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Towards a Structure-Function Relationship for Vascular Endothelial Growth Factor-B (VEGF-B)

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Abstract The vascular endothelial growth factor (VEGF), or VEGF-A, is intimately involved in both physiological and pathological forms of angiogenesis. VEGF-A is now recognized as the founding member of a family of growth factors that has expanded to include VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). This family of cytokines binds differentially to at least three receptor tyrosine kinases, however, the extent to which family members other than VEGF-A contribute to physiological and pathological angiogenesis remains unclear. Issues that are of relevance include uncertainty regarding the consequences of signaling through VEGF-R1 in particular, and the ability of some family members to heterodimerize, leading to the possibility of heterodimeric receptor complexes. Structural characterization is one approach that can be used to address these issues, however, the vast majority of previous structure-function studies have only focused on VEGF-A. While these studies may provide some clues regarding the structural basis of the interaction of other family members with their receptors, studies using the ligands themselves are clearly required if highly specific interactions are to be revealed. With the recent progress toward refolding and purifying substantial quantities of other VEGF family members, such structural studies are now possible. Here, these issues are addressed with a particular emphasis on VEGF-B and its receptors.

Key words: VEGF-B, refolding, inclusion body, angiogenesis, recombinant protein, high-level expression

Blood vessels form through the process of vasculogenesis, where endothelial cells (EC) proliferate and migrate to assemble into capillary tubes and then form a mature vessel [30]. New vessels are also capable of sprouting from existing vessels *via* angiogenesis, which requires a similar sequence of endothelial cell proliferation, migration, and cellular interactions [30]. While vasculogenesis is restricted to the embryo, angiogenesis occurs both during embryonic development and following birth. Both processes appear to be tightly regulated by a set of soluble factors or cytokines that interact with receptors that are expressed on, and in some cases restricted to, EC and pericytes. Angiogenesis, and the cytokines that regulate this process, also play a central role in a number of significant pathologies, including the growth of solid and dispersed tumors [24], the development and progression of rheumatoid arthritis [13, 39], and visual impairment found in diabetics and the aged [3].

While angiogenesis is a characteristic feature of some pathologies, a number of others are characterized by a chronic and severe deterioration in blood flow. Ischemic heart and/or limb disease can be the result of artery occlusion caused by atherosclerotic lesions, and represents a leading cause of morbidity and mortality worldwide. Currently, there is considerable interest in using the cytokines that regulate angiogenesis as novel therapeutics for the treatment of ischemic disease, with a number of clinical trials either in progress or recently completed [23]. In support of this approach, the recent analysis of gene targeted mice suggests a critical role for these cytokines in the protection of the heart under hypoxic conditions. Mice that express only the shortest of the VEGF-A splice forms ($VEGF\text{-}A_{121}$) have impaired postnatal myocardial angiogenesis, which ultimately leads to ischemic cardiomyopathy [11]. In another example, the recovery from myocardial ischemia in mice with a targeted deletion of the gene coding for VEGF-B is substantially impaired [6]. Not surprisingly, expression of the VEGF-A and VEGF-B receptor tyrosine kinase VEGF-R1, is also upregulated under these conditions.

Here, the functional and biological characteristics of VEGF-B are compared and contrasted with related members that bind to the same set of receptors, and the biological significance of ligand heterodimerization is explored. Structure-

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function studies represent a valuable approach to addressing these important issues. However, for such studies to occur, it is essential that pure and active forms of both homodimeric and heterodimeric species are available. While VEGF family members share common structural motifs, differences in sequence and structure are likely to influence their physicochemical properties. Methods that have been used to overexpress and refold recombinant forms of VEGF family members are described, as well as strategies that will aid in the production of presently uncharacterized homodimeric and heterodimeric species.

