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Identification of two novel alternatively spliced Neuropilin-1 isoforms

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Abstract

Neuropilin-1 (NRP1) is a coreceptor to a tyrosine kinase receptor for both the vascular endothelial growth factor (VEGF) family and semaphorin (Sema) family members. NRP1 plays versatile roles in angiogenesis, axon guidance, cell survival, migration, and invasion. NRP1 contains three distinct extracellular domains, a1a2, b1b2, and c. We report here the identification of two novel soluble human NRP1 isoforms, which we named s_{III}NRP1 and s_{IV}NRP1. These soluble NRP1 isoforms were generated by alternative splicing of the NRP1 gene, a common regulatory mechanism occurring in cell surface receptor families. Both s_{III}NRP1 and s_{IV}NRP1 contain a1a2 and b1b2 domains, but no c domain, and the rest of the NRP1 sequence. Additionally, s_{III}NRP1 is missing 48 amino acids within the C-terminus of the b2 domain. Both s_{III}NRP1 and s_{IV}NRP1 are expressed in human cancerous and normal tissues. These molecules are capable of binding to VEGF₁₆₅ and Sema3A. Furthermore, recombinant s_{III}NRP1 and s_{IV}NRP1 proteins inhibit NRP1-mediated MDA-MB-231 breast cancer cell migration. These results indicate the multiple levels of regulation in NRP1 function and suggest that these two novel NRP1 isoforms are useful antagonists for NRP1-mediated cellular activities.

Keywords

Neuropilin-1; VEGF receptor; Angiogenesis; Cell migration

Neuropilin-1 (NRP1),¹ first characterized in *Xenopus*, is a cell surface receptor that is important in both growth of blood vessels and guidance of neuronal axons [1–3]. The role of NRP1 in these processes is mediated by its interaction with numerous ligands, most notably, vascular endothelial growth factor (VEGF) in angiogenesis and semaphorin (Sema) in neuronal guidance [4,5]. NRP1 has no known enzymatic activity and therefore participates in signal transduction events through the complex formation with tyrosine kinase receptors. To carry out its axon guidance function, NRP1 binds to Sema3A or other class III semaphorins and

forms a complex with a coreceptor of the plexin family to form a functional Sema3 receptor [6,7]. To promote vessel growth, NRP1 interacts with several members of the VEGF family, including the 165-amino-acid isoform of VEGF-A (VEGF₁₆₅), VEGF-B, VEGF-E, and placental growth factor 2 but not diffuse VEGF-A isoform, VEGF₁₂₁ [5,8–10]. The biological effects of VEGF are mediated by a family of VEGF tyrosine kinase receptors, Flt-1 (VEGFR-1), Flk-1/KDR (VEGFR-2), and Flt-4 (VEGFR-3) [11,12]. NRP1 has been shown to enhance the binding of VEGF₁₆₅ to VEGFR-2 and increase endothelial cell chemotaxis [5]. The interaction of NRP1 with its various ligands and coreceptors has been shown to be important for the development of the nervous and vascular systems. NRP1-deficient mice die because of severe defects in the development of the cardiovascular system, vascularization, and peripheral nervous system [13,14]. NRP1 also plays important roles in tumor angiogenesis, growth, survival, and invasiveness [15]. For example, overexpression of NRP1 by tumor cells increased angiogenesis and tumor growth in a rat prostate carcinoma model [16]. Autocrine VEGF₁₆₅ was shown to increase cell survival in MDA-MB-231 breast carcinoma cells [17]. Treatment of MDA-MB-231 cells by an anti-NRP1 antibody or VEGF antisense oligonucleotides inhibited cell migration and invasion induced by the conditioned medium of fibroblast (NIH 3T3) cells [18].

NRP1 is known to exist as a membrane-bound form (full-length) as well as two soluble isoforms. The NRP1 gene consists of 17 exons and 16 introns. The full-length form of the protein contains all 17 exons, whereas two previously reported soluble NRP1 isoforms, s₁₂NRP1 and s₁₁NRP1, are created by reading through into introns [19,20]. The extracellular domain of full-length NRP1 is composed of CUB motifs homologous to complement components C1r/C1s (also called a1 and a2 domains), two domains that are homologous to coagulation factors V and VIII (also called b1 and b2 domains), and a Meprin A5 μ -phosphatase domain (also called the c domain) [21,22]. The 40-amino-acid positively charged carboxyl-terminal basic domain does not display homology to any known proteins [23]. The mRNA for s₁₂NRP1 contains the first 12 exons of the gene followed by a sequence derived from intron 12 and a polyadenosine (poly(A)) tail. The protein encoded by this mRNA contains the a1a2 and b1b2 domains and most of the b/c linker followed by 3 novel amino acids [20]. The mRNA for s₁₁NRP1, another soluble isoform, contains the first 11 exons of the gene followed by a sequence derived from intron 11 and a poly(A) tail. The resulting protein contains the a1a2 and b1b2 domains, followed by the portion of the b/c linker encoded by exon 11 and 83 novel amino acids [19]. Full-length NRP1 is expressed in endothelial cells and tumor cells [5,20], whereas s₁₂NRP1 and s₁₁NRP1 are expressed in a variety of tissues but not in endothelial cells [19,20]. s₁₂NRP1 and s₁₁NRP1 are thought to function as VEGF₁₆₅ antagonists [20]. s₁₂NRP1 has been shown to inhibit VEGF₁₆₅ binding to cells expressing NRP1 and also inhibit VEGF₁₆₅-induced tyrosine phosphorylation of VEGFR-2 *in vitro*. When overexpressed in a rat prostate carcinoma model, s₁₂NRP1 caused a higher percentage of apoptotic cells, hemorrhaging within the tumors, and a lower number of blood vessels [20]. The antagonistic effects of s₁₂NRP1 can be explained by the domains of the protein responsible for the competitive binding of various NRP1 ligands. Recent studies have shown that the a1a2 and b1 domains are required for Sema3A binding [24]. The VEGF₁₆₅ binding site is located in the b1b2 domains [25]. The s₁₂NRP1 and s₁₁NRP1 isoforms contain the entire a1a2 and b1b2 domains but not the c domain. Therefore, both soluble NRP1 proteins have the protein structure necessary to bind and sequester VEGF₁₆₅. Although the physiological effects of s₁₁NRP1 have not been studied, the protein would be expected to have biochemical and physiological properties similar to those of s₁₂NRP1 because of the domains present [19].

It seems possible that there could exist other soluble NRP1 isoforms that have not been characterized. Indeed, neuropilin-2 (NRP2), the homologue of NRP1, has a soluble isoform that is different in cDNA and protein structure from both s₁₂NRP1 and s₁₁NRP1 [19]. In this study, we report the discovery and characterization of two novel soluble NRP1 isoforms that

we named s_{III}NRP1 and s_{IV}NRP1. These two new NRP1 isoforms are naturally occurring and are generated by alternative splicing. The s_{III}NRP1 and s_{IV}NRP1 molecules displayed binding capacities to Sema3A and VEGF₁₆₅ similar to that of s₁₂NRP1. Furthermore, the soluble NRP1 isoforms including s₁₂NRP1 are able to inhibit the full-length NRP1-mediated MDA-MB-231 breast carcinoma cell migration.

