

Neuropilins: a versatile partner of extracellular molecules that regulate development and disease

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1. ABSTRACT

Neuropilins are a vertebrate-specific family of membrane multidomain proteins. They are crucial for the embryonic development of neural and vascular systems, whereas in the adult organism they are implicated in many processes, such as angiogenesis and the immune response. Additionally, it has been shown that they are overexpressed in numerous types of tumours, which results in higher microvessel density and correlates with poor prognosis. Their functions have been linked to their binding partners: semaphorins/collapsins, vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), hepatocyte growth factor/scatter factor and heparin/heparan sulfate (HS). Multiplicity of ligands alongside complex formation with several membrane receptors makes neuropilins potential 'hub' proteins, which act as a scaffold for multimeric associations. This review focuses on the structural features of neuropilins that underpin their multiple molecular interactions and hence their functions.

2. INTRODUCTION

Neuropilin 1 (NRP-1) was first identified as antigen A5, which was proposed to be a recognition molecule in the visual centre's neural cells (1) and a promoter of neural overgrowth (2). Subsequently, it was found to be a receptor for a family of semaphorins/collapsins responsible mainly for chemorepulsive neuronal responses, causing the collapse of the growth cone in nervous system development (2-4), however, a mediation of chemoattractive stimuli was also suggested (5). Additionally, the interaction of semaphorins with neuropilin-1 was linked to conveying an apoptotic response in neurons (6), which was dependent on the presence of neuropilin-1. A homologue to the neuropilin-1 receptor for semaphorins was identified and named neuropilin-2 (NRP-2) (7-9). Similarly to neuropilin-1, neuropilin-2 was identified as playing a part in nervous system development (10).

2.1. Neuropilins' splice variants

In humans the gene encoding neuropilin-1 maps to chromosome 10 and that encoding neuropilin-2 to chromosome 2 (11). Both proteins are around 140 kDa and can be glycosylated. The two neuropilins, although encoded by distinct genes, arose from gene duplication and are structurally related, and consist of the same set of domains, the a1, a2, b1, b2 and c extracellular domains, a transmembrane domain and a short intracellular domain (12), where the a1 and a2 domains belong to the CUB (for complement C1r/C1s, Uegf, Bmp1) family, the b1 and b2 domains belong to the FA58C (for coagulation factor 5/8 C-terminal domain) family and the c domain belongs to the MAM (for meprin/A5-protein/PTPmu) family.

As a result of alternative splicing, there are several splice variants of both neuropilins (7, 11, 13, 14). Human neuropilin-1 has six splice variants, where four of them are soluble forms (Figure 1), while neuropilin-2 has five splice variants, but only one encodes a soluble form (Figure 2) (11, 15, 16). The highest diversity in sequence is observed in the C-terminal part of the proteins (starting where the b2 domain ends), which results in soluble forms that lack fragments of sequence or possess different sequence as a consequence of alternative splicing or the use of different open reading frames. In the case of neuropilin-1, all isoforms share the a1, a2 and b1 domains. The longest one (923 amino acids, isoform a) has also the b2 and c domains. Similar to isoform a is neuropilin-1 (906 amino acids, delta exon 16), which lacks small fragment between the c and the transmembrane domains. Among the soluble isoforms, there are two truncated neuropilin-1 proteins, lacking the C-terminus, including the c domain. These are *s12*neuropilin-1 (644 amino acids, isoform b) and *s1V*neuropilin-1 (609 amino acids, isoform c), where the latter also lacks a small fragment within the b-c linker, but they both contain intron 12 derived C-terminal 3 amino acid sequence (GIK). The other two soluble neuropilin-1s differ substantially in C-terminal sequences with respect to the other isoforms. The *s11*neuropilin-1 isoform shares with other neuropilin-1 isoforms a fragment in the b-c linker, while further sequence is intron 11 derived, although it still shows some sequence similarity to the linker and c domain. The last soluble form (551 aa, *sIII*neuropilin-1) is the only one lacking part of the b2 domain and the rest of the C-terminal sequence, and it uses a different reading frame in exon 12, which results some sequence similarity with the b2 domain.

Among the neuropilin-2 isoforms it is interesting that all but one share all 5 domains (a1, a2, b1, b2, c). The shortest isoform (555 amino acids, *s9*, isoform 6) is soluble, lacks part of b2 domain together with the rest of the C-terminus and similarly to *s11*neuropilin-1, it has an intron 9 derived C-terminus, which shows similarity with the b-c linker. The longest isoform (931 amino acids, 2a22, isoform 1) has the full set of domains with transmembrane and intracellular domains. The 926 amino acids isoform (2a17, isoform 2) is missing 5 amino acids between the c domain and the transmembrane domain. The remaining two isoforms (grouped as isoforms b in contrast to the previously described two full-length isoforms a) have a

distinct C-terminus, which is a result of alternative splicing and is suggested to encode a functional transmembrane and intracellular domain. The difference in length is due to a short fragment after the c domain that the longer isoform encodes (906 amino acids, 2b5, isoform 4), but not this shorter isoform (901 amino acids, 2b0, isoform 5).

Analysis of mRNA expression patterns of variants has revealed that the isoforms are not equally expressed in human tissues. The tissue specific expression pattern of neuropilin-1 isoforms seems to be largely overlapping (brain, heart, kidney, liver, lung, pancreas, placenta, trachea), however, the mRNA level of isoforms IIIs and IVs of neuropilin-1 is 3 and 10 times less abundant, respectively (11, 15). Contrary to neuropilin-1, the isoforms of neuropilin-2 seem to have a more diversified pattern of expression. While isoforms a of neuropilin-2 are dominating in liver, small intestine and placenta, the isoforms b dominate in heart, skeletal muscles and lung. Interestingly, difference in expression levels between the two known isoforms a (2a22 and 2a17) was also observed with significant predominance of 2a17 isoform and 2a22 isoform expressed in smaller amounts in lung, placenta and trachea (11). The mechanism of the control of the expression of specific isoforms is not known.

The protein sequence differences between neuropilins indicate putative modes of action. Firstly, the multiplicity of truncated/soluble isoforms suggests competition and titration mechanisms, where soluble isoforms would be responsible for binding of potential ligands and, therefore, would diminish the local concentration of the ligand available to form signalling complexes with membrane-bound neuropilins. The ligands of neuropilins titrated out by competing soluble isoforms are not known. However, this has been questioned, as the level of these isoforms may not always be physiologically significant (15). Nevertheless, a competition related antitumour property was confirmed *in vivo* with a *s12*neuropilin-1 overexpression system. In addition, the difference between transmembrane and cytoplasmic fragments gives rise to a potentially greater multiplicity of interacting partners. The various short length insertions could possibly disrupt domain structure and consequently structure-dependent interactions, e.g., dimerisation. However, in several studies, no difference in binding features between the short and full length isoforms was detected. Thus, *sIII/sIV/s12*neuropilin-1s interaction with VEGF165 and SEMA3A was confirmed in binding assays (15), independently interaction of VEGF165 with *s12*neuropilin-1 in crosslinking experiments was demonstrated (16) and neuropilin-1(deltaexon16) VEGF165 interaction was confirmed as well (13).

Together with the varied expression pattern, these features expand the possibilities of the mechanism of action of these related proteins. Moreover, it seems that a similar set of functional domains is not only characteristic of neuropilins. One other protein was discovered in human coronary arterial cells, ESDN (endothelial and smooth muscle cell-derived neuropilin-like molecule), which contains CUB and FA58C domains and it was suggested to play a variety of roles akin to those of neuropilins (17).

