP1 and NRP2 on the cell surface**

VEGF-A165a/NRP binding can be experimentally measured in endothelial cells expressing only NRP1 or
NRP2, VEGF-A121a, however, does not appear to bind to porcine aortic endothelial cells expressing only NRP1. Shraga-Heled et al showed that VEGF-A121a binding affinity to VEGF2R is ~10 fold higher in the presence of NRPs, and coexpression of NRPs and VEGFRs on the same endothelial cell may affect VEGF-A121a association with NRPs. As discussed later, HSPG binding to VEGF2R and NRP1 may also affect VEGF-A121a association.

VEGF-A165a binds NRP1 with higher affinity than to NRP2 (k₄ ~100–300 pM for NRP1, ~700 pM for NRP2), as noted in the previous section, the measured cell surface affinities are stronger than in cell-free systems. VEGF2R phosphorylation on Y951 and Y1175 is lower for VEGF-A121a-ligated receptor complexes than for VEGF-A165a-ligated complexes in the presence of NRP1, but higher for VEGF-A121a-ligated complexes in the presence of NRP2 (Figure 5). This suggests that there are distinct mechanisms by which NRP1 and 2 modulate VEGF-A121a-mediated VEGF2R activation.

**Evidence supporting Neuropilins modulating VEGF-A121a-induced signaling**

**Anti-NRP1 antibodies inhibit VEGF-A121a-induced EC migration and sprouting**

In a cell-free system, Pan et al showed that an anti-NRP antibody blocks NRP1 binding to VEGF-A165a and VEGF-A121a immobilized on a surface (Figure 4 E-F). The same anti-NRP antibody also inhibits VEGF-A165a-induced and VEGF-A121a-induced HUVEC migration and sprouting, and it did not affect VEGF-A109-induced migration (Fig. 2C).

**Extracellular ‘Bridging’ of VEGF2R and NRP1 by VEGF is not necessary for NRP1 modulation of VEGF2R activation**

In a 2007 paper, Shraga-Heled, Neufeld, and colleagues characterized VEGF-A165aKF, a VEGF-A165a mutant that does not bind to VEGF1R or VEGF2R but does bind to NRP1, NRP2 and HSPGs. This mutant induced significant phosphorylation of VEGF2R on Y951 and Y1175, and ERK1/2 phosphorylation, in PAE/VEGFR2/NRP1 cells even at low VEGF-A165KF concentrations; but did not activate VEGF2R in cells that lacked NRP1 (Fig. 2). Thus, VEGF-A165a activation of VEGF2R appears to be possible without the simultaneous binding (so called ‘extracellular bridging’) of VEGF-A165a to NRP1 and VEGF2R. This is important, as it appears that VEGF-A121a cannot bridge these two receptors extracellularly.

VEGF-A165aKF also induced substantial angiogenesis in vitro in PAE/VEGFR2/NRP1 cells and in HUVECs (Fig. 3). Similarly, using PAE/VEGFR2, PAE/VEGFR2/NRP1 and PAE/VEGFR2/NRP2 cell lines expressing similar VEGF2R levels, the authors showed that both VEGF-A165a and VEGF-A121a substantially enhanced VEGF2R activation (Y951 and Y1175 phosphorylation) when NRP1 is present (Figure 5). Furthermore, siRNA knockdown of NRP1 inhibited VEGF-A121a-induced, VEGF-A165aKF-induced, and VEGF-A165a-induced HUVEC proliferation (Figure 6). A combination of siRNA against both NRP1 and NRP2 inhibited EC proliferation induced by either isoform more potently than NRP1-only siRNA, demonstrating that NRP2 also contributes to VEGF-A121a- and VEGF-A165a-induced VEGF2R signaling in ECs (Figure 6). VEGF-A165a promoted formation of stable VEGF2R-VEGF-A-NRP1 complexes (Figure 7). According to the authors, while the VEGF2R-VEGF-A165a-NRP1 complexes survive immunoprecipitation, the VEGF2R-NRP1-VEGF-A165aKF and VEGF2R-NRP1-VEGF-A121a only do so at higher concentrations of ligand (1μg/ml). Thus, it appears that NRP1 can enhance VEGF-A signaling – including VEGF-A121a-induced VEGF2R signaling – through mechanisms other than VEGF2R-NRP extracellular bridging. We note that in that paper, the authors state that “VEGF-A121a does not bind to Neuropilins,” however, based on their own results and those of other publications described above, it is more likely that it can (certainly it does in cell-free systems), and that the mechanisms may be different than for VEGF-A165a.