Results

Identification of EST clones that encode novel soluble NRP1 isoforms

As depicted in Fig. 1A, previous studies report that human NRP1 exists as a full-length membrane receptor and two naturally occurring soluble receptors, s₁₂NRP1 and s₁₁NRP1, both generated by reading through into the intron of the NRP1 gene [19,20]. To determine whether there are other naturally occurring NRP1 isoforms, we searched the human EST clone database at the NCBI Web site and discovered two classes of sequences that encode two types of novel soluble NRP1 isoforms apparently generated by alternative splicing. One class of alternatively spliced soluble NRP1 EST clones contain full-length sequences upstream of the beginning of exon 10 (1614 of the full-length sequence). Exons 10 and 11 are deleted (position 1614 to 1864 of the full-length sequence), while exon 12 (position 1864 to 1924) is present. After exon 12, a sequence of 28 bp identical to the beginning of NRP1 intron 12 is present. This class of EST clones will be referred to as s_{III}NRP1 (Fig. 1B, top). The protein produced from this class of EST clones would contain all amino acids derived from exons 1–9 of the gene. It is missing the last 48 amino acids of the b2 domain and all domains between the b2 domain and the C terminus but contains 13 novel amino acids derived from a different reading frame of exon 12. The stop codon TAG was discovered within the exon 12 sequence (position 1904 of the full-length NRP1 sequence). Four different EST clones of this class were identified in the human EST clone database, Accession Nos. AA976114 derived from lung neuroendocrine carcinoid, AA987327 and AA973607 derived from lung carcinoid, and AI953301 derived from ovary normal epithelial tissue. Clones AA976114 and AI953301 were purchased from Incyte Genomics, Inc. (St. Louis, MO, USA) and completely sequenced. AA976114 was over 1 kb in length. Homology with the full-length NRP1 began at position 699 of the full-length NRP1 open reading frame (ORF) sequence. Thirty-two base pairs identical to NRP1 intron 12 were present before the poly(A) signal. In AI953301, 27 bp were present before the poly(A) signal. Thus, sequencing confirmed that a soluble NRP1 sequence missing exons 10 and 11 was present in EST clones derived from two different tissue types (lung neuroendocrine carcinoid and ovary normal epithelium).

Another class of alternatively spliced soluble NRP1 EST clones, which will be referred to as s_{IV}NRP1, was also found (Fig. 1B, bottom). In this class, NRP1 sequence was present before exon 11 (ORF position 1759) but not as far back as the stop codon. Exon 11 (position 1759 to 1864) was missing. Exon 12 was present (position 1864 to 1924). No exons after exon 12 were present. After exon 12, 28 bp identical to the beginning of intron 12 were present. The protein produced by this EST clone would be identical to full-length NRP1 for the first 586 residues (encoded by exons 1–10), would be missing the 35 amino acids encoded by exon 11, but would include the wild-type amino acids encoded by exon 12, followed by the sequence G I K derived from intron 12. The splicing out of exon 11 does not change the reading frame. The last 23 amino acids are the same as in the previously reported soluble NRP1 isoform, s₁₂NRP1 [20]. Two EST clones of this class were found: Accession No. BG120342 derived from a liver adenocarcinoma cell line and Accession No. BF939225 from adult prostate. An agar stab of bacterial transformed BG120342 was purchased. This clone was sequenced completely from the 5' end, and its authenticity was thus verified.

The novel soluble NRP1 isoforms are naturally occurring in normal and cancerous human tissues

To determine whether the two novel soluble NRP1 isoforms are expressed in human normal or cancerous tissues, we first performed RT-PCR analyses using PCR primers to amplify s_{III} NRP1, s_{IV} NRP1, or s_{12} NRP1 cDNA in the same reaction. The expression of all three isoforms was observed in normal human brain tissue, human glioma cell lines (Fig. 2A, top), and a human lung adenocarcinoma cell line (data not shown). The intensity of the bands for s_{III} NRP1 and s_{IV} NRP1 was consistently less than the band corresponding to s_{12} NRP1 (3- to 10-fold less than that of s_{12} NRP1 quantified using the expression level of β -actin for normalization), indicating low abundance of these two NRP1 isoforms in various tissues and cell lines. Next, the expression of s_{III} NRP1 and s_{IV} NRP1 was further examined using primers specific for each isoform. s_{III} NRP1 mRNA was consistently expressed in human glioma cell lines and normal brain (Fig. 2A, bottom), human glioma tissues at various WHO grades (Fig. 2B), human lung adenocarcinoma tissue, 784T (a human lung adenocarcinoma cell line), and 128-88T (a human squamous carcinoma cell line) (data not shown). The expression of s_{IV} NRP1 mRNA was found in most normal and cancerous human tissues that express s_{12} NRP1 and s_{III} NRP1 isoforms (Fig. 2C and data not shown). In addition to those tissues shown, s_{IV} NRP1 expression was also observed in normal human spleen and pancreas. The authenticity of the PCR product for each NRP1 isoform was completely verified by automated sequencing after subcloning the PCR products into a TA cloning vector (TOPO 2.1).

Expression of the two novel soluble NRP1 isoform proteins

We performed Western blot analyses to determine the protein expression of s_{12} NRP1, s_{III} NRP1, and s_{IV} NRP1 in total cell lysates prepared from various glioma and lung cancer cell lines shown in Fig. 2A using an anti-NRP1 antibody (N-18; Santa Cruz Biotechnology) that was raised against a peptide epitope at the N-terminus of NRP1 protein and recognizes both full-length and various soluble forms of NRP1 proteins. However, we could not detect the expression of the three NRP1 isoform proteins or full-length NRP1 protein in various human tumor cell lines. We also examined the expression of full-length NRP1 protein in these cells by Western blot analyses using several other anti-NRP1 antibodies that recognize full-length NRP1 protein (A-12 and C-19 from Santa Cruz Biotechnology, Ab-1 from Oncogene Science, or NP1ECD1A [26]). No NRP1 proteins could be detected by Western blot assays. This was probably due to protein expression in the cells that was below the detectable levels of the anti-NRP1 antibodies.

Next, after assembling each novel soluble NRP1 isoform into a mammalian expression vector (pcDNA 3.0) and completely verifying the DNA sequences of various expression constructs, we determined the expression of various soluble NRP1 isoforms in mammalian cells. We separately transfected COS-7 cells with expression vectors encoding s_{12} NRP1, s_{III} NRP1, or s_{IV} NRP1 proteins and assessed the expression of the soluble NRP1 isoforms using Western blot analyses. As shown in Fig. 3, the NRP1 isoform proteins were expressed in the COS-7 cells as s_{12} NRP1 (90 kDa), s_{III} NRP1 (60 kDa), or s_{IV} NRP1 (67 kDa).

The novel soluble NRP1 isoforms bind natural NRP1 ligand VEGF₁₆₅ and Sema3A

We next determined whether the novel NRP1 isoforms are capable of binding the NRP1 ligand VEGF₁₆₅. Recent studies have shown that a recombinant membrane-bound NRP1 variant with a deleted b2 domain had decreased VEGF₁₆₅ binding with no effects on Sema3A binding [24]. However, another recombinant NRP1 variant composed of the b1b2 domain only and missing 30 amino acids at the C terminus of the b2 domain had no binding affinity for VEGF₁₆₅ [25]. Interestingly, s_{III} NRP1 contains a1a2 and b1b2 domains missing 48 amino acids at the C terminus of the b2 domain, whereas s_{IV} NRP1 has intact a1a2 and b1b2 domains. Therefore, we hypothesized that s_{III} NRP1 may have decreased VEGF₁₆₅ binding and that

s_{IV} NRP1 would have unaffected binding for VEGF₁₆₅. As expected, similar to s_{12} NRP1, s_{IV} NRP1 is capable of binding VEGF₁₆₅ (Fig. 4A). However, s_{III} NRP1 was also found to be able to bind VEGF₁₆₅ (Fig. 4A). In addition, we assessed the abilities of s_{12} NRP1, s_{III} NRP1, and s_{IV} NRP1 to bind Semaphorin 3A by performing similar assays. Since s_{III} NRP1 and s_{IV} NRP1 contain a1a2 and b1 domains necessary for binding to Semaphorin 3A, as expected from previous studies [24], both soluble NRP1 isoforms were capable of binding Semaphorin 3A (data not shown).