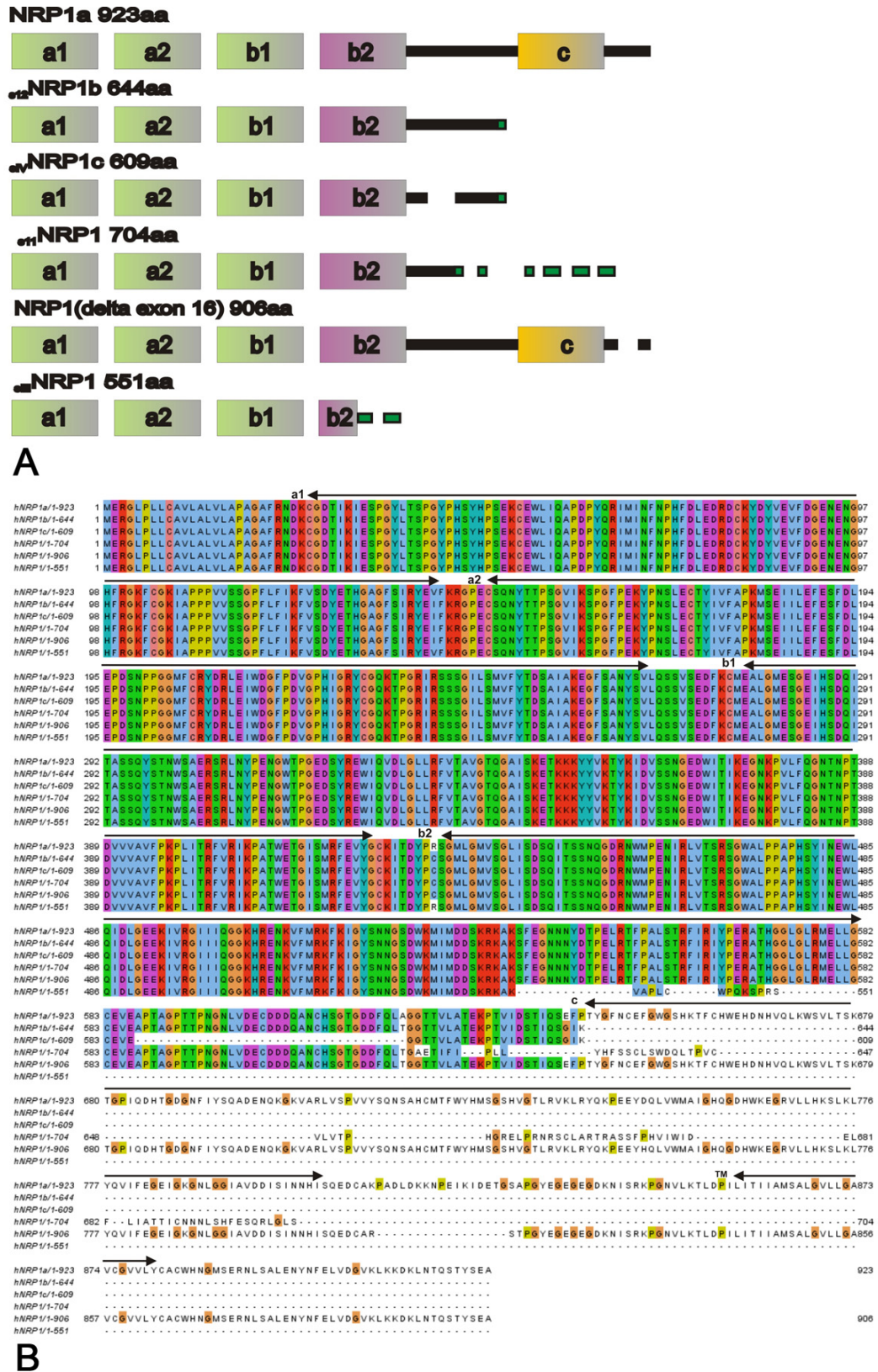


Figure 1. Human neuropilin-1 isoforms analysis; a) schematic representation of multiple alignment, the names of isoforms and domains are indicated, alternative sequences are labelled in green; b) detailed multiple alignment in ClustalW color mode, the sizes of isoforms are indicated together with their symbols and the domains are marked above the sequence.

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NRP215 (2b0) 901aa



NRP216 (s9) 555aa



NRP21 (2a22) 931aa



NRP212 (2a17) 926aa



A

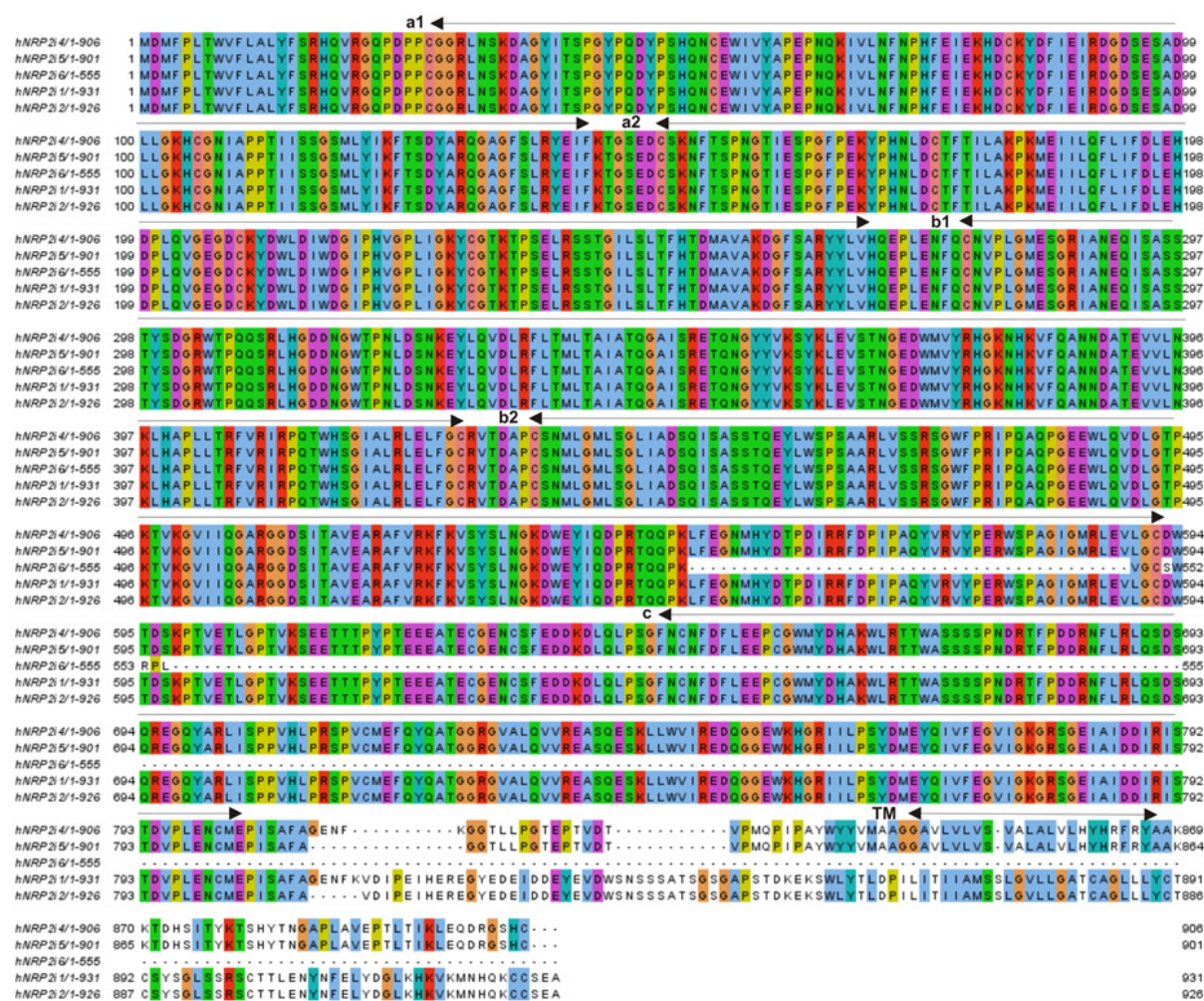


Figure 2. Human neuropilin-2 isoforms analysis; a) schematic representation of multiple alignment, the names of isoforms and domains are indicated, alternative sequences are labelled in green; b) detailed multiple alignment in ClustalW color mode, the sizes of isoforms are indicated together with their symbols, and the domains are marked above the sequence.

2.2. Neuropilins' expression and function

Genetic studies aiming to elucidate functions of neuropilins have been carried out. In mice, deletion of neuropilin-1 is embryonically lethal in E12.5, and causes defective neural patterning and vascular regression (2, 18). Interestingly, the neural phenotype resembled the knockout of *Sema3A*, while the vascular phenotype in comparison to *VEGFR2* knockout suggested a requirement for neuropilin-1 in late embryonic vasculogenesis and the early development of the cardiovascular system. Neuropilin-1 overexpression also has severe consequences, namely heart and blood-vessel formation abnormalities such as excess capillaries and vessels and hemorrhaging and malformation of heart, as well as defects in the nervous system and limbs (19). Significantly, it was shown that neuropilin-1 and neuropilin-2 knockouts do not have the same phenotypes. Neuropilin-2 knockouts have a 40% death rate occurring close to birth and survivors, apart from being smaller in size, have reduction of lymphatic vessels and capillaries or their missposition and neuronal defects (10, 20, 21). In these mutants no change in the blood vessel system was observed. Interestingly, it was shown that both knockouts, neuropilin-1 and neuropilin-2, became unresponsive towards their semaphorin ligands, *Sema3A* and *Sema3F*, respectively (2, 10). Simultaneous knockouts of both genes causes E8.5 embryonic lethality (22).

Although these most severe defects after interference with native levels of neuropilins affect mainly blood-vessel and neural systems, neuropilins have been confirmed to be expressed in many other tissues, often in a specific manner. Generally, both neuropilins have been shown to be expressed by several types of organs and tissues, which makes them a wide ranging interacting partner, however, their mRNA expression patterns are often not overlapping (7, 23). This emphasizes the issue of the likelihood of neuropilin-1 and neuropilin-2 possessing distinct functions. In the case of endothelial cells it has even been suggested that, while arterial areas express neuropilin-1, veins produce mainly neuropilin-2, and that this pattern of expression might affect tissue identity (24). A hormone driven interplay between neuropilin-1 and neuropilin-2 in human endometrium was also observed. Under the natural menstruation cycle an estradiol-dependent upregulation of neuropilin-1 in the proliferation phase was followed by neuropilin-2 upregulation in the secretion phase, which is also indicative of distinct functions for the two proteins (25).

Information about the function of neuropilins can be also deduced from data on the regulation of the expression of their mRNAs. There have been several transcription and growth factors identified that influence their expression (reviewed in (23)). *Ets-1* (26), *dHAND* (27), *SP1* (28) and *AP1* (28) increase expression of neuropilin-1 mRNA. *Ets-1* and *dHAND* upregulation of neuropilin mRNA is generally linked to vascularisation processes, whereas the upregulatory effects of *SP1* and *AP1* have been obtained from promoter sequence analysis in cell culture. Growth factors identified that upregulate neuropilin-1 mRNA include *TNF α* (29), *VEGF*, *EGF* (30-

32) and *IL-6* (33). *TNF α* has angiogenic properties *in vivo*, and at the molecular level it has been shown to upregulate both *VEGFR2* and neuropilin-1, which has been suggested as the mechanism whereby it potentiates *VEGF* action though, in another study this effect was not observed (30). Upregulation of neuropilin-1 has also been observed to be associated with *VEGF* and *EGF* – presence of these growth factors in tissue is associated with increases in the amount of mRNA encoding neuropilin-1. In pancreatic cancer cells *IL-6* has been found to upregulate neuropilin-1 mRNA. An orphan receptor, *Nurr1*, was shown to upregulate the levels of neuropilin-1, in the course of the formation of dopamine neurons in midbrain (34). *Cyclophilin A*, a protein known to be involved in the regulation of vascularisation and cell growth, was shown to upregulate neuropilin-1 mRNA levels in aorta smooth muscle cells (35). Among the transcription factors responsible for downregulation of mRNA encoding neuropilin-1 are *COUP-TFII* (36), *Prox-1* (37), *HEX* (38) and *NRSF/REST* (39). *NRSF/REST* was found to suppress the expression of neuropilin-1 mRNA in keratinocytes. *COUP-TFII*, by suppressing neuropilin-1 mRNA expression within the vasculature, permits arterial-venial differentiation. In this process, *COUP-TFII* dependent downregulation of neuropilin-1 mRNA in presumptive veins enables them to acquire the characteristics of vein. *Prox1* controls an analogous switch in differentiation of blood and lymphatic endothelium. Here, the downregulation of neuropilin-1 is a characteristic of developing lymphatic vasculature from classical blood vessels. Altogether, these results indicate that the expression of mRNA encoding neuropilin-1 is subjected to a variety of regulatory inputs, though a regulatory signalling network responsible for the control of the levels of neuropilin mRNA has yet to emerge. The extent to which these changes in expression of mRNA encoding neuropilin-1 may be affected by neuropilin-2 is not known. Moreover, in the event of co-expression of the two neuropilins changes in their relative expression may also affect the cellular response, though this has yet to be documented directly.