VEGF-B and Related Proteins

Based on sequence homology, the VEGF family of cytokines now encompasses six proteins including the founding member, VEGF or VEGF-A, VEGF-B [17, 18], placenta-derived growth factor (PlGF; [44], VEGF-C [35], VEGF-D [1, 54], and VEGF-E, which has been identified in the genome of the Orf poxvirus [47, 51, 79]. Each subunit of the functional homodimer possesses eight conserved cysteines, six of which are involved in three intramolecular disulfide bonds that constitute a cystine knot motif characteristic of PDGF superfamily members. While VEGF-C and VEGF-D form noncovalent homodimers [36, 66], the remaining members possess two intermolecular disulfide bonds that bring the monomeric subunits together in a head-to-tail fashion, as depicted in Fig. 1 [49].

There are at least five different isoforms of VEGF-A that arise from alternative mRNA splicing, ranging in length from 121 to 206 residues per monomeric subunit [32, 40, 57, 72]. Each isoform shares a common N-terminal receptor binding domain of 115 residues per monomer while each of the three longer forms (165, 189 and 206) contains a C-terminal heparin binding domain encoded within exons 6 and 7 [21, 38], or in the case of VEGF-A_{145}, exon 6 [57]. Similarly, three alternative splice variants of PlGF have been identified [9, 45]. PlGF-1 is the smallest, with the mature form resulting from the cleavage of a 20 amino acid signal peptide. PlGF-3 possesses a 216-nucleotide insertion between exons 4 and 5, and results in a 72-residue C-terminal extension relative to PlGF-1 [9]. For PlGF-2, an additional 21-residue insertion at exon 6 is highly basic and imparts the heparin binding affinity that is lacking in both PlGF-1 and PlGF-3 isoforms.

There are two isoforms of VEGF-B (167 and 186 amino acids) that arise through the use of an alternate splice acceptor site within exon 6, resulting in an insertion of 101 base pairs and a concomitant frameshift [29]. Although both isoforms share the same 115 N-terminal amino acids, their carboxyl-terminal domains differ substantially. As such, these isoforms exhibit distinct biological characteristics, including their respective affinity for heparin (167 isoform only) and subsequent bioavailability.

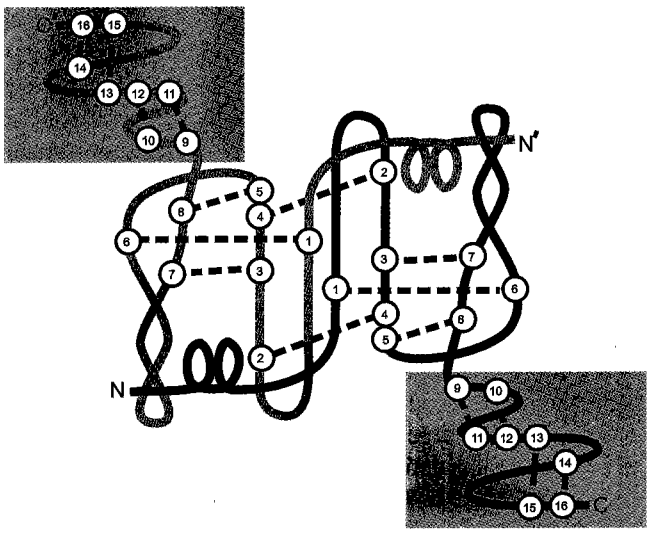

Fig. 1. Diagrammatic representation of folding and dimer formation within the VEGF family of cytokines.
The disulfide pattern highlighted by the shaded areas was determined in VEGF-A_{165} [21, 37] and predicted for VEGF-A_{189}, VEGF-A_{206}, and VEGF-B_{167} by similarity. VEGF-C and VEGF-D are non-disulfide-linked heterodimers.