To determine whether the binding affinity for VEGF₁₆₅ is different for s_{III} NRP1 relative to that displayed by s_{12} NRP1, we performed a semiquantitative binding assay using normalized concentrations of each soluble NRP1 isoform. Various concentrations (12.0, 6.0, 2.4, or 1.2 nM) of s_{III} NRP1 or s_{12} NRP1 were mixed with VEGF₁₆₅-AP at a fixed VEGF₁₆₅-AP protein concentration of 3.0 nM [25,27]. Soluble NRP1 bound to VEGF₁₆₅-AP was coimmunoprecipitated with an anti-AP antibody conjugated to agarose beads and analyzed by Western blot analysis using an anti-NRP1 antibody (N-18; Santa Cruz Biotechnology) reactive against all soluble NRP1 isoforms. There was no significant difference in binding for VEGF₁₆₅-AP between s_{III} NRP1 and s_{12} NRP1 (Fig. 4B). Similar amounts of s_{12} NRP1 and s_{III} NRP1 proteins were coprecipitated with the VEGF₁₆₅-AP fusion proteins at each dilution. Neither s_{12} NRP1 nor s_{III} NRP1 was detected at 1.2 nM dilution (data not shown). We also found that VEGF₁₆₅ binding for s_{IV} NRP1 was similar to that displayed by s_{12} NRP1 (data not shown). Thus, these data suggest that although the s_{III} NRP1 and s_{IV} NRP1 isoforms lack certain amino acids present in s_{12} NRP1, these two novel soluble isoforms have similar capacities for binding to their cognate ligands.

The novel soluble NRP1 isoforms prevent VEGF₁₆₅ from binding to membrane-bound VEGF receptors

To evaluate further the binding properties of the novel soluble NRP1 isoforms, we performed competition assays to determine whether each soluble NRP1 isoform can inhibit VEGF₁₆₅-AP binding to cell surface VEGF receptors (Figs. 5A and 5B). Human MDA-MB-231 breast carcinoma cells were used since these cells lack VEGFR-2 [28] but express NRP1 at high levels on their surface [5,17]. As shown in Fig. 5A, the binding of VEGF₁₆₅-AP to the cells measured by AP activity was concentration-dependent in the assay. However, when various soluble NRP1 isoforms and Ig-Basic-AP (a negative control) are separately included in the binding assays, the novel soluble NRP1 isoforms, but not Ig-Basic-AP, prevented VEGF₁₆₅-AP from binding to the cells to an extent similar to that displayed by s_{12} NRP1 (Fig. 5B). The amount of AP activity bound to the MDA-MB-231 cells as a percentage of the control was 12, 13, and 24% for s_{12} NRP1, s_{IV} NRP1, and s_{III} NRP1, respectively, at the highest concentration of soluble NRP1 protein (24.0 nM). These data suggest that s_{III} NRP1, s_{IV} NRP1, and s_{12} NRP1 are capable of competing with VEGF receptors for VEGF₁₆₅ binding.

The novel soluble NRP1 isoforms act as NRP1 antagonists

Since the novel s_{III} NRP1 and s_{IV} NRP1 isoforms can bind and sequester VEGF₁₆₅ as effectively as s_{12} NRP1 does, we postulated that they would be able to antagonize the biological functions of full-length NRP1 in promoting cell migration. It has been previously reported that autocrine VEGF₁₆₅ signaling is responsible for the invasive behavior of MDA-MB-231 breast carcinoma cells. The proposed mechanism is that autocrine VEGF₁₆₅ prevents the MDA-MB-231 cells from entering apoptosis and promotes NRP1-mediated cancer cell migration [18]. We determined whether the two novel soluble NRP1 isoforms inhibit NRP1-mediated human breast cancer cell migration. As shown in Fig. 6, the two novel soluble NRP1 isoforms, but not Ig-Basic-AP, were able to inhibit MDA-MB-231 cell migration toward NIH 3T3 CM to an extent similar to that of s_{12} NRP1. MDA-MB-231 cell migration was inhibited 51% by s_{12} NRP1, 52.5% by s_{IV} NRP1, and 55.5% by s_{III} NRP1, respectively, when calculated as a percentage of the control at the highest concentration of soluble NRP1 protein. These data

demonstrate that the two novel soluble NRP1 isoforms can act to sequester VEGF₁₆₅, thus inhibiting autocrine-mediated breast cancer cell migration.

Discussion

NRP1 plays critical roles in neuronal guidance, cardiovascular development, angiogenesis, and tumor progression. Studies show that regulation of NRP1 function in these processes is at multiple levels. One mechanism is the modulation of NRP1 gene expression. The expression of full-length NRP1 has been found in normal and cancerous tissues and in endothelial, hematopoietic, and cancerous cell lines [15]. NRP1 is regulated by tumor necrosis factor- α [29] or VEGF [30] in EC and by epidermal growth factor in human gastric or pancreatic cancer cells [31,32]. It is likely that the regulation of NRP1 by these growth factors is mediated by transcription factors such as Sp1 [33]. Another mechanism for the regulation of NRP1 function is the expression of naturally occurring soluble NRP1 isoforms, which often act as antagonists. Klagsbrun and colleagues have previously reported two naturally occurring soluble NRP1 isoforms, s₁₂NRP1 and s₁₁NRP1. Both s₁₂NRP1 and s₁₁NRP1 are generated during pre-mRNA processing by reading through into the introns of the NRP1 gene [19,20]. The s₁₂NRP1 and s₁₁NRP1 isoforms are expressed in multiple normal human tissues [19], whereas s₁₂NRP1 was also found in cancerous tissues [5,15]. Our results show that two novel NRP1 isoforms, s_{III}NRP1 and s_{IV}NRP1, are produced by an alternative splicing mechanism that often occurs in cell surface receptors such as sFlt-1 [34], NRP2 [19], and FGFR [35]. Although s_{III}NRP1 and s_{IV}NRP1 were initially found in several human EST clones through searching the NCBI human EST database, these novel isoforms are indeed naturally occurring. s_{IV}NRP1 was present in most normal human and cancerous tissues that were analyzed, whereas s_{III}NRP1 was detected only in cancerous and normal brain tissues and not in other normal human tissues that were analyzed. However, our semiquantitative RT-PCR analyses using β -actin as an internal control showed that the expression levels of s_{III}NRP1 and s_{IV}NRP1 were lower than that of s₁₂NRP1 in the tissues and cells analyzed (Fig. 2A). We detected the protein synthesis of full-length NRP1 in human U87MG glioma and MDA-MB-231 breast cancer cells using an anti-NRP1 antibody against C-terminal amino acids (clone C-19; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). However, this antibody and several other anti-NRP1 antibodies including commercially available and two well-characterized anti-NRP1 antibodies [4,16] could not detect the expression of s₁₂NRP1, s_{III}NRP1, and s_{IV}NRP1, probably due to the lack of C-terminal amino acid sequences in these soluble NRP1 isoforms or low sensitivities of the antibodies in this type of analyses (data not shown). This suggests that the protein expression of these soluble NRP1's was below the detectable levels by direct Western blot analyses. Low levels of expression of s_{III}NRP1 and s_{IV}NRP1 isoforms also suggest that these two soluble NRP1 molecules may not achieve functional inhibitory levels under physiological and pathological conditions. This would also undermine the relevance of these NRP1 splice variants in various pathophysiological processes. Moreover, we found that the expression level of s_{III}NRP1 mRNA was higher in GBM (WHO grade IV) than in lower grade gliomas when the expression level of s_{III}NRP1 was normalized to the expression of β -actin in RT-PCR analyses (Fig. 2B and data not shown). At present, we cannot explain why the level of s_{III}NRP1 mRNA in malignant GBM was higher than that in their less aggressive counterparts. A possible explanation is that in GBM increased levels of VEGF expression are much higher than the elevated levels of its antagonists such as sFlt-1 and sNRP1. Active tumor angiogenesis has otherwise been ensured by predominant bioavailable VEGF proteins within the tumors during the glioma progression [36].