Much less is known about mRNAs encoding neuropilin-2. High levels of neuropilin-2 mRNA were suggested to be major drive of axonal regeneration. Consistently, neuropilin-2 blocking antibodies prevented first step of regeneration, which is axonal aggregation. Moreover, forskolin, the axon aggregation mimetic, was able to downregulate neuropilin-2 mRNA and thus, confirm its role in regeneration process (40).

It is worth noting that expression of neuropilins is also dependent on the cell microenvironment. Hypoxia, which results in acidic pH has been found to upregulate neuropilin mRNA. This is important, as hypoxia is a major driver of angiogenesis and commonly occurs upon tumour development, when a shortage of oxygen results in the accumulation of lactate and *CO₂* in the extracellular compartment and causes pH values to be as low as 5.5 (41, 42). Hypoxic conditions upregulate several mRNAs of angiogenesis-related proteins, including neuropilin-1 and neuropilin-2, which lead to enhanced vascularisation (21, 43-45). However, it is noteworthy that so far a direct link

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between the expression of the neuropilins' mRNA and HIF (hypoxia-inducible factor), a key molecular regulator of the hypoxic response, has not been confirmed.

Initial studies aiming to characterise mouse gene expression during embryonic and early postnatal development show that neuropilin-1 mRNA is expressed in the cardiovascular system, nervous system and mesenchymal tissues surrounding them (19, 46). A general pattern is that rather than being generally expressed in these tissues, neuropilin-1 expression is instead focused to certain types of cells and this localised expression is often temporally regulated. Interestingly, initial observations suggested that neuropilin-1 might be an auto-recognition molecule, as it was abundant in actively growing axons and the target of these axons also expressed neuropilin-1. Subsequently, interactions with other molecules were identified. Consequently, another function postulated for neuropilin was neuronal circuit formation (46). In the cardiovascular system high levels of neuropilin-1 are found in vessel system epithelium and also in the surrounding blood vessel mesenchymal cells. Additionally, expression is detected in endocardial cells in developing heart. Interestingly, in adult mouse the expression drops and is mainly localised to heart atria. Neuropilin-1 expression is also pivotal in limb development, where initial high levels in mesenchymal cells are replaced by only connective tissue expression (19). In human adult tissues neuropilin-1 is highly expressed in heart and placenta and at lower levels in lung, skeletal muscles, kidney and pancreas (14). Mouse neuropilin-2 expression is also dynamically regulated and is largely separable from that of neuropilin-1 in the nervous system. Additional locations of neuropilin-2 expression are limb bud muscle masses, bones, smooth muscle of the gut, intestinal epithelium, kidney, lung, inner ear, submandibular glands and whisker follicles of the snout (7).

Neuropilin mRNAs share a feature of being overexpressed in a number of cancers, although usually not in a redundant manner (reviewed in (23, 47, 48)). High levels of neuropilin-1 or neuropilin-2 often correlate with increased tumour size, neovascularisation, decreased tumour apoptosis, tumour cell migration and clinically is often associated with poor prognosis (49, 50). However, the association between the levels of expression of neuropilin-1 and patient prognosis is somewhat contradictory. Thus, in colon cancer one study that measured the level of expression of mRNA encoding both soluble and membrane bound neuropilin-1 suggested that neuropilin-1 expression correlated with a better patient prognosis (51), whereas another study that used immunocytochemistry and presumably was biased towards the detection of cell-associated neuropilin-1 protein suggested that neuropilin-1 was associated with a poor disease outcome (52).

3. SEQUENCE ANALYSIS

3.1. Origins of the domains of neuropilins

Both neuropilins are comprised of the same set of domains, a1, a2, b1, b2 and c, where a1 and a2 belong to the CUB (for complement C1r/C1s, uEGF, BMP1) family (53), b1 and b2 belong to the FA58C (coagulation factor

5/8 C-terminal domain) family (54) and the c domain belongs to the MAM (for meprin/A5-protein/PTPmu) family. CUB domains have been for some time recognised as important elements of developmentally significant proteins, e.g., bone morphogenic protein (BMP1), sea urchin endothelial growth factor (uEGF) and subcomponents of complement (C1r/C1s), spermadhesins, some vertebrate proteases and mammalian hyaluronate-binding protein TSG-6. The function of CUB is related to binding sugars, dimerisation (55) and protein-protein interactions (56). The FA58C domains are characteristic of coagulation factors and of discoidin proteins. They are found in milk fat globule membrane proteins, receptor tyrosine kinases and contactin-associated proteins. The function suggested for these domains is binding of anionic phospholipids on the surface of cells, and a consequent role in adhesion and cell-cell recognition. The MAM domain was found in a surface glycoprotein called meprin and a receptor-like tyrosine protein phosphatase (RPTP mu). MAM domains have been suggested to play a role in protein dimerisation (57) and cell-cell adhesion (58). Importantly, none of the neuropilins has an intracellular domain with clear interaction or signalling motif.

According to the Blast search engine the organisation the domains found in neuropilins evolved relatively late and is not present in any group of organisms other than vertebrates. However, the individual domain families are commonly represented in many systematic groups. CUB-like domains are found in viruses, bacteria, euglenozoa and across metazoa; FA58C is found in wide range of bacteria, archaea, metazoa, mycetozoa, fungi, parabasalidea, viridiplantae, haptophyceae; MAM domain is not found in viruses, but is present in many groups of bacteria, in alveolata and metazoa. Therefore, neuropilins arose from old evolutionary motifs that in this particular juxtaposition formed a protein of new functions related to vertebrate-specificity.

3.2. Neuropilins' *in silico* sequence analysis

Although neuropilins show domain structure similarity, comparison of the amino acid sequence between human neuropilin-1a (923 amino acids, Fig.1) and neuropilin-2 (2a17, isoform 2, Fig.2) reveals 44% identity (Figure 3). Moreover, the level of conservation is not uniform throughout the sequence. The highest similarity is observed in the transmembrane region, whereas the lowest is in the c domain and its flanking regions that link it to adjacent domains. In fact, analysis of the C-terminal part of the sequence (the transmembrane and intracellular domains) reveals that neuropilin-2a isoforms show more sequence similarity with neuropilin-1 than neuropilin-2b isoforms (11). Interestingly, analysis of charged residues in sequences of both human neuropilins shows, that although some of the charged residues are similar in the alignment, there are a good number of residues of opposite charges in the two proteins, which may be associated with important differences in function (Figure 4). Comparison of human, mouse, rat and zebrafish sequences of most similar length gives interesting insights into evolutionary conservation. The zebrafish sequences of both neuropilins vary substantially from the others, which is apparent in the

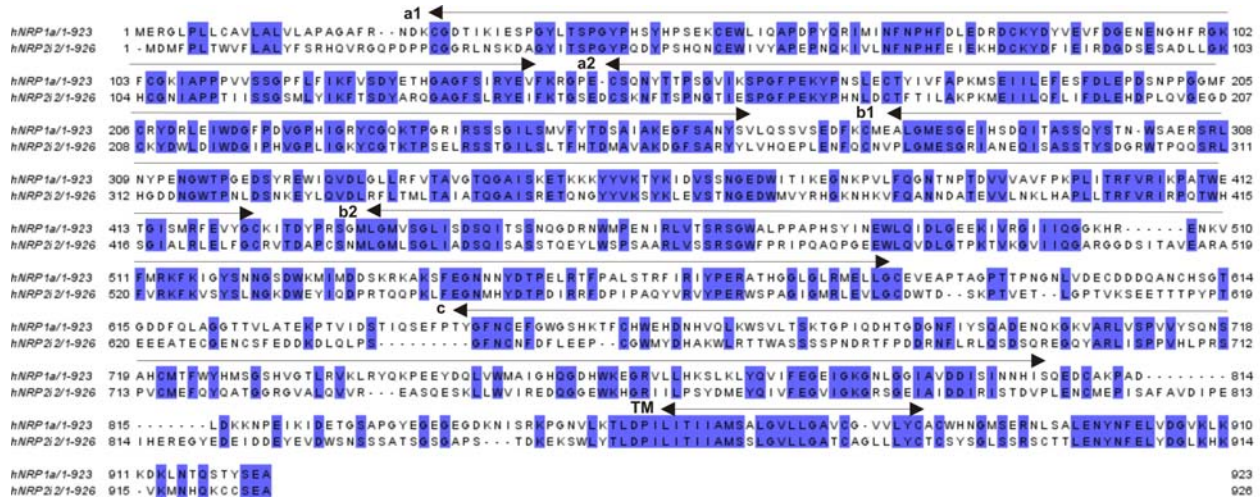


Figure 3. Comparison of most similar human isoforms of neuropilin-1 (isoform a) and neuropilin-2 (isoform 2); the sizes of isoforms are indicated together with their symbols, the domains are marked above the sequence, the residues marked in blue are identical.

number of mismatches, insertions and deletions, especially in the neuropilin-2 b-c linker and c domain and to lesser extent in the same region in neuropilin-1. However, another isoform of zebrafish neuropilin-1, isoform b, has several larger insertions, e.g., 23 amino acids in the b-c linker and 19 amino acids in the c domain (not shown). Overall, the transmembrane and cytoplasmic fragments seem to be most conserved between species, which suggests that the intracellular part of neuropilins may interact with intracellular signalling cascade proteins (59), despite this region having no obvious signalling motifs. When the number of mismatches is considered, the a and b domains are more conserved in neuropilin-2 (21 mismatches versus 55 in the a and b domains in neuropilin-1), while in neuropilin-1 it is c domain that has the lowest number of substitutions (8 mismatches versus 20 in the same region in neuropilin-2) (Figure 5, 6). To summarise, sequence analysis shows that the sequence of zebrafish variants of neuropilins differ to some extent from those of other organisms. Comparison between human, mouse and rat sequences and also between human neuropilin-1 and neuropilin-2 show distinct conservation patterns in different parts of the protein, which is a good indication of putative diverse functions.