VEGF-R1 and Neuropilin-1 are Receptors for VEGF-B

Three tyrosine kinase receptors have been found to bind members of the VEGF family: VEGF-R1, VEGF-R2, and VEGF-R3 [50]. VEGF-B binds specifically to VEGF-R1 [52], which is expressed on endothelial cells, vascular smooth muscle cells [75], and blood monocytes [12]. VEGF-A also interacts with VEGF-R1 [18, 64], and in addition, binds to the kinase insert domain-containing receptor VEGF-R2 [69, 70], the expression of which appears to be restricted to endothelial cells [18]. Whereas the biological functions of VEGF-R2 have been well characterized, the consequences of signaling through VEGF-R1 remain controversial [22]. The targeted deletion of either the VEGF-R1 or VEGF-R2 gene results in embryonic lethality with a subtle difference in phenotype leading initially to the suggestion that, while -R2 signals migration and proliferation, -R1 signals capillary tube formation and endothelial cell-cell interaction [25, 30]. However, in subsequent "knock-in" studies where the VEGF-R1 gene was replaced with a version encoding only the transmembrane and extracellular domains (i.e., non-signaling), the lethal phenotype was reversed. This has led to the suggestion that, in the embryo at least, VEGF-R1 acts as a non-signaling decoy receptor that plays a role in regulating the access of VEGF-A to VEGF-R2 [31]. Interestingly, of these two receptors, only VEGF-R1 appears to be upregulated in direct response to hypoxic conditions [27].

The neuronal cell-guidance receptor, neuropilin-1 (NP-1), has also been identified as a receptor for both isoforms of VEGF-B [46]. Although NP-1 binds tightly to full-length VEGF-B_{167}, its interaction with VEGF-B_{186} occurs only after proteolytic processing of the C-terminal domain

exposes a basic NP-1 recognition site [46]. Interestingly, the addition of heparin enhances the interaction between NP-1 and its ligands, including VEGF-B_{186}. This suggests that at least a proportion of the effect of heparin may be at the level of the receptor since VEGF-B_{186} does not contain a heparin binding domain. Where the ligand does retain a heparin binding domain, for example VEGF-B_{167}, VEGF-A_{165}, and PLGF-2, the extent to which this interaction influences NP-1 binding remains to be determined. The application of X-ray crystallography or NMR spectroscopy will be useful in shedding light on the actual mechanisms operating in these binding processes. X-ray crystallography has recently been used to demonstrate heparin induced conformational changes critical to the interaction of fibroblast growth factor-1 (FGF-1) with FGF-R2 [55].

The extent to which the binding of VEGF family members to NP-1 contributes to the signaling process remains unclear. However, NP-1 has been found to coexpress with VEGF-A and VEGF-R2 [33, 34, 59], while there is evidence to suggest that complex formation with VEGF-R1 occurs [26, 56]. Clearly, further work is required to fully characterize the significance of these interactions in terms of both their neuroprotective effects and signaling processes within the cell.

Structural Aspects of Receptor Binding

The receptor tyrosine kinases VEGF-R1 and VEGF-R2 share similar structural characteristics and belong to the platelet-derived growth factor (PDGF) family of receptors [73]. Their extracellular regions consist of seven immunoglobulin-like domains, each approximately 100 residues in length. Alanine scanning mutagenesis and structural characterization have revealed that two symmetrical surfaces located at each pole of VEGF-A are required for binding to VEGF-R2 [49]. Domain deletion studies with VEGF-R1 have shown that the ligand-binding region resides within the first three domains [4, 14, 15]. A modified VEGF-R1 construct containing only domains 2 and 3 binds VEGF-A with near-native affinity, and only a 60-fold decrease in binding affinity results when VEGF-A binds to domain 2 of VEGF-R1 (VEGF-$R1_{D2}$) [78]. The crystal structure of VEGF-A (residues 10–109) bound to VEGF-$R1_{D2}$ shows that VEGF-A does not change appreciably upon binding, suggesting that interaction is predominantly hydrophobic in nature [78].

Mutations of key residues involved in VEGF-R1 binding to VEGF-A [38] also reduce the affinity of VEGF-B binding to VEGF-R1 [52], and suggest that both VEGF-A and VEGF-B share a common VEGF-R1 binding domain. A VEGF-A mutant with a VEGF-R2 binding domain substituted with the homologous PlGF-2 sequence is still capable of inducing vascular permeability, albeit with reduced VEGF-R2 binding affinity [67]. Furthermore, comprehensive mutational studies of VEGF-A have shown that selective affinity for either VEGF-R1 or VEGF-R2 can be maintained even when nonconservative mutations of residues previously considered crucial for receptor binding are made [41]. Consequently, modeling the receptor binding of related molecules based on