The binding capacity of NRP1 molecules to Sema3A and VEGF has been studied in great detail. It has been demonstrated that the extracellular domains of the NRP1 molecule, a1a2 and b1, bind to Sema3a, while the b1b2 domains are the docking site for VEGF₁₆₅ [37–39]. Recently, two reports further dissected the binding properties of NRP1 to VEGF₁₆₅ using

recombinant NRP1 variants that contained various extracellular domains [24,25]. While one study showed that the a1a2 and b1 domains are required for Semaphorin 3A binding [24], the other investigation demonstrated that the VEGF₁₆₅ binding site is located in the b1b2 domains [25]. However, there were slightly different observations between these two studies in terms of VEGF₁₆₅ binding. When the b2 domain was deleted in a recombinant soluble NRP1 that has only a1a2 and b1 domains, no VEGF₁₆₅ binding was found [25]. In contrast, when the b2 region was removed in a membrane-bound NRP1 variant that contained a1a2, b1, and c domains, the capacity of binding to VEGF₁₆₅ was only partially reduced [24]. This suggests that although the a1a2 domain by itself does not bind to VEGF₁₆₅, the a1a2 domain is able to modulate the binding of VEGF₁₆₅ to b1b2. This conclusion was supported by the observation that the a1a2/b1b2 recombinant proteins were 50 times more potent than the b1b2 domain alone in inhibiting VEGF₁₆₅ binding to cell surface receptors. A truncated b1b2 domain lacking 30 amino acids from its C-terminus and also missing the a1a2 domains did not bind to VEGF₁₆₅ [25]. The binding properties of s_{III}NRP1 and s_{IV}NRP1 proteins to Semaphorin 3A and VEGF₁₆₅ assessed under the indicated conditions (Figs. 4 and 5) corroborate these conclusions. Despite slightly differing structures compared to previously discovered isoforms, the novel s_{III}NRP1 and s_{IV}NRP1 isoforms have biochemical and biological properties similar to those displayed by s₁₂NRP1 and s₁₁NRP1 molecules. Both s_{III}NRP1 and s_{IV}NRP1 bind to Semaphorin 3A and VEGF₁₆₅ with affinities similar to those displayed by the s₁₂NRP1 protein containing the entire a1a2 and b1b2 domains [20]. Although the s_{III}NRP1 protein has more amino acids missing (48 aa) from its b2 domain C-terminus than the truncated b1b2 NRP1 variant lacking 30 amino acids and also the a1a2 domain [25], the s_{III}NRP1 isoform binds VEGF₁₆₅ at a capacity similar to that of s_{IV}NRP1 as well as s₁₂NRP1 proteins. This may suggest that the a1a2 domains present in s_{III}NRP1 indeed contribute to VEGF₁₆₅ binding. Moreover, due to expression levels of s_{III}NRP1 and s_{IV}NRP1 proteins in our experimental system, these results may not accurately reflect the binding affinities of the various NRP1 splice variants.

Previous studies have also shown that either naturally occurring s₁₂NRP1 isoform or a recombinant a1a2 b1b2 NRP1 variant could inhibit VEGF₁₆₅ binding to cell surface VEGF receptors and suppress the stimulatory activities of VEGF₁₆₅ on cell migration and proliferation [20,25]. Since our data suggest that s_{III}NRP1 and s_{IV}NRP1 are capable of binding to VEGF₁₆₅, we predicted that the presence of either of these novel NRP1 isoforms would inhibit NRP1-mediated cell migration. The inhibition of human MDAMB-231 breast carcinoma cell migration by s_{III}NRP1 and s_{IV}NRP1 supports this hypothesis. Furthermore, since the MDA-MB-231 cells express only NRP1 and VEGFR-1 at their cell surface [17,18], the presence of various soluble NRP1 isoforms suppressed NRP1-mediated breast cancer migration.

One of the important functions of soluble cell surface receptors is to sequester the cognate ligands of the full-length receptor, thus inhibiting the physiological processes mediated by the full-length receptor. For example, when overexpressed in a rat prostate carcinoma AT2.1 cell model, s₁₂NRP1 has been shown to increase apoptosis and decrease blood vessel number and integrity, thus strongly inhibiting rat prostate tumor angiogenesis and progression [20]. We attempted to demonstrate that s_{III}NRP1 and s_{IV}NRP1 have similar inhibitory effects displayed by s₁₂NRP1 on tumor angiogenesis and growth. We separately introduced s₁₂NRP1, s_{III}NRP1, and s_{IV}NRP1 isoform or Ig-Basic-AP cDNAs into different types of mammalian expression vectors and expressed them in human U87MG glioma cells individually to determine their inhibitory functions on angiogenesis and tumor growth in our U87MG glioma xenograft models in nude mice [18,26,40,41]. However, although strong expression of each of these soluble NRP1 proteins was detected in the transfected U87MG cells 3 days after the transfection, we failed to isolate any drug-resistant U87MG cell clones that stably expressed one of these soluble NRP1 isoforms. Because NRP1 has been shown to mediate autocrine survival effects displayed by VEGF [17], it is possible that overexpression of one of the three soluble NRP1 isoforms in U87MG glioma cells disrupted such antiapoptotic function

mediated by the full-length NRP1 receptor, thus causing the death of U87MG glioma cells that expressed exogenous soluble NRP1 isoform proteins.

In summary, we have identified, expressed, and initially characterized two novel alternatively spliced soluble human NRP1 isoforms that we have designated s_{III}NRP1 and s_{IV}NRP1. These two NRP1 spliced isoforms were initially found in the NCBI human EST database and they are naturally occurring in normal human and cancerous tissues. In addition, our data show that despite slight structural differences in the b2 domain and linker region between s_{III}NRP1, s_{IV}NRP1, and the previously reported s₁₂NRP1 isoform, the three NRP1 variants showed similar abilities to bind to their cognate ligands, Sema3A and VEGF₁₆₅, and inhibited NRP1-mediated MDA-MB-231 breast cancer cell migration. Our results demonstrate that s_{III}NRP1 and s_{IV}NRP1 are functional antagonists against VEGF receptors, suggesting that these soluble NRP1 molecules could be useful pharmacological entities to regulate angiogenesis and neuronal development during physiological and pathological processes.

Materials and methods

Cell lines and reagents

Human glioma cell lines U251MG, U87MG, U373MG, and T98G; human embryonic kidney (HEK) 293T; human breast carcinoma MDA-MB-231 cells; and COS-7 cells were from American Type Culture Collection (ATCC; Manassas, VA, USA). Human glioma cell lines LN2-308, LN18, LN235, LN428, and LN443 were previously described [42]. COS-7 cells were cultured in DMEM with glutamine (2.0 mM; Mediatech, Herndon, VA, USA), 10% FBS (Sigma, St. Louis, MO, USA), and 1% penicillin/streptomycin at 37°C in 5% CO₂. Glioma cell lines, HEK 293T cells, and MDA-MB-231 cells were cultured under the same conditions except that FBS from HyClone (Salt Lake City, UT, USA) was used. The following reagents were used for this study: goat polyclonal anti-NRP1 antibody (N-18, 1:300), mouse monoclonal anti-NRP1 antibody (A-12, 1.0 µg/ml; Santa Cruz Biotechnology), rabbit anti-NRP1 antibody (Ab-1, 1:500; Oncogene Sciences, San Diego, CA, USA), rabbit anti-NRP1 antibody (NP1ECD1A [26]), rabbit anti-goat immunoglobulins conjugated to horseradish peroxidase (1:500, P0449; DAKO, Carpinteria, CA, USA), monoclonal anti-human placental AP antibody conjugated to agarose beads (A2080; Sigma), AP Assay Reagent A (Q501), cell lysis buffer (Q504; GenHunter, Nashville, TN, USA), FastStart *Taq* DNA polymerase (Roche, Indianapolis, IN, USA), Lipofectamine PLUS, Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), total RNA from normal human tissues (Clontech, Palo Alto, CA, USA), VEGF₁₆₅-AP/pCEP4 DNA construct from Dr. J. Raper, University of Pennsylvania (Philadelphia, PA, USA), Sema3A-AP/pCEP4 DNA construct from Dr. A. Kolodkin, Johns Hopkins University (Baltimore, MD, USA). All other reagents were from Invitrogen, Sigma, or Fisher Scientific (Hanover Park, IL, USA).