Interestingly, the *in silico* analysis of neuropilin sequences in search of putative N-glycosylation and O-glycosylation sites reveals one possible important feature of the multiple splice variants (Figure 7, 8) (60, 61). Most N-glycosylation sites are located in the regions shared by all neuropilin-1 and neuropilin-2 isoforms. Thus, among neuropilin-1 isoforms, four out of six N-glycosylation sites are shared, whereas the sites in the flanking regions of transmembrane domain are shared only by the two longest variants of neuropilin-1. In neuropilin-2 isoforms, two out of four sites are common, one site is absent only in the most truncated isoform 6, and another is only present in the two longest variants of neuropilin-2. Analysis of potential O-glycosylation sites reveals that there are two such sites in

both proteins and they localise in both cases to the b-c linker, the least conserved part of the sequence. In neuropilin-2 both predicted sites are shared by all isoforms except for isoform 6. In neuropilin-1 there are also two potential O-glycosylation sites and the shortest isoform sIII lacks both, while the s11 and sIV isoforms have just one, but each of them has a different one. Isoforms a, b (s12) and delta exon 16 share both putative sites. Serine 612 of neuropilin-1 isoform a, which has been suggested to be facultatively glycanated by the glycosaminoglycans heparan sulfate and chondroitin sulfate (62), is located very closely to the region containing the predicted O-glycosylation sites. It is important to note that the interdomain linkers in both neuropilins have no recognised structure. These are likely to be important in mediating domain orientation, interactions with other proteins and contain posttranslational modification sites (63). Thus, these putative posttranslational modification sites could be a part of the regulation of the function of neuropilins.

4. NEUROPILINS' INTERACTOME

The number of different molecular interactions that neuropilins make describe an ever-increasing catalogue of partners. These interactions are, where it is known, associated with particular domains of neuropilin. Alongside the large number of normal and pathological events where neuropilins have been suggested to play important regulatory functions, this suggests that the domains of neuropilins may provide a set of modules involved in multiple molecular interactions. Thus, one view of neuropilins is as a scaffold for cell-cell and cell guidance signalling.

Initially, molecules related to the function of neuropilins were discovered in the neural system, e.g., plexins, semaphorins (firstly SEMA3A) (64). Subsequently, more interacting partners were discovered, like L1-CAM, which is an adhesion molecule that can

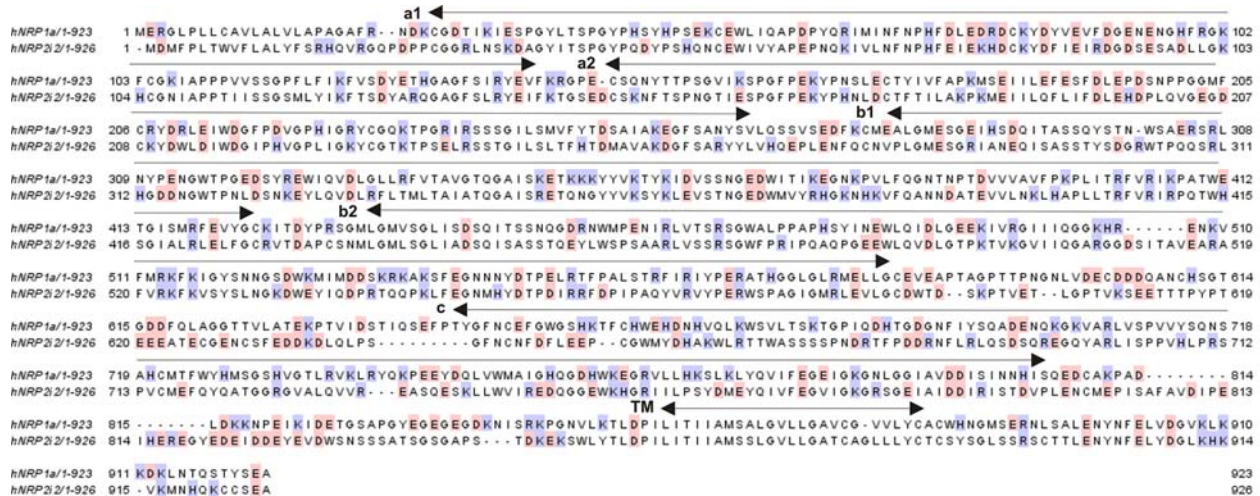


Figure 4. Comparison of charged residues pattern in human neuropilin-1 (isoform a) and neuropilin-2 (isoform 2); the sizes of isoforms are indicated together with their symbols, the domains are marked above the sequence, the basic and acidic residues are marked blue and red, respectively.

modify the SEMA3 repulsive signals (65). The next group of partners of neuropilins were the glycosaminoglycans heparan sulfate, the dominant scaffold and long-range integrator of extracellular signals (66) and members of the VEGF family that bind to heparan sulfate (67). The latest group of partners of neuropilins are prion protein, several members of the FGF family (fibroblast growth factor) and HGF/SF (hepatocyte growth factor/scatter factor) and receptors such as FGF receptor 1 (68), VEGF receptors (69-71) and integrins (72). These proteins are structurally unrelated and apart from integrins they possess in common only one feature: binding to sulfated glycosaminoglycans such as heparan sulfate. Not typical was the discovery of NIP (neuropilin-1 interacting protein), a protein containing the common protein interaction motif PDZ that binds the intracellular part of neuropilin-1 (59). Adding to this complexity are dimerisation and possibly higher order oligomers of neuropilins that have been observed (9, 68, 73, 74). Interaction with heparan sulfate may modify binding affinities and is likely to bring neuropilin into proximity with many complexes involved in cell adhesion and cell-cell communication. In some cases the protein-protein interactions were suggested to occur upon heparin binding, e.g. interaction of neuropilin-1 with VEGFR2 (75) or neuropilin-1/2 with VEGFD (76). Additionally, it has been hypothesised that heparin/heparan sulfate might cause multimerisation of neuropilin-1 (69), however, a mechanism whereby heparin/heparan sulfate serves as a docking molecule for multivalent interactions with neuropilin-1 has also been suggested (77).

4.1. Structural features of neuropilin interactions

The structure of neuropilins suggests they might be a scaffold for protein-protein interactions. This idea is supported by an increasing body of evidence from studies using a variety of approaches such as deletion analysis, mutagenesis, crystallography and biophysics.

Investigation of semaphorin binding until recently had no underpinning from structural biology. Thus, what was initially suggested from deletion studies was that the sema domain of semaphorins bound the a1a2 domains and that these interactions defined the specificity of binding, whilst basic C-terminal region of semaphorins binds to a1a2 and b1b2 (4, 78, 79). In another study the semaphorin specificity was attributed to both regions of a1a2 and b1b2 domains (9). In parallel, it was shown for neuropilin-2 that binding SEMA3F required both a1a2 and b1b2 domains (80). Subsequent work refined the identification of potential semaphorin binding sites by mutational analysis. Basing on structural alignment of the neuropilin-1 a1 domain and bovine spermadhesin CUB domain, residues likely to be exposed to solvent in the a1 predicted loop regions were identified and mutated into residues of opposite charge (residues 46, 47, 51, 52, 53, 79, 80, 128, 129, 130). The introduced mutations inhibited binding of semaphorin, but not that of VEGF. Importantly, these mutations had inhibited the binding of both SEMA3C and F, indicating that the discrimination between these two semaphorins was lost. Also, no effect of these mutations was observed on the interactions with plexin and VEGFR2 (79). Recently, this body of data could be compared to a crystallographic model of neuropilin-2 domains a1 to b2 in complex with a semaphorin-specific blocking antibody (77). The region where the antibody is binding neuropilin-2 is in a1 (residues 39, 45-47, 72-77, 107, 138) and is highly conserved in the neuropilin-1 a1 domain. The antibody binding area appears to be adjacent to the region characterised by Gu et al. as a semaphorin binding region, therefore, together these data span the semaphorin binding site on a larger interface of the a1 domain. Moreover, as a putative calcium binding motif was discovered in crystal models of the a1 domain in close proximity to the characterised semaphorin binding site, it was shown that the interaction with semaphorins is indeed calcium dependent (77).