Whether or not VEGF-A121 binds neuropilins in appreciable quantities on cells in vitro, or on cells in vivo where VEGF concentrations are lower, is less certain. Regardless, the impact of NRP on VEGF-A121a signaling suggests that, in the absence of VEGF-A121a mediating a weak extracellular bridging of VEGF2R and NRP1 not captured by immunoprecipitation or cross-linking, then either: 1) VEGF-A121a binds to NRP1 and affects VEGF2R signaling without bridging; and/or 2) VEGF-A121a binds only VEGF2R and its signaling is modulated by NRP1 without bridging. In either case, VEGF2R-NRP1 interactions at the transmembrane and intracellular level could explain this (see next section).

NRP1 and NRP2 both enhance and modulate VEGF-A121a- and VEGF-A165a-induced VEGF2R activation and signaling in HUVECs. Interestingly, Shraga-Heled et al further demonstrated that VEGF-A165aKF (a VEGF-A165a mutant with impaired VEGFR binding but intact NRP and HSPG binding) did not modulate VEGF2R or ERK phosphorylation in the presence of NRP2, suggesting that extracellular bridging by VEGF-A165a might be
required for NRP2 to enhance VEGFR2 signaling. NRP2 appears to present VEGF-A165a to VEGFR2 (i.e. to act as a concentrator/reservoir of VEGF-A), since VEGF-A165aKF does not induce VEGFR2 or ERK1/2 phosphorylation in the presence of NRP2.\textsuperscript{15}

**Evidence for modulation of VEGF and NRPs by HSPGs**

Heparan sulfate proteoglycans (HSPGs) consist of a core protein and two or three attached heparan sulfate (HS) or chondroitin sulfate (CS) chains.\textsuperscript{36} The abundant negatively-charged HS/CS chains of endothelial-cell-surface proteoglycans bind VEGF-A165a and, as with HS/CS chains in the extracellular matrix, can differentially regulate accessibility/storage of this isoform (and other isoforms with a heparin-binding domain, with affinities depending on the exon 6–8 combinations\textsuperscript{37} and tertiary fold). These HS/CS chains can also modify the ability of VEGF isoforms to bind to VEGFR2 and NRPs, which themselves interact with and colocalize with cell surface HSPGs (VEGFR2 was shown to directly interact with HS chains on HSPGs expressed in endothelial cells via a stretch of residues between D6-D7 of VEGFR2\textsuperscript{38,41}; heparin also binds to VEGFR1\textsuperscript{42-44}, NRP1\textsuperscript{17} and NRP2\textsuperscript{45} independent of VEGFs).

Heparin and HS are known to increase the affinity of VEGF-A165a for VEGFRs and NRPs\textsuperscript{46}. In contrast, VEGF-A121a lacks both exons 6 and 7 and does not bind to HS, as demonstrated by multiple assays.\textsuperscript{10,32} However, HS appears to be required for binding of VEGF-A121a to VEGFR1.\textsuperscript{43} There are effects downstream of receptor binding too: \textit{in vivo} alteration of HS biosynthesis, or inhibition of the HS-VEGFR interaction, inhibit VEGF-A121a-induced hyperpermeability; and removal of the HS chains decreases VEGF-A121a/VEGFR2/NRP1 and VEGF-A165a/NRP1/VEGFR2 assembly level and attenuates effective VEGFR2 phosphorylation in endothelial cells.\textsuperscript{39}

NRP1 was shown to interact with HS via a streak of positive residues stretching over both extracellular b1-b2 domains\textsuperscript{45}; a 3-O sulfated modification specifically facilitates this binding.\textsuperscript{38} Additionally NRP1-specific GAG modification of HS/CS chain on Serine 612\textsuperscript{30} can enhance VEGF-A165a binding to the NRP core protein, which may play a role in the VEGF-A165a-responsive-ness of endothelial cells.