Search of the NCBI database for EST clones that encode human NRP1

The NCBI human EST clone database was searched by BLAST using the entire coding sequence of full-length NRP1. We looked for EST clone sequences encoding human NRP1 cDNA and also containing polyadenylation signals. Six clones with the following accession numbers were identified: BF939225, BG120342, AA976114, AI953301, AA987327, AA973607. The cDNA for the discovered IMAGE clones with Accession Nos. AA976114, AI953301, and BG120342 were purchased in bacteria transformed agar stabs from Incyte Genomics, Inc. (St. Louis, MO, USA) or ATCC. DNA from each EST clone was confirmed by full-length automated sequencing.

Assembly of the full-length and soluble NRP1 isoforms

First, full-length NRP1 was cloned by RT-PCR from the total RNA isolated from human U87MG glioma cells. Two RT-PCR products containing NRP1 bases 1–935 and 880–2772 were produced and then TA separately cloned into a TOPO 2.0 vector (Invitrogen). To assemble the full-length NRP1 sequence, NRP1 bases 1–904, cut with *Bam*HI and *Pst*I, and NRP1 bases 905–2772, cut with *Pst*I and *Sac*I, were ligated into the *Bam*HI/*Sac*I cut of the pBluescript SK⁽⁻⁾ vector (Stratagene, San Diego, CA, USA). *Pst*I was used to cut at the naturally occurring site at 904 of NRP1. To assemble a s₁₂NRP1 construct, the sequence between base 880 and the end of exon 12 (1924) was amplified by PCR, with the downstream primer introducing the intron-derived amino acids (GIK), a stop codon, and a *Sac*I and an *Eco*RI site. Bases 1–904 digested with *Bam*HI and *Pst*I and the PCR product of the C-terminal portion of s₁₂NRP1 digested with *Pst*I and *Sac*I were then ligated into the *Bam*HI/*Sac*I cut of pBluescript SK⁽⁻⁾ to assemble the complete s₁₂NRP1 sequence. Both s_{III}NRP1 and s_{IV}NRP1 were then assembled. For each isoform, the 5' portion of full-length NRP1 was added since the EST clones did not contain the 5' portion of NRP1. For s_{III}NRP1, AA976114 was used for cloning. The construct was first assembled in pBluescript SK⁽⁻⁾ by PCR using primers upstream, 5'-CTTCCCAGTATAGCACC-3' (880–896), and downstream, 5'-TATAGAATTCTGTACATTTTCGTATTTTA-3', specific for intron 12 sequence and including an *Eco*RI restriction site. The PCR product digested with *Pst*I and *Eco*RI and the first 904 bases of the full-length NRP1 digested with *Bam*HI and *Pst*I were then ligated together into a *Bam*HI/*Eco*RI-digested pBluescript SK⁽⁻⁾. s_{IV}NRP1 was also first assembled in pBluescript SK⁽⁻⁾. BG120342 was first cut with *Sal*I and *Not*I, followed by *Bsp*HI, at the naturally occurring site at 1531 of the NRP1 full-length sequence. This digestion yielded a fragment containing the 3' region of s_{IV}NRP1 (the alternatively spliced region) including the poly(A) stretch. The s_{IV}NRP1 construct was assembled by ligating the pBluescript SK⁽⁻⁾ vector digested by *Bam*HI and *Not*I, the first 1531 bp of NRP1, and the EST clone sequence containing the 3' region of s_{IV}NRP1 cDNA. After assembly, full-length NRP1, s₁₂NRP1, s_{IV}NRP1, and s_{III}NRP1 were subcloned into a pcDNA3.0 vector (Invitrogen). Later, to remove the poly(A) tail included in the EST clone and increase mammalian expression, s_{IV}NRP was resubcloned into the pcDNA 3.0 vector. This was done using PCR amplification as was described in the assembly of s_{III}NRP1. DNA sequences of all four expression constructs, full-length NRP1, s₁₂NRP1, s_{IV}NRP1, and s_{III}NRP1 in pcDNA3.0, were completely verified by automated sequencing. No changes in amino acid sequences of the various NRP1 proteins were found.

Determination of mRNA expression of the soluble isoforms by RT-PCR

Total RNA was isolated from various snap-frozen human tumor tissues using the TRIZOL reagent (Invitrogen/Life Technologies). Human glioma tissues were from Dr. R. Nishikawa, Saitama Medical School (Saitama, Japan). Human lung cancer tissues were from Dr. J. Siegfried, University of Pittsburgh Cancer Institute. Identities and grades of glioma and lung cancer tissues were separately verified using hematoxylin and eosin-stained tumor tissue sections by pathologists at Saitama Medical School and the University of Pittsburgh. Total RNA from various normal human tissues was purchased from Clontech. Reverse transcriptase reactions were performed with RT and without RT. PCR was performed on the various RT reactions to determine the expression of each isoform. Primers were designed either to be specific for each isoform or to amplify s₁₂NRP-1, s_{III}NRP-1, and s_{IV}NRP-1 in the same reaction. The downstream primer (5'-TATAGAATTCTGTACATTTTCGTATTTTA-3', matching from 9 to 28 bp from the 5' end of intron 12) was used for all PCR. The upstream primer was used to cause the specificity of each reaction. To amplify s₁₂NRP-1, s_{III}NRP-1, and s_{IV}NRP-1 simultaneously, the upstream primer (5'-GTGTTTCATGAGGAAGTTC-3', nucleotide positions 1528–1545) was used (open reading frame position 1528–1545, within exon 9). To amplify s_{III}NRP-1 specifically, the upstream primer (5'-GCAAACGCAAGGCGAAGGT-3', 5' end at NRP1 open reading frame position 1597) was

designed across exons 9 and 12 with 17 bp in exon 9 and 2 bp in exon 12. The identity of the 114-bp s_{III}NRP-1 PCR product was completely verified by cloning into a TOPO 2.1 vector (Invitrogen) and automated sequencing. To amplify s_{IV}NRP-1 specifically, the upstream primer (5'-GGGCTGTGAAGTGAAGGTG-3', 5' end at NRP1 open reading frame position 1742) was designed across exons 10 and 12, with 17 bp in exon 10 and 3 bp in exon 12. Primers for human β -actin (5'-CGGGAAATCGTGCGTGACAT-3', upstream, and 5'-GGAGTTGAAGGTAGTTTCGTG-3', downstream) were included in some reactions at a 15-fold lower primer concentration than NRP1 primers. PCR conditions for reactions amplifying all three isoforms and reactions specific for s_{III}NRP-1 were 95°C for 4 min, (95°C for 30 s, 56°C for 60 s or 58°C for 50 s, 72°C for 30 s) for 40 cycles and for s_{IV}NRP-1, 95°C for 4 min (95°C for 1 min, 58°C for 1 min, 72°C for 1 min) for 35 cycles and then 72°C for 10 min. To quantify the relative levels of gene expression among different NRP1 isoforms, the captured images of gel electrophoresis were imported into the Image Pro Plus program (version 4.1, Media Cybernetics, Silver Spring, MD, USA) and analyzed.

Western blot analyses

The proteins of cell lysates or CM were separated on 7.5% SDS polyacrylamide gels and then transferred to a nitrocellulose membrane. The rinsed and blocked membranes were then incubated with a goat polyclonal anti-NRP1 antibody (1:300, N-18; Santa Cruz) at room temperature for 1 h. The blot was washed and probed with a rabbit anti-goat antibody, conjugated with horseradish peroxidase (DAKO), at room temperature for 1 h. The blot was washed again and developed with enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA).