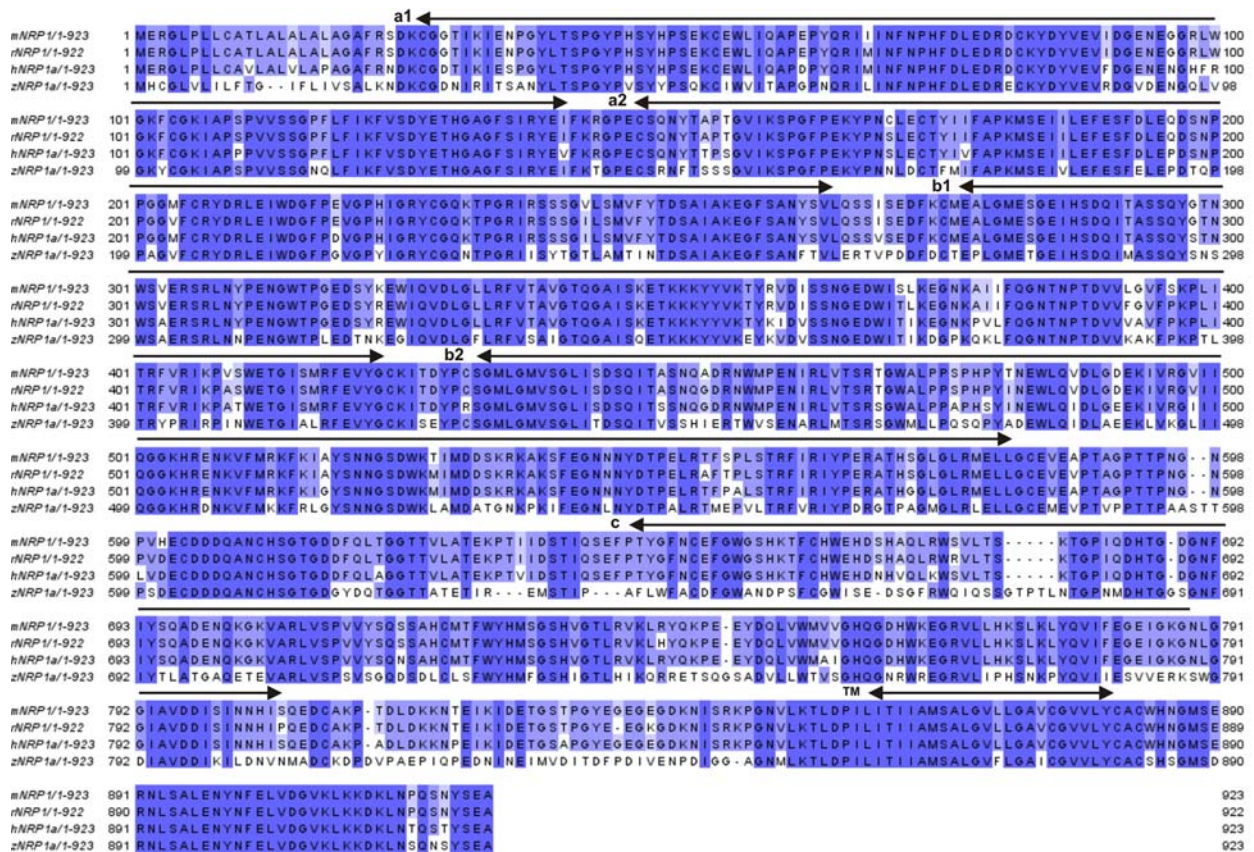


Figure 5. Comparison of most similar human (isoform a), mouse, rat, zebrafish (isoform a) neuropilin-1 sequence; the sizes of isoforms are indicated together with their symbols, the domains are marked above the sequence, the residues marked in blue are identical.

By and large the analysis of VEGF165 binding structures in neuropilins has been supported by subsequent crystallographic models, initially of the human b1 domain and then of rat b1b2 domains (81, 82). Similarly to semaphorins, it was shown by deletion analysis that the b1b2 domain is crucial for the binding of VEGF165 to neuropilin and that the additional presence of the a1a2 domain enhanced the binding (79, 83). Similar results were obtained for the interaction of VEGF165 and neuropilin-2 (80). On the other hand the related PIGF was suggested to bind to only the b1b2 domain of neuropilin-1 and its binding sites in the b domains was thought to overlap with that of VEGF165 (83). Mutational analysis of neuropilin-2, based on sequence similarity with the known neuropilin-1 b1 domain crystal structure, indicated the electronegative loop in the b1 domain as the putative binding site of VEGF165 (residues 284, 287, 290, 291). As the interaction between neuropilin-1 and VEGF165 was suggested to occur via the positively charged heparin binding domain of VEGF (68), the mutation of electronegative residues reduced the binding of VEGF165, whilst mutations introducing more electronegative residues enhanced binding of the growth factor. No change in the K_d for SEMA3F binding was observed as a consequence of these mutations (80). The crystal of the b1b2 domain with tuftsin, a peptide analogue of the basic heparin binding in

the C-terminus of VEGF, suggests a binding site for this domain of VEGF 165 in a part of b1 (residues 297, 301, 320, 353, 346, 349) adjacent to the area mutated in neuropilin-2 (80). Interestingly, the binding pocket identified in the crystallographic model fits the basic tail that of VEGF165, tuftsin and SEMA3A, however, the latter lacks the C-terminal arginine, which seems to be crucial for the binding. Consequently, it has been suggested that several modes of ligand binding are possible (82). This binding site was further confirmed in a study where a crystal of neuropilin-1 b1 domain with VEGF165 blocking antibody was obtained, however, a broader interface between these molecules was suggested, spanning the b1b2 domain of neuropilin-1 towards putative heparin binding site (77).

The physical and functional relationship of the binding sites semaphorins and VEGF in neuropilin is contentious. A competition effect of SEMA3A and VEGF165 was observed in cell migration and growth cone collapse assays (84). Functionally, in lung cancer VEGF was suggested to promote tumour development while semaphorins were suggested to act as inhibitors of this process (85). Additionally, mechanism of VEGF dependent neuropilin-1 internalisation was observed and explained as a support of preferential VEGF signalling inhibiting

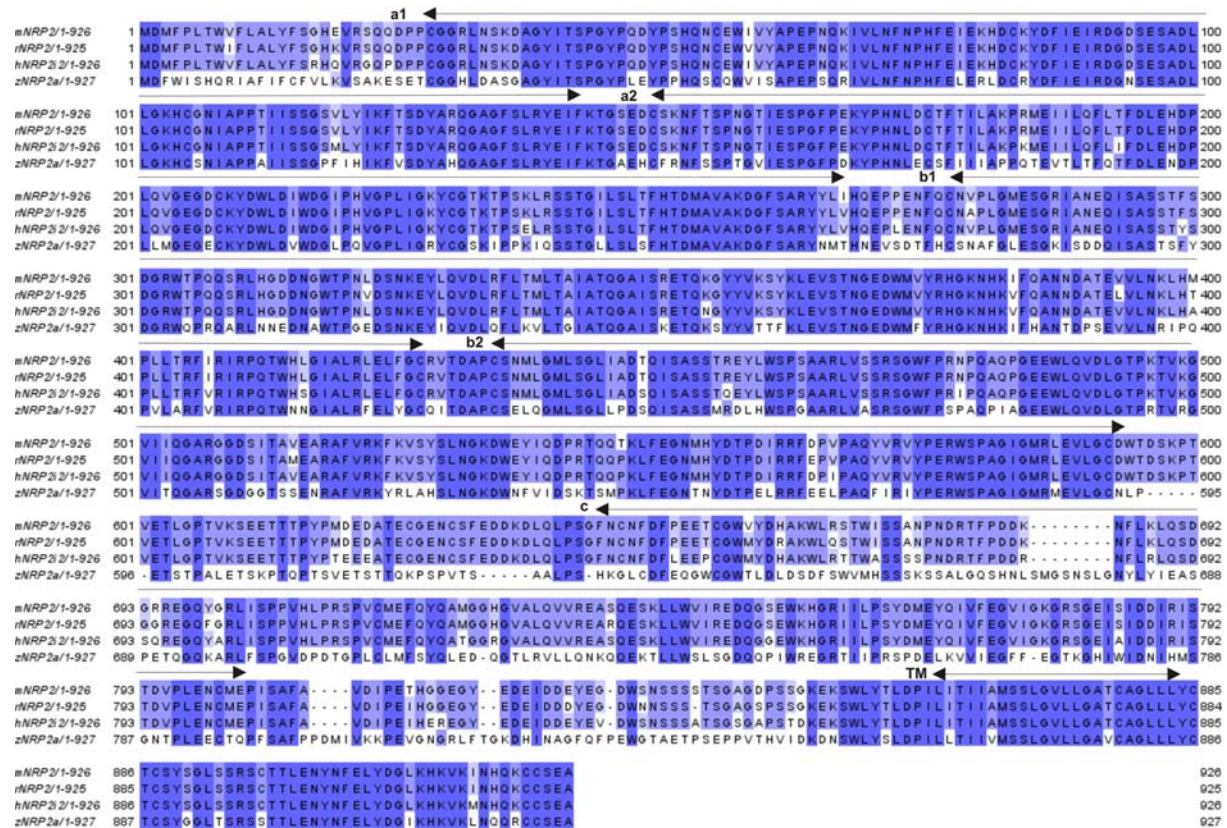


Figure 6. Comparison of most similar human (isoform 2), mouse, rat, zebrafish (isoform a) neuropilin-2 sequence; the sizes of isoforms are indicated together with their symbols, the domains are marked above the sequence, the residues marked in blue are identical.

neuropilin-semaphorin interactions (86). Interestingly, competition with semaphorins was not observed for VEGF121, VEGFB and FGF2 (86). Similarly, it was shown that neuropilin-2 complexed with VEGFR2/3 promotes cell survival by interaction with VEGFA/C, which is functionally inhibited by interaction of neuropilin with SEMA3F (87). This idea was supported by a crystallographic study of the b1 domain of neuropilin-1, and it was suggested that a pocket binding C-terminal arginine of VEGF analog tuftsin can possibly accommodate also basic tail of semaphorins (82). Nevertheless, recent studies argue against a physical overlap of the VEGF and semaphorin binding sites. Firstly, mutation and deletion studies aimed at characterising the VEGF and the semaphorin binding sites indicate that these are located within different domains of neuropilin, i.e. while VEGF binds mainly b1b2 domains, the semaphorins interact with a1a2 domains (79, 80). Secondly, the ability of semaphorin binding to the same pocket as VEGF was excluded because of a lack of the highly conserved arginine as the very C-terminal residue, which is crucial for interaction with the VEGF analog tuftsin (77). Thirdly, crystal structure study depicting binding sites for both ligands by analysis of structures of neuropilin domains with ligand binding blocking antibodies suggests that the binding sites are separated by 65 Å. This independence of binding is also supported by optical biosensor experiments in which

footprinting of SEMA3A and VEGF165 binding sites in neuropilin was attempted and interpreted as independent event (77). Finally, functional independence is indicated by experiments in mouse development. A study in branchiomotor neurons expressing neuropilin-1 showed that the axon and somata have distinct affinities for VEGF165 and semaphorins and it was suggested that the somata is controlled by VEGF165, while the axon by semaphorins in a neuropilin-1 dependent manner without competition between these ligands (88). In other mouse developmental studies only selective preference for distinct ligands was observed during development of vascular and neural systems without direct competition (89). Taken together, it seems as if physical competition for binding to neuropilin may not occur, but that it is possible to engineer a situation where instances functional competition does take place. A comprehensive and quantitative analysis of the interactions of neuropilin with these and other ligands would certainly help to determine the circumstances necessary for competition (functional or otherwise) between VEGF and semaphorins to occur.