**A new model: VEGFR-NRP interactions outside of the extracellular domain**

The extracellular domains of VEGFR2 and NRP1 interact very weakly with one another or not at all\textsuperscript{27,47}. This supported the concept that VEGF needed to ‘extracellular bridge’ VEGFR2 and NRP1 in order for VEGFR2 activation. However, while NRP1 substantially increases VEGF-A121a affinity to VEGFR2 and VEGF-A121a activation of VEGFR2\textsuperscript{15,27}, as described earlier, VEGF-A121a cannot bridge the extracellular domains of VEGFR2 and NRP1 as VEGF-A165a can. Therefore, ‘extracellular bridging’ appears not to be required for NRP1 enhancement of VEGFR2 activation, suggesting that NRP1 and VEGFR2 also associate in a different, ligand-independent, non-extracellular domain way. Thus, we need a new model. Interactions of NRP1 with VEGFR2 via transmembrane (TM) and intracellular (IC) domain contacts are a strong possibility.\textsuperscript{9,15,48} Evidence of functional cytoplasmic interactions between VEGFR2 and NRP1 was presented by Prahst \textit{et al} in 2008. Blockade of VEGFR2 phosphorylation disrupts formation of VEGFR2-NRP1 complexes, and removal of the IC domain of NRP1 reduces the number of VEGFR/NRP/VEGFA165a complexes.\textsuperscript{49} If intracellular domain contacts can supplement or replace extracellular ligand bridging, then NRP1 can impact VEGF-A121a activation of VEGFR2. This mechanism may even occur in the absence of VEGF-A121a binding to NRP1, as it would be the interaction between VEGFR2 and NRP1 that would be key. Sequence-specific contacts at the transmembrane and intracellular domains in VEGF2-NRP interactions\textsuperscript{29,48-50} can provide a mechanistic interpretation for the Shraga-Heled \textit{et al} results demonstrating enhancement of VEGFR2 activation by VEGF-A165a, VEGF-A121a, and VEGF-A165aKF (a VEGF-A165a mutant with impaired VEGFR binding but intact NRP and HSPG binding).

Assembly via homo- and heterodimerization is common among integral membrane proteins as demonstrated for multiple receptor tyrosine kinase families.\textsuperscript{51} All 58 RTK transmembrane domains show a self-association propensity as measured by the TOXCAT assay.\textsuperscript{52} VEGFR2 homodimerizes in the absence and presence of VEGFs.\textsuperscript{50} VEGFRs form heterodimers as well: intact VEGFR2/VEGFR3 and VEGFR2/VEGFR1 dimers form as demonstrated by a number of biochemical techniques including PLA and co-immunoprecipitation.\textsuperscript{53-56} NRP1, though not an RTK, homodimerizes in the absence of VEGFs\textsuperscript{29}, and the transmembrane domain plays a role in NRP-NRP interactions. The NRP1 transmembrane domain includes two GxxxG dimerization motifs in the form of GxxxGxxxG, (where G is an amino acid with a small side chain such as Glycine, Serine or Alanine); this motif is long recognized to promote higher order structures of alpha-helical transmembrane domains.\textsuperscript{57,58} There is also evidence for the formation of VEGFR2-NRP1 dimers on endothelial cells, in the absence and presence of VEGFs. Prahst \textit{et al} and others have shown
by co-immunoprecipitation that NRP1 and VEGFR2 heterodimerize in the absence and presence of VEGFs. A number of studies have used other techniques to report evidence for spontaneously formed (pre-formed) VEGFR2-NRP1 complexes. In addition, NRP1 D320K (NRP1 with impaired VEGF binding) is found to be capable of regulating VEGFR2 activation, and NRP1 modulates VEGF2 levels independent of VEGF binding to NRP1. Dimerization propensity of NRP1 TM domains has been measured using reporter genes in ToxLuc and FRET assays in detergent micelles. Roth et al. demonstrated that the TM domain drives NRP1 homodimerization as mutation of the GxxxG motifs in the TM domains diminished NRP1-induced Sema3A activation. Thus, this motif is important for NRP1-involved oligomerization with VEGFR and Semaphorin family receptor complexes in the plasma membrane and biological function of NRP1 requires the GxxxGxxxG motif integrity. NRP1-NRP1 and NRP1-VEGFR2 complexes potentially modulate signaling upon differential binding to ligands and/or formation of distinct VEGFR2-NRP1 dimer conformations (dimer structures) upon binding to VEGF-A121a and VEGF-A165a (Fig. 3).