Expression of s₁₂NRP1, s_{III}NRP1, and s_{IV}NRP1 in mammalian cells

s_{III}NRP1, s_{IV}NRP1, and s₁₂NRP1 constructs in pcDNA 3.0 vectors were separately transfected into COS-7 cells using the Lipofectamine PLUS reagent. The cells were lysed 48 h posttransfection. Expression of the expected sized proteins was confirmed by immunoblotting. To collect CM containing secreted proteins used in binding experiments, expression constructs of s_{III}NRP1, s_{IV}NRP1, and s₁₂NRP1 as well as Sema3A-AP, VEGF₁₆₅-AP, or Ig-Basic-AP fusion proteins were transfected into HEK 293T cells separately by calcium phosphate precipitation as previously described [39]. The medium was changed after transfection with serum-containing medium. For serum-containing CM, the CM was collected after the cells were allowed to grow for an additional 48 h. For serum-free CM, the cells were allowed to recover for 24 h in the presence of serum and then grown for an additional 48 h in medium without serum.

Coimmunoprecipitation/binding assay

The binding capacities of s_{III}NRP1, s_{IV}NRP1, and s₁₂NRP1 to Sema3A-AP or VEGF₁₆₅-AP were determined by immunoprecipitation for the ligand (Sema3A-AP or VEGF₁₆₅-AP) followed by immunoblotting for the soluble NRP1 receptors. To assess abilities of binding to each ligand, equal volumes of the various soluble NRP1's containing CM (with serum) were mixed with VEGF₁₆₅-AP, Sema3A-AP, or Ig-Basic-AP containing CM (with serum). As negative controls, soluble NRP1 containing CM was mixed with CM from mock (no DNA expression construct)-transfected cells. Protease inhibitors (100 μ g/ml PMSF, 1.5 μ g/ml leupeptin, 1.0 μ g/ml pepstatin A, 1.0 μ g/ml aprotinin) were added to each mixture. The mixtures of the CM were rotated at 4°C for 4 h for VEGF₁₆₅-AP binding or 6 h for Sema3A-AP binding. Then, 50 μ l of a 50/50 slurry of an anti-human placental AP antibody conjugated to agarose beads was added. The CM was rotated for an additional 6 h at 4°C for VEGF₁₆₅-AP binding or 12 h at 4°C for Sema3A-AP binding. The beads were spun down and then washed with 1

ml of cold PBS. The samples were then eluted in sample buffer, separated on a 7.5% SDS–polyacrylamide gel, and immunoblotted for NRP1 as described above.

To perform semiquantitative binding assays of s_{III}NRP1 or s_{IV}NRP1 and VEGF₁₆₅, CM containing VEGF₁₆₅-AP was added at a final concentration of 3.0 nM. Recombinant s₁₂NRP1, s_{III}NRP1, s_{IV}NRP1, or Ig-Basic-AP proteins were added separately at concentrations of 12.0, 6.0, 2.4, and 1.2 nM. CM from mock-transfected cells was used in negative controls instead of VEGF₁₆₅-AP CM and also added to bring the volume up to 6 ml in other reactions. CM was rotated, immunoprecipitated, and immunoblotted as described above. The amount of the recombinant s_{III}NRP1, s_{IV}NRP1, s₁₂NRP1, or Ig-Basic-AP protein in each CM was normalized by SDS–polyacrylamide gel electrophoresis, followed by Coomassie blue staining. A known amount of bovine serum albumin was used as a standard for protein quantification. The concentration of VEGF₁₆₅-AP in the CM was calculated by assaying the AP activity with Gen-Hunter AP Assay Reagent A, yielding a concentration in U/ml, and then using the activity of pure human placental AP of 1500 U/mg to calculate the VEGF₁₆₅-AP molar concentration. One unit of AP hydrolyzes 1.0 μmol/min *p*-nitrophenyl phosphate at 37°C.

Cell-based receptor binding assay

Binding of VEGF₁₆₅-AP fusion proteins to cell-bound VEGF receptors was performed using human MDA-MB-231 breast carcinoma cells, which express NRP1 at high levels [5], according to the manufacturer's protocol (GenHunter Corp.). Briefly, VEGF₁₆₅-AP, Sema3A, or Ig-Basic-AP fusion proteins were preincubated in the presence of 1.0 μg/ml heparin for 30 min on ice prior to cell binding. The cell-binding assay was carried out in 12-well plates for 2 h at 4°C with gentle agitation followed by five washes with ice-cold PBS. Cells were lysed with cell lysis buffer (GenHunter). The endogenous AP activities were inactivated by incubating the cell lysates at 65°C for 10 min. Cell-associated VEGF₁₆₅-AP or Ig-Basic-AP was quantified by measuring the generation of yellow color at 420 nm using *p*-nitrophenyl phosphate as a substrate (AP Assay Reagent A; GenHunter). In the competition assay, VEGF₁₆₅-AP was preincubated with various soluble forms of NRP1 or Ig-Basic-AP in the presence of 1.0 μg/ml heparin for 30 min on ice, followed by incubation with cells for 2 h at 4°C and quantitation of cell-bound AP activity as described above.

Chemotaxis assay

The cell migration assay was carried out in Boyden chambers as previously reported [18]. Briefly, MDA-MB-231 cells were harvested with trypsin and resuspended in DMEM containing 0.1% BSA, 1.0 μg/ml heparin, and 20.0 μg/ml ZVAD-FMK. The inhibitory effects of a mouse monoclonal anti-NRP1 antibody (A-12, 1.0 μg/ml; Santa Cruz) or various soluble forms of NRP1 were determined by preincubating cells with the indicated reagents for 30 min on ice. Cells incubated with antibody or soluble forms of NRP1 or Ig-Basic-AP were placed in the upper chamber and the lower chamber was filled with serum-free CM from NIH 3T3 cells as a chemoattractant. The assembled Boyden chambers were incubated at 37°C in 5% CO₂ for 4 h. At the end of the incubation period, cells were fixed and stained. Nonmigrating cells on the upper surface were removed. The number of migrating cells was quantified by counting 10 random high-powered fields (200× total magnification) per filter.

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References

- [1]. Neufeld G, Cohen T, Shrager N, Lange T, Kessler O, Herzog Y. The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. *Trends Cardiovasc. Med* 2002;12(1):13–19. [PubMed: 11796239]
- [2]. Puschel AW. The function of neuropilin/plexin complexes. *Adv. Exp. Med. Biol* 2002;515:71–80. [PubMed: 12613544]
- [3]. Takagi S, Tsuji T, Amagai T, Takamatsu T, Fujisawa H. Specific cell surface labels in the visual centers of *Xenopus laevis* tadpole identified using monoclonal antibodies. *Dev. Biol* 1987;122(1):90–100. [PubMed: 3297854]
- [4]. Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD. Neuropilin is a semaphorin III receptor. *Cell* 1997;90(4):753–762. [PubMed: 9288754]
- [5]. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;92(6):735–745. [PubMed: 9529250]
- [6]. Takahashi T, Fournier A, Nakamura F, Wang LH, Murakami Y, Kalb RG, Fujisawa H, Strittmatter SM. Plexin–neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* 1999;99(1):59–69. [PubMed: 10520994]
- [7]. Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* 1999;99(1):71–80. [PubMed: 10520995]
- [8]. Migdal M, Huppertz B, Tessier S, Comforti A, Shibuya M, Reich R, Baumann H, Neufeld G. Neuropilin-1 is a placenta growth factor-2 receptor. *J. Biol. Chem* 1998;273(35):22272–22278. [PubMed: 9712843]
- [9]. Makinen T, Olofsson B, Karpanen T, Hellman U, Soker S, Klagsbrun M, Eriksson U, Alitalo K. Differential binding of vascular endothelial growth factor B splice and proteolytic isoforms to neuropilin-1. *J. Biol. Chem* 1999;274(30):21217–21222. [PubMed: 10409677]
- [10]. Wise LM, Veikkola T, Mercer AA, Savory LJ, Fleming SB, Caesar C, Vitali A, Makinen T, Alitalo K, Stacker SA. Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. *Proc. Natl. Acad. Sci. USA* 1999;96(6):3071–3076. [PubMed: 10077638]
- [11]. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 1999;13(1):9–22. [PubMed: 9872925]
- [12]. Ferrara N. Vascular endothelial growth factor: molecular and biological aspects. *Curr. Top. Microbiol. Immunol* 1999;237:1–30. [PubMed: 9893343]
- [13]. Kitsukawa T, Shimizu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T, Fujisawa H. Neuropilin– semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* 1997;19(5):995–1005. [PubMed: 9390514]
- [14]. Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, Yagi T, Fujisawa H. A requirement for neuropilin-1 in embryonic vessel formation. *Development* 1999;126(21):4895–4902. [PubMed: 10518505]
- [15]. Soker S. Neuropilin in the midst of cell migration and retraction. *Int. J. Biochem. Cell Biol* 2001;33(4):433–437. [PubMed: 11312112]
- [16]. Miao HQ, Lee P, Lin H, Soker S, Klagsbrun M. Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. *FASEB J* 2000;14(15):2532–2539. [PubMed: 11099472]
- [17]. Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G, Mercurio AM. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res* 2001;61(15):5736–5740. [PubMed: 11479209]