Qualitative binding studies have suggested that a tetradecasaccharide is the minimal structure able to bind the b1b2 domain of neuropilin-1. It has been further suggested that a mechanism of dimerisation of neuropilin is mediated by heparin at a 2:2 ratio of heparin:neuropilin.

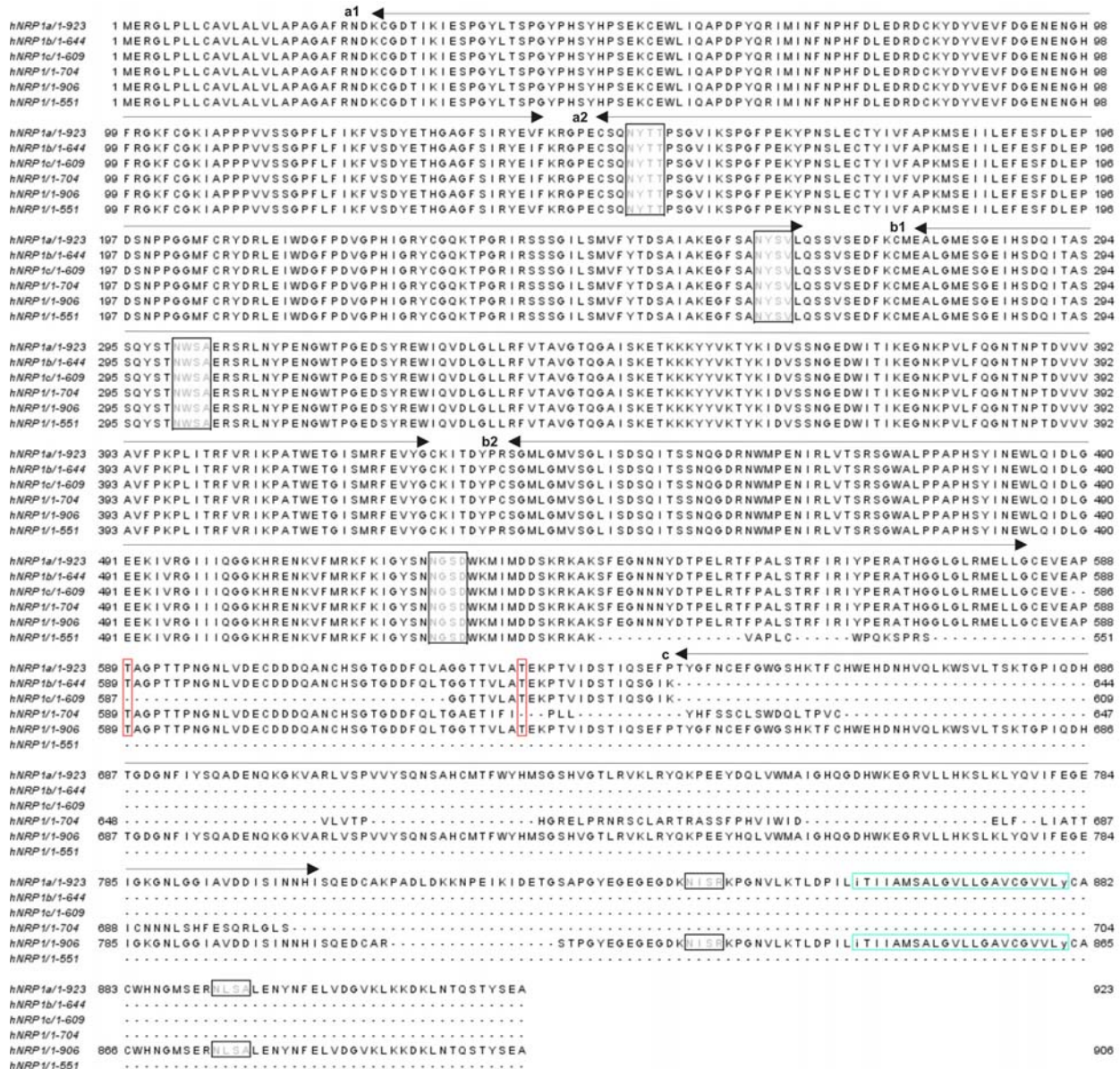


Figure 7. *In silico* analysis of human isoforms of neuropilin-1; the sizes of isoforms are indicated together with their symbols, the domains are marked above the sequence, sequences in black frame are putative N-glycosylation sites, sequences in red frame are putative O-glycosylation sites, sequence in green frame is transmembrane region.

However, given that there is no direct evidence for an interaction between neuropilin molecules in such polysaccharide-protein complexes, it remains to be seen whether heparin does in fact cause neuropilin dimerisation or whether, by virtue of this polysaccharide possessing multiple overlapping binding sites it simply “bridges” neuropilins. The residues involved in heparin interaction based on the crystal structure are 359, 373, 513, 514, 516 and locate on the surface of b1b2 adjacent to tuftsin binding pocket, therefore, such proximity may support a mechanism of increased affinity for VEGF165 mediated by neuropilin-1 (77, 82).

Dimerisation of neuropilins has been also suggested to occur through the c domain. However,

mutants without c domains still show a degree of dimerisation and, therefore, another domain may also mediate neuropilin homophilic interactions (4). Moreover, it has been shown that interactions between neuropilins are likely to be driven by ionic bonding (68). In crystallographic studies an additional dimerisation role was ascribed to the a1 domain, due to its conserved putative interface and flexible character. A model where dimerisation via the a1 and c domains together with heparin interaction take place was suggested. Consistent with this model is enhanced VEGF binding and independent location of semaphorin binding site in such a dimer (77).

As neuropilins mediate cellular responses, requirements for signal transduction were studied. It was

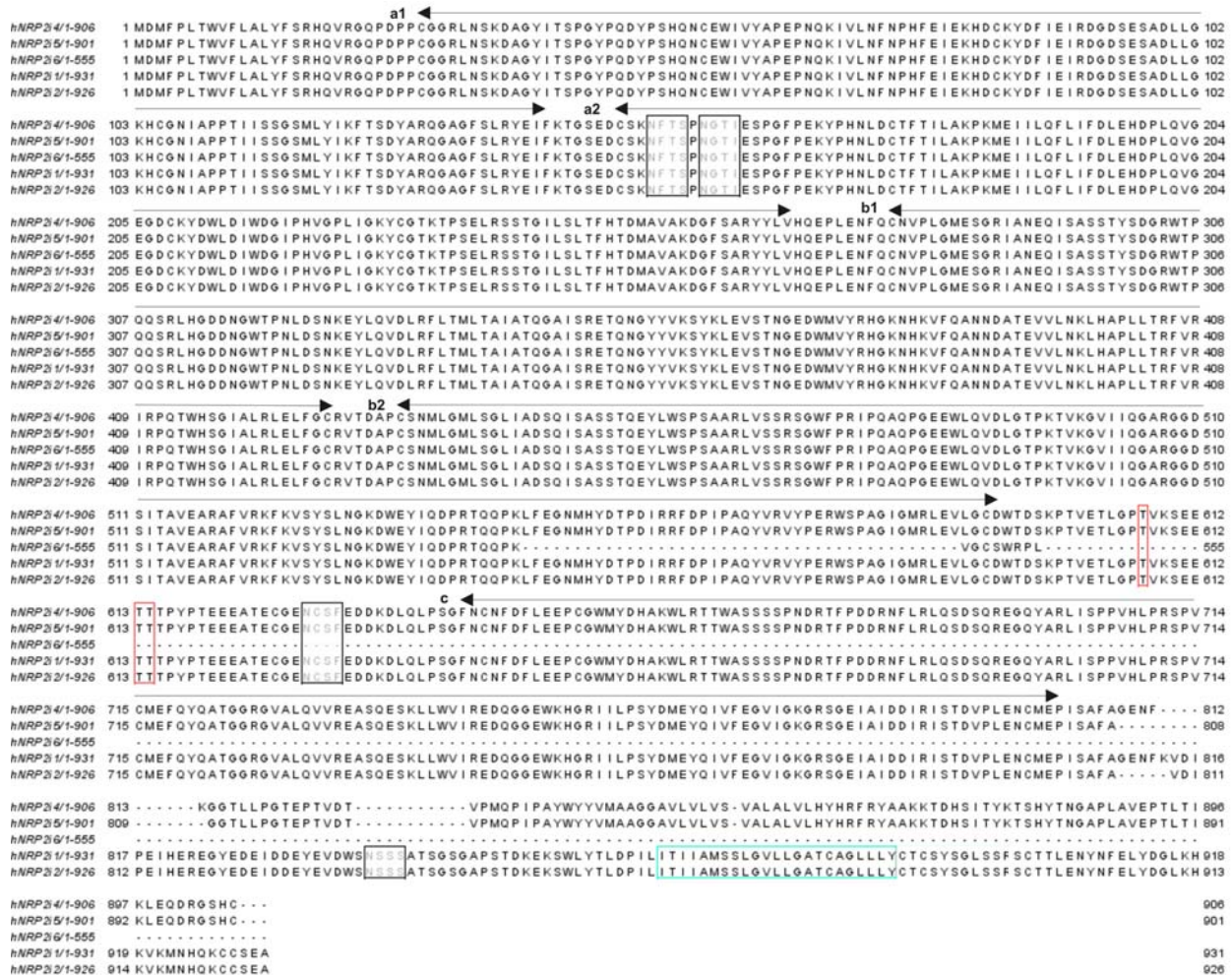


Figure 8. *In silico* analysis of human isoforms of neuropilin-2; the sizes of isoforms are indicated together with their symbols, the domains are marked above the sequence, sequences in black frame are putative N-glycosylation sites, sequences in red frame are putative O-glycosylation sites, sequence in green frame is transmembrane region.

shown in chick neural growth cone assay that the elicited response is strongly dependent firstly on the presence of the a1a2 semaphorin binding domain, secondly on the c domain, and to lesser extent it was observed that full potency of signal requires the b1b2 domain. Interestingly, it was also shown that transmembrane and intracellular parts of neuropilins, even upon deletion, were completely irrelevant for signal transduction in this model (3, 4).