Conclusions

Binding of the VEGF-A165a C-terminal exon 8a-encoded domain to NRP1 has been captured via crystallography (Figure 6 of, Fig. 2 of). NRP1 binding to VEGF-A is isolated to C-terminal portion of VEGF-A165a and VEGF-A165a exon 8a encoded residues are indispensable for NRP1 binding. Crystallographic studies are not yet available for intact VEGF-A121a, but this ligand has the entire exon 5 and exon 8a encoded sequence intact and is shown by the SPR technique (cell-free) to bind NRP1, with lower affinity compared to VEGF-A165a and to NRP2 with similar affinity as NRP1. Modulation of VEGF-A121a-VEGFR2 signaling by the expression of (or interference with) NRP1 on cells in vitro suggests that these components are interacting (or at least interdependent), although it is also established that VEGF-A121a cannot form the extracellular VEGF2-VEGFR-NRP1 bridge that VEGF-A165a is capable of forming (or if a weak extracellular VEGF2-VEGFR2-NRP1 bridge exists, it can not be measured experimentally).

A VEGF-A165a mutant (VEGF-A165aKF) that binds to HSPGs and NRPs but not VEGFRs, demonstrates that ‘extracellular bridging’ of VEGFR2 and NRP1 by VEGF-A, previously thought central to the modulation of VEGFR2 signaling by NRP1, is not required for that modulation. Here, we proposed an alternate model for NRP modulation of VEGFR2 signaling, through transmembrane and intracellular association (specific contacts) rather than extracellular bridging (Fig. 3). Interestingly, although the evidence points to the ability of VEGF-A121a to bind NRP1 and NRP2 in a cell-free system, this model does not require that this binding interaction occur on the endothelial cells in vitro; rather than a VEGF2-VEGFR-NRP1 extracellular bridge, this would be a VEGF-VEGFR2-NRP1 complex, with the VEGF-A-VEGFR2 and/or VEGF-A-NRP binding extracellularly and the VEGFR2-NRP1 binding via transmembrane and intracellular domains.

Based on this proposed model, the interactions between VEGFR2 and NRP1 at the transmembrane and intracellular domains joins ligand binding specificity, HSPG interactions and priming of VEGFRs and NRPs towards VEGF-A121a binding, receptor dimerization propensity, and conformational changes in the VEGFR kinase domain as key regulators of active VEGF2-NRP1-VEGF-A signaling complexes on the cell surface (Fig. 3). If this interaction mechanism mediated by transmembrane domains and intracellular domains exists, it may be important for VEGF-A165a signaling as well.

Abbreviations

RTK Receptor Tyrosine Kinase
VEGF Vascular endothelial growth factor
ECD Extracellular domain
TMD Transmembrane domain
ICD Intracellular domain
TKD Tyrosine kinase domain
HBD heparin binding domain
GAGs Glycosaminoglycans
HSPG heparin sulfate proteoglycan
VEGFR Vascular endothelial growth factor receptor
NRP Neuropilin
PIGF Placenta growth factor
SEMA3A Semaphorin-3A
ECM Extracellular matrix
DSS Disuccinimidyl suberate
SPR Surface Plasmon Resonance
PLA proximity ligation assay
CO-IP co-immunoprecipitation
HUEs or HUVECs Human umbilical vascular endothelial cells
ECs Endothelial cells
PAECs or PAOECs Porcine aortic endothelial cells

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