- [18]. Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res* 2002;62(24):7203–7206. [PubMed: 12499259]
- [19]. Rossignol M, Gagnon ML, Klagsbrun M. Genomic organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. *Genomics* 2000;70(2):211–222. [PubMed: 11112349]
- [20]. Gagnon ML, Bielenberg DR, Gechtman Z, Miao HQ, Takashima S, Soker S, Klagsbrun M. Identification of a natural soluble neuropilin-1 that binds vascular endothelial growth factor: in vivo expression and antitumor activity. *Proc. Natl. Acad. Sci. USA* 2000;97(6):2573–2578. [PubMed: 10688880]
- [21]. Takagi S, Hirata T, Agata K, Mochii M, Eguchi G, Fujisawa H. The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. *Neuron* 1991;7(2):295–307. [PubMed: 1908252]
- [22]. Kawakami A, Kitsukawa T, Takagi S, Fujisawa H. Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. *J. Neurobiol* 1996;29(1):1–17. [PubMed: 8748368]
- [23]. Goodman CS, Kolodkin AL, Lou Y, Puschel AW, Raper JA. Unified nomenclature for the semaphorins/collapsins. Semaphorin Nomenclature Committee. *Cell* 1999;97(5):551–552. [PubMed: 10367884]
- [24]. Gu C, Limberg BJ, Whitaker GB, Perman B, Leahy DJ, Rosenbaum JS, Ginty DD, Kolodkin AL. Characterization of neuropilin-1 structural features that confer binding to semaphorin 3A and vascular endothelial growth factor 165. *J. Biol. Chem* 2002;277(20):18069–18076. [PubMed: 11886873]
- [25]. Mamluk R, Gechtman Z, Kutcher ME, Gasiunas N, Gallagher J, Klagsbrun M. Neuropilin-1 binds vascular endothelial growth factor 165, placenta growth factor-2, and heparin via its b1b2 domain. *J. Biol. Chem* 2002;277(27):24818–24825. [PubMed: 11986311]
- [26]. Guo P, Xu L, Pan S, Brekken RA, Yang ST, Whitaker GB, Nagane M, Thorpe PE, Rosenbaum JS, Su Huang HJ, Cavenee WK, Cheng SY. Vascular endothelial growth factor isoforms display distinct activities in promoting tumor angiogenesis at different anatomic sites. *Cancer Res* 2001;61(23):8569–8577. [PubMed: 11731444]
- [27]. Van Valckenborgh E, Bakkus M, Munaut C, Noel A, St Pierre Y, Asosingh K, Van Riet I, Van Camp B, Vanderkerken K. Upregulation of matrix metalloproteinase-9 in murine 5T33 multiple myeloma cells by interaction with bone marrow endothelial cells. *Int. J. Cancer* 2002;101(6):512–518. [PubMed: 12237890]
- [28]. Soker S, Fidler IJ, Neufeld G, Klagsbrun M. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. *J. Biol. Chem* 1996;271(10):5761–5767. [PubMed: 8621443]
- [29]. Giraudo E, Primo L, Audero E, Gerber HP, Koolwijk P, Klagsbrun S, Klagsbrun M, Ferrara N, Bussolino F. Tumor necrosis factor- α regulates expression of vascular endothelial growth factor receptor-2 and of its co-receptor neuropilin-1 in human vascular endothelial cells. *J. Biol. Chem* 1998;273(34):22128–22135. [PubMed: 9705358]
- [30]. Oh H, Takagi H, Otani A, Koyama S, Kemmochi S, Uemura A, Honda Y. Selective induction of neuropilin-1 by vascular endothelial growth factor (VEGF): a mechanism contributing to VEGF-induced angiogenesis. *Proc. Natl. Acad. Sci. USA* 2002;99(1):383–388. [PubMed: 11756651]
- [31]. Akagi M, Kawaguchi M, Liu W, McCarty MF, Takeda A, Fan F, Stoeltzing O, Parikh AA, Jung YD, Bucana CD, Mansfield PF, Hicklin DJ, Ellis LM. Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells. *Br. J. Cancer* 2003;88(5):796–802. [PubMed: 12618892]
- [32]. Parikh AA, Liu WB, Fan F, Stoeltzing O, Reinmuth N, Bruns CJ, Bucana CD, Evans DB, Ellis LM. Expression and regulation of the novel vascular endothelial growth factor receptor neuropilin-1 by epidermal growth factor in human pancreatic carcinoma. *Cancer* 2003;98(4):720–729. [PubMed: 12910515]
- [33]. Rossignol M, Pouyssegur J, Klagsbrun M. Characterization of the neuropilin-1 promoter: gene expression is mediated by the transcription factor Sp1. *J. Cell. Biochem* 2003;88(4):744–757. [PubMed: 12577308]

- [34]. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem. Biophys. Res. Commun* 1996;226(2):324–328. [PubMed: 8806634]
- [35]. Johnson DE, Lu J, Chen H, Werner S, Williams LT. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. *Mol. Cell. Biol* 1991;11(9):4627–4634. [PubMed: 1652059]
- [36]. Lamszus K, Ulbricht U, Matschke J, Brockmann MA, Fillbrandt R, Westphal M. Levels of soluble vascular endothelial growth factor (VEGF) receptor 1 in astrocytic tumors and its relation to malignancy, vascularity, and VEGF-A. *Clin. Cancer Res* 2003;9(4):1399–1405. [PubMed: 12684411]
- [37]. Giger RJ, Urquhart ER, Gillespie SK, Levensgood DV, Ginty DD, Kolodkin AL. Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. *Neuron* 1998;21(5):1079–1092. [PubMed: 9856463]
- [38]. Nakamura F, Tanaka M, Takahashi T, Kalb RG, Strittmatter SM. Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. *Neuron* 1998;21(5):1093–1100. [PubMed: 9856464]
- [39]. Renzi MJ, Feiner L, Koppel AM, Raper JA. A dominant negative receptor for specific secreted semaphorins is generated by deleting an extracellular domain from neuropilin-1. *J. Neurosci* 1999;19(18):7870–7880. [PubMed: 10479689]
- [40]. Guo P, Hu B, Gu W, Xu L, Wang D, Huang HJ, Cavenee WK, Cheng SY. Platelet-derived growth factor-B enhances glioma angio-genesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment. *Am. J. Pathol* 2003;162(4):1083–1093. [PubMed: 12651601]
- [41]. Hu B, Guo P, Fang Q, Tao HQ, Wang D, Nagane M, Huang HJ, Gunji Y, Nishikawa R, Alitalo K, Cavenee WK, Cheng SY. Angiopoietin-2 induces human glioma invasion through the activation of matrix metalloprotease-2. *Proc. Natl. Acad. Sci. USA* 2003;100(15):8904–8909. [PubMed: 12861074]
- [42]. Furnari FB, Lin H, Huang HS, Cavenee WK. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc. Natl. Acad. Sci. USA* 1997;94(23):12479–12484. [PubMed: 9356475]

Fig. 1.

Structure of the full-length NRP1, the previously discovered soluble isoforms, and the novel soluble isoforms. (A) Genomic structure of the NRP1 gene as well as cDNA and protein structure of the full-length NRP1 and two previously identified soluble isoforms, s₁₂NRP1 and s₁₁NRP1 [19,20]. In the genomic structure, filled boxes represent translated exons, open boxes represent untranslated exons, lines represent introns. In the cDNA structures, each exon is represented by an open box. Intron-derived sequence is represented by a filled box. In the protein structures, each subdomain is represented by an oval or a rectangular box. Intron, exon, and subdomain sizes are not to scale. (B) cDNA and protein structures of novel soluble isoforms s_{III}NRP1 and s_{IV}NRP1. Sequences at splice sites are shown, with exon-derived sequences in capitals and intron-derived sequences in lowercase. Splice donor and acceptor sites and each stop codon are underlined. The cleavage/polyadenylation site (aataaa) is in bold.

Fig. 2.

Expression of s_{III}NRP1 and s_{IV}NRP1 mRNA in various human normal and cancerous tissues and glioma cell lines. Expression of the NRP1 isoforms was examined in human tissues by RT-PCR using either primers capable of amplifying s₁₂NRP1, s_{III}NRP1, and s_{IV}NRP1 simultaneously (A, top) or primers specific for each isoform (A, bottom, B, and C). Total RNA was isolated from various frozen human cancerous tissues and normal brains, or total RNA from various normal human tissues was purchased from Clontech. (A, top) Expression of three NRP1 isoforms (s₁₂NRP1, s_{III}NRP1, and s_{IV}NRP1) in human glioma cell lines and normal brain. β-Actin primers as well as primers capable of amplifying s₁₂NRP1, s_{III}NRP1, and s_{IV}NRP1 simultaneously were used. Sizes of RTPCR products are β-actin, 214 bp; s₁₂NRP1, 433 bp; s_{IV}NRP1, 328 bp; s_{III}NRP1, 183 bp. (Bottom) Expression of s_{III}NRP1 in identical glioma cell lines and normal brain tissues examined using a primer pair specific for s_{III}NRP1. RT-PCR product of s_{III}NRP1 is 114 bp. (B) Expression of s_{III}NRP1 in various human primary glioma specimens, examined using a primer pair specific for s_{III}NRP1. RT-PCR product of s_{III}NRP1 is 114 bp. Glioma tissues are A, astrocytoma (WHO grade II); ODG, oligodendroglioma (WHO grade II); A + ODG, tumors showing morphology of both astrocytoma and oligodendroglioma (WHO grade II/III); PX, pleomorphic xanthoastrocytoma (WHO grade II/III); AA, anaplastic astrocytoma (WHO grade III); GBM, glioblastoma multiforme (WHO grade IV). (C) Expression of s_{IV}NRP1 in various human normal and cancerous tissues using a primer pair specific for s_{IV}NRP1. The RT-PCR product of s_{IV}NRP1 is 114 bp. Tissues used for analyses are lung carcinoid, lung adenocarcinoma (AdenoCA), lung squamous carcinoma (Squamous CA), AA, GBM, and normal kidney, lung, pancreas, and placenta tissues. All experiments shown in (A) to (C) were done at least two independent times with similar results.

Fig. 3.

Expression of soluble NRP1 isoforms in COS-7 cells. Each soluble NRP1 isoform was assembled in a pcDNA 3.0 vector. The constructs were transfected separately into COS-7 cells with LipofectAMINE (Invitrogen). The lysates of various transfected cells were transferred to a membrane and immunoblotted with an anti-human NRP1 antibody (N-18; Santa Cruz Biotechnology). Protein of the correct size for each isoform was observed (s_{III} NRP1, 60 kDa; s_{IV} NRP1, 67 kDa; and s_{12} NRP1, 90 kDa).

Fig. 4. Binding assays of soluble NRP1 isoforms and VEGF₁₆₅. (A) Qualitative assay of s_{III} NRP1/VEGF₁₆₅ binding. CM containing s_{III} NRP1, s_{IV} NRP1, s_{12} NRP1, VEGF₁₆₅-AP, proteins and Ig-basic-AP were produced by calcium phosphate transfection with various expression vectors into HEK 293T cells. CM containing serum was collected 48 h after transfection. CM containing soluble NRP1 was mixed with either CM containing VEGF₁₆₅-AP or CM from non-VEGF₁₆₅-AP-transfected cells (mock) and the assays for binding capacities of the soluble NRP1 isoforms were performed as described under Materials and methods. The sizes of the NRP1 proteins are as follows: s_{III} NRP1, 60 kDa; s_{IV} NRP1, 67 kDa; s_{12} NRP1, 90 kDa. (B) Semiquantitative s_{III} NRP1/VEGF₁₆₅-AP binding assay. Serum-free CM containing s_{III} NRP1, s_{12} NRP1, and VEGF₁₆₅-AP proteins were produced in HEK 293T cells and quantification of the concentration of NRP1 or VEGF₁₆₅-AP proteins was done as described under Materials

and methods. 3.0 nM VEGF₁₆₅-AP was mixed with various concentrations (12.0, 6.0, 2.4, and 1.2 nM) of s₁₂NRP1 or s_{III}NRP1 proteins along with protease inhibitors. The proteins were incubated at 4°C. An anti-AP antibody conjugated to agarose beads was added followed by further incubation. The precipitated proteins were transferred to a membrane and immunoblotted with an anti-NRP1 antibody. The sizes of soluble NRP1 proteins are s_{III}NRP1, 60 kDa; and s₁₂NRP1, 90 kDa.

Fig. 5. Soluble forms of NRP1 inhibit VEGF₁₆₅-AP binding to MDA-MB-231 breast carcinoma cells. (A) VEGF₁₆₅-AP binding to MDA-MB-231 cells. Increasing amounts of VEGF₁₆₅-AP fusion proteins were preincubated in the presence of 1.0 μ g/ml heparin for 30 min on ice followed by 2 h incubation with the MDA-MB-231 cells at 4°C. Cell-associated VEGF₁₆₅-AP was quantified by measuring the AP activity (absorbance at 420 nm) in the cell lysates. The VEGF₁₆₅-AP molar concentration was calculated from the AP activity as described under Materials and methods. (B) Soluble forms of NRP1 inhibit VEGF₁₆₅-AP binding to MDA-MB-231 cells. Equal concentrations (0.96, 4.8, or 24.0 nM) of s₁₂NRP1, s_{III}NRP1, s_{IV}NRP1, or Ig-Basic-AP proteins were mixed with 0.48 nM VEGF₁₆₅-AP protein in the presence of 1.0 μ g/ml heparin for 30 min on ice prior to cell binding. The change in bound VEGF₁₆₅-AP was calculated as the percentage of untreated control that has no soluble forms of NRP1 protein. The data shown are the mean values, and error bars indicate the SD. Experiments were performed in triplicate at two separate times and similar results were obtained.

Fig. 6.

Soluble forms of NRP1 inhibit NRP1-mediated MDA-MB-231 breast carcinoma cell migration. MDA-MB-231 cells were preincubated with isotype-matched mouse IgG (25.0 mg/ml), anti-NRP1 antibody (A-12, 25.0 mg/ml), or equal concentrations of s₁₂NRP1, s_{III}NRP1, s_{IV}NRP1, or Ig-Basic-AP (2.4 or 24.0 nM) in the presence of heparin (1.0 µg/ml) and ZVAD-FMK (an apoptosis inhibitor, 20.0 µg/ml) for 30 min on ice. Cells were allowed to migrate toward serum-free CM of NIH 3T3 cells. Inhibitory effects of soluble forms of NRP1 were indicated as percentage decrease in the number of migrating cells compared to that of the control cells in the absence of soluble forms of NRP1 protein. The data shown are the mean values, and error bars indicate the SD. Data were statistically significant (as determined by power analyses using one-way ANOVA, $p < 0.001$). Experiments were performed in triplicate two independent times and similar results were obtained.