An important feature of neuropilin-1 is its so-called adhesive function (90, 91), which structural requirements were also studied (78). This has been mapped by deletion experiments and synthetic peptide binding assays. The adhesion region was found within the b1b2 domain (residues 347-364 in b1 and 504-521 in b2) and its function was neither enhanced nor competed by VEGF165, SEMA3A and plexins. Intriguingly, this suggested region responsible for the adhesion function of neuropilin-1 overlaps with the putative heparin binding site of the protein (82), suggesting that neuropilin interactions with

proteoglycans may be responsible for at least part of the neuropilin's adhesion function.

4.2. Functional aspect of neuropilin interactions

The main functions of neuropilins that have been identified with certainty are associated with vessel and neural systems. In mouse development, both neuropilins are essential in embryonic angiogenesis (18, 22) and similarly for neural system development (92, 93). Also, both of them were confirmed to be important for neural migration (93-95) and they are involved in endothelial cell migration (87, 96), cell survival (87, 97) and vascular permeability (25, 98). Endothelial neuropilin-1 in adult organisms is also involved in wound angiogenesis (99). Related to neuropilins' endothelial localisation is a developmental role in kidney morphogenesis, where they seem to provide a morphogenetic guide plan (100). Similar developmental roles of neuropilin-1 were observed in salivary gland formation and lung development (101, 102). This shows that neuropilins have pivotal functions in development. Another field where neuropilins seem to play

Table 1. Neuropilins expression

Localisation	neuropilin-1	s12 neuropilin-1	neuropilin-2
Vessel systems	18		24
Retina	149		21
Neural system	90		7, 10, 40
Hepatocytes		16	
Kidney	100, 150	16	100
Liver	16		
Keratinocytes	16, 39		
Melanocytes	151		
Chondrocytes	152		
Osteoblasts	153		
Schwann cells			40
T cells	103, 154		
Basophils	106		106
Bone marrow	107		
Dendritic cells			155
Intervertebral disc			156
Aorta smooth muscle cells	35		

an important role is the immune system (103). Neuropilin-1 was shown to be involved in the primary immune response (104) and the migration of thymocytes (105). The presence of neuropilin-1 in basophils is interpreted as a possible means whereby basophils regulate angiogenesis (106). Other studies suggest that neuropilin-1 is involved in the regulation of hematopoiesis (107). A tissue/organ specific summary table presents neuropilin expression data (Table 1). Despite such widespread expression and a wealth of data suggesting that neuropilins play important roles in vertebrate development and homeostasis, the mechanisms whereby neuropilins exert these functions are not well defined. What is known is largely focused around the molecular partners of neuropilins and the association of these interactions of neuropilins with biological activities.

The original partners of neuropilins, the class III semaphorins, require neuropilin co-receptors such as plexins or L1 subfamily molecules to transduce intracellular signals (65, 108). Semaphorins are a versatile group of membrane-associated or soluble proteins classified into eight families, where class 3 in humans consists of 7 members, named A to G. It is noteworthy that neuropilins do not interact with all isoforms equally and within those that interact, the affinities have different values. Thus, the main ligand for neuropilin-1 is SEMA3A, while for neuropilin-2 it is SEMA3F (109). Additionally, proteolytic modifications may influence the binding properties of semaphorins (110). The expression pattern, interactions and signalling mechanism of semaphorins has been thoroughly studied (reviewed in (111)). The interaction with neuropilins seems to be crucial for neural development by causing a chemorepulsive signal in axon guidance (reviewed in (64)) and the expression of semaphorins and neuropilins in neural systems reveals high level of complementarity, which suggests semaphorin function in defining repulsive regions (8, 9). Importantly, it is suggested in different subpopulations of cells at different stages of embryonic and postnatal development the same semaphorins (SEMA3A/E/F) could have either repulsive or no effect on axonal response due to differential expression of partners such as neuropilins (112).

In the neural system several functions of class III semaphorins were observed. The main function related to neuropilins acting as receptors for semaphorins is to generate a repulsive signal for growing axons, which is best studied for SEMA3A (3, 74, 113). Also for SEMA3E/F mediation of a repulsive function was suggested though no direct link with neuropilins was identified for this activity (113, 114). Semaphorins have diverse functions and in fact they are able to antagonise each other, e.g., SEMA3A inhibits neural overgrowth via neuropilin-1, whereas other semaphorins, such as SEMA3C and B, cannot induce such a response and may even block it. Interestingly, while SEMA3B/C act as antagonists of neuropilin-1, they are agonists of neuropilin-2 and via this molecule they can induce growth cone collapse (74, 115). It is noteworthy in the latter respect that only SEMA3A does not interact with neuropilin-2 (7). Similarly, it was shown in zebrafish that SEMA3D elicits both repulsive or attractive signals, depending on subset of neuropilins expressed in cells (116). Other important developmental roles have been observed for SEMA3B, which together with neuropilin-2 is involved in positioning the anterior/posterior orientation of the anterior commissure, a major brain commissural projection (71). Non neural developmental function is ascribed to SEMA3A/C and SEMA3C/F, where they are involved in regulating the processes of salivary gland formation and lung branching, respectively (101, 102). Also SEMA3G in zebrafish was shown to have a function in heart formation (117). Additionally, SEMA3C has been observed to elicit prosurvival responses in neurons, which correlates with the presence of neuropilins (118). SEMA3F, the main interacting ligand of neuropilin-2, was shown in rat cerebellar cone cells, which only express neuropilin-2, to elicit a chemoattractive rather than a repulsive signal (119). SEMA3F was also shown to be important for axonal wiring in guanylate cyclase-D expressing olfactory neurons together with neuropilin-2 (120). SEMA3F is also suggested to control neural crest cells migration together with neuropilin-2 (93). Therefore, a set of functions of semaphorins related to their interaction with neuropilins emerges, as they can elicit not only antagonising guidance signals, but direct also important developmental functions. The complexity of the signalling network, which is a result of multiple members of semaphorin family, permits a very

Table 2. Neuropilins interacting partners

Name	References
Neuropilin-1	
VEGFR1/Flt-1	69
VEGFR2/KDR/Flk-1	70
FGFR-1	68
Plexins	157
L1-CAM	158
Integrin beta-1	72
c-Met	147
SEMA3A	3
SEMA3C, D, E	159
VEGFA121	138
VEGFA165	69, 83, 160
VEGFB	144, 160
VEGFC/D	76
FGF-1,2,4,7	68
PIGF-2	83, 143, 160
HGF/SF	68, 148
NIP	59
FGFBP	68
Prion protein	68
Neuropilin-1/2	68, 73
Heparin	82, 83
Neuropilin-2	
VEGFR2	87
VEGFR3	76, 87
SEMA3C, F	7
VEGFA145 (160
VEGF165	160
VEGFC	76, 160
VEGFD	76
PIGF-2	160
Neuropilin-1	73

fine mechanism of control and, thus, implicates an important role for neuropilins.

As neuropilins are implicated in the formation and progression of tumours, the impact of semaphorins in these processes has been studied. In human pancreatic cancer, high levels of SEMA3A were suggested to be associated with an increase in the malignancy of the tumours and also correlated with higher levels of expression of neuropilin-1 (121). In contrast, in endothelial cells SEMA3A was suggested to decrease cell proliferation in a neuropilin-dependent manner and the inhibition of expression of both neuropilins resulted in the cells losing their sensitivity to pro-apoptotic signals caused by Sema3A (122). SEMA3F expression has been linked to reduced tumorigenicity and tumour formation, which was attributed to its interaction with neuropilin-2 and the inhibition of the activities of VEGF165 and FGF-2 (123-125). Another semaphorin, SEMA3B, was shown to enhance apoptosis and reduce mitosis in a neuropilin dependent way in lung and breast cancer cells (126). This finding is important, as it relates neuropilins to the cancer field where sets of proteins of given properties can drive pathological process, and therefore expands the potential complexity of the molecular networks that drive tumour progression and metastasis.

Another large group of neuropilin interacting ligands are members of the VEGF family. This family consists of five members in mammals, VEGFA, B, C, D and PIGF (reviewed in (127)). The functional importance of the interaction of neuropilins and VEGFs is related to angiogenesis (128-130) and neural development (131).

Several mechanisms such as alternative splicing and proteolytic processing (by matrix metalloproteinases or plasmin) diversify the number of bioavailable VEGF isoforms (132-134). Interactions with neuropilins involve a specific subset of these variants (Table 2), while signal transduction is thought to occur via the canonical VEGF receptor tyrosine kinases (127). It is noteworthy that much less is known about neuropilin-2 function and VEGF family members than about neuropilin-1. The feature that seems to be required for VEGF to bind to both neuropilins are exons 7-8, where exon 7 encodes the heparin binding site in VEGF. Thus, the VEGF121 isoform that lacks the ability to bind heparin does not interact with neuropilins (14, 135). An absence of interaction with neuropilin-1 was also shown for isoforms lacking only exon 8, such as VEGF165b and VEGF159 (136). Another unusual isoform is VEGF145, which also lacks exon 7, however, it can bind heparin and is able to interact with neuropilin-2, but not neuropilin-1 (137). In one study VEGF121 was found to interact with neuropilin-1 with an affinity similar to that of VEGF165, which was explained by the fact that commercial VEGF121 lacks the C-terminus, which is crucial for the interaction with neuropilin-1 (138). However, not all studies with VEGF121 have used C-terminally truncated protein, for example, full-length VEGF121 produced in baculovirus also fails to bind to neuropilins (70). Nevertheless, in two independent studies similar concentration of VEGF121 was observed to elicit either similar effect as VEGF165 or much weaker (138-140).

Next, neuropilins can bind to VEGF receptors and it was shown that neuropilin-1 can interact with VEGFR2 (141) and VEGFR1 (69), while neuropilin-2 can interact with VEGFR1, VEGFR2 and VEGFR3 (76, 87, 142). Apart from VEGFs which can bind both VEGFR1/2, neuropilin-1 can also bind VEGFR1 specific PIGF and VEGFB, and VEGFR2 specific viral VEGFE (143-145). The interesting feature of neuropilins is that they may participate in the formation of signalling complexes not only in *cis*, but also in *trans*, between two cells (75). The formation of complexes between neuropilins and ligands and receptors for VEGF has been suggested to have several effects. Thus, it was shown that VEGF165 may possess a higher signalling potency, compared to that of VEGF121 although they both bind VEGFR2 with similar affinity, due to interaction with neuropilin-1 and formation a ternary complex neuropilin-1-VEGF165-VEGFR2 (141). Moreover, it was shown that mutagenesis of VEGF165, which blocked its binding to VEGFR2, but not to neuropilin-1, resulted in the phosphorylation of VEGFR2 (70). Interaction of neuropilin-1 with VEGFR2 was suggested to result in an enhancement of signalling potency, however two possible mechanisms explaining this were proposed. In one this enhancement was thought to be due to the formation of a complex without affecting the complex affinity for ligand (14, 141). Whereas in a different study, a mechanism whereby neuropilin enhanced ligand affinity was proposed (69). Interestingly, in case of neuropilin-1 and VEGFR1 complex no influence on VEGF165 complex affinity was observed (69). Importantly, the formation of complexes between

Neuropilins

neuropilin-1 and VEGFR2 was questioned and a VEGF165-dependent, but not VEGF121, mechanism was suggested (75).

The formation of complexes between neuropilins, VEGF ligand and receptor is complicated by the fact that all three types of proteins also interact with heparan sulfate/heparin. Some work has focused on identifying the relationship between these four partners. In one study it was suggested that neuropilin-1-VEGF binding is strongly enhanced by the addition of heparin, in a manner dependent on the size of the heparin oligosaccharides. This was explained by longer fragments of heparin affecting the avidity of the complex (69, 135). Heparin may also be important for the interaction of neuropilin-2 and VEGF165 (76). It is also suggested to inhibit binding of neuropilin-1 and VEGFR1, which is consistent as the VEGFR1:neuropilin-1 binding site overlaps with the heparin binding site. Interestingly, an interplay between VEGF165 and VEGFR1 to bind neuropilin-1 was shown, which implicates overlapping binding sites for these two targets in neuropilin. Consequently heparin was suggested to have negative regulating effect as it interferes with VEGFR1 binding but enhances VEGF165 binding (69). A related study demonstrated that glycanlated neuropilin-1 may increase VEGF165 binding and signalling, thus showing the importance of the sugar-modified isoforms in the mechanism of signalling control (62). Another heparin function is possibly to mediate neuropilin-1 multimerisation (69).

Other VEGFs were also shown to be important interacting partners of neuropilins. VEGFB similarly to VEGFA possesses a heparin binding site and via this region it interacts with neuropilin-1 (144). VEGFC and VEGFD bind both neuropilins, however, only interaction of VEGFC with neuropilin-2 occurs in the absence of heparin, while the others require heparin. Importantly, VEGFC/D do not contain typical VEGFA heparin binding sites, which is thought to mediate interaction with neuropilins. Indeed, it was shown that VEGFC uses its N-terminal to bind neuropilins. On the other hand, in order to bind VEGFC neuropilin-1 requires the b1b2 domain and heparin, while neuropilin-2 requires the b1b2 domain and either heparin or a1a2, which may make the a1a2 domain functional equivalent to heparin in this respect (76).

The role of neuropilins' interactions with other growth factors is much less understood. PIGF-2 is suggested to potentiate VEGF signalling (146) and increased motility (143). FGF2 together with neuropilin-1 has stimulating effect on endothelial cells (68). Another neuropilin interacting partner, HGF/SF, is suggested to promote cancer progression in two independent studies (147, 148). It was shown that neuropilin-1 is essential for successful signalling and response in both cases and thus, enhances cell survival and invasion through activation of c-MET pathway through direct c-MET interaction.

Collectively, the structural data reviewed here together with a set of interactions and associated functions enriched by genetic studies enable this initial compilation

of facts about neuropilins that present contemporary knowledge about this family of proteins. According to the historical profile of neuropilins the first publication concerning neuropilin-1 (1) was in 1991, and at the moment there are around 700 available publications. However, the complexity of the action of neuropilins seem to grow proportionally to the number of studies devoted to these proteins. Therefore, the current approaches for elucidating the function of neuropilins needs to be replaced by novel approaches to characterise protein functions which would accommodate the flexible and multifunctionality of proteins such as neuropilins.

4.3. Functional significance of neuropilins' interactions

Neuropilins have established cellular functions such as cell guidance, angiogenesis and cell adhesion. In the simplified models the cell guiding function is ascribed to the interaction of neuropilin with semaphorins and angiogenic function to the interaction with VEGF. The basis of the adhesive property of neuropilin are still not known. Contemporary knowledge of neuropilins permits their schematised characterisation, however, there are still many missing elements in the puzzle.

A first key element still not fully understood is the issue of neuropilin oligo/multimerisation. The question of its functional importance and a putative switch between the action of monomeric and oligo/multimeric forms could play a significant role in the regulation of responses elicited via neuropilins. Moreover, this mechanism could be allied to the hypothesised competition between soluble and membrane bound neuropilins. Evidently, such a switch would be dependent on protein interactions that involve neuropilin and, therefore, could determine their ability to form homophilic associations.

Another mystery is the influence of heparin on neuropilin function. Interactions with glycosaminoglycans (heparin being a common experimental proxy) is a hallmark of many extracellular regulatory molecules, yet the diversity of data presented so far on neuropilin is substantial and leaves open any discussion of the functional significance of the interaction of neuropilin with the polysaccharide. Firstly, the issue of the structural role of heparin as a molecule involved in the formation of signalling complexes in which neuropilin is involved needs resolution. Secondly, the question of heparin-dependent signal transduction and modulation of signalling requires clarification. Many of the partners of neuropilins bind heparin/heparan sulfate and the polysaccharide is an integral part of their ligand-receptor complex. How neuropilin fits into such complexes and the role of its interaction with the polysaccharide is not known. Thirdly, as a holistic functional consequence *in vivo*, the relation between neuropilin dependent responses and the differential expression of specific protein-binding structures by heparan sulfate observed in tissues may provide a means to specify particular signalling outcomes and the selection of partners by neuropilin.

Next, the membrane localisation of neuropilins indicates that they possess interactions in three different

compartments, namely the extracellular, intracellular and transmembrane environments. Therefore, neuropilin is a target of extracellular signalling molecules, an integral part of the signalling complexes that are formed at the cell surface and a molecule that triggers directly intracellular signalling cascades.

Last but not least is the involvement of neuropilin in cell-cell contact. Since it is confirmed that neuropilins can act *in trans* between distinct cells, another field of neuropilin function emerges, which ascribes to neuropilins the important function of maintaining the physical and functional connectivity of tissue. Therefore, neuropilins apart from bridging cells may also play a part in communication between them.

5. PERSPECTIVES: NEUROFILINS IN MOLECULAR NETWORKS

Neuropilins have a large number of structurally unrelated molecular partners, though the biological functions of a number of these interactions remain to be elucidated. Neuropilins should thus perhaps be considered as multifunctional proteins. The functions of neuropilins at any given time will depend on the localisation of the neuropilins in plasma membrane domains, and their association with membrane bound and pericellular proteins and glycosaminoglycans or glycans, thus their functions depend ultimately on the cellular proteome and glycome. However, individual neuropilin molecules are likely to partition between different partners or groups of partners. Thus, on a single cell, not all neuropilin molecules may be engaged in the same functionality. This complexity is founded on a protein structure, which seems able to accommodate multiple partners. Different parts of the neuropilin protein interact with members of VEGF family, semaphorins, signalling receptors and heparin. Neuropilins possess considerable unstructured regions in the interdomain linkers and loops joining secondary structural elements. Such unstructured regions are a hallmark of sites of interaction, which often become structured in the molecular complex. The discovery of the the first partner of the intracellular domain of neuropilin-1, the PDZ motif containing NIP, suggests that, like other transmembrane proteins with small cytoplasmic stubs, such as syndecans, neuropilins may possess a reasonably complex intracellular interactome. However, a complete description of the partners of the intracellular domain remains to be established. The picture of neuropilin function is perhaps somewhat confounding, which is a consequence of its multifunctionality and complexity. Thus, it is expressed by many cell types; as a co-receptor of several other receptors it modifies their signalling potency; it interacts with and is probably regulated by heparan sulfate; its developmental role is clearly significant, but there is no obvious single molecular mechanism that can explain, for example, the phenotypes of neuropilins knockout mice. Collectively, these data suggest that a simple "A interacts with B, causing signals X, Y and Z" does not explain the biological functions we can observe. This problem requires a modification of our models of

molecular function that incorporate the idea of a protein interactome. Thus, instead of focusing on one protein, a model where sets of proteins cooperating in the same moment and in the same place determine the functionality of the components of the complexes may provide a more adequate explanation of molecular function. Therefore, the overall cell response is a result of the cooperation of multiple molecules working in concerted networks to generate intracellular signals.

A major challenge for this field, as for much of postgenome biology, is to define molecular function. However, molecules such as neuropilins, which seem to represent complex regulatory nodes in molecular networks pose major analytical problems. Thus, the challenge is one of resolving complexity in the context of different individual neuropilin molecules performing different functions at the same time in a single cell. Current approaches will simply average the functions across the population of neuropilins. It seems likely that an individual molecule approach may be required to resolve the intricacies of the functions of neuropilins.

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Abbreviations: FGF: Fibroblast Growth Factor; HGF/SF: Hepatocyte Growth Factor/ Scatter Factor; HS: Heparan Sulfate, NRP-1/-2: Neuropilin-1/-2; PlGF: Placenta Growth Factor; VEGF: Vascular Endothelial Growth Factor; VEGFR: VEGF Receptor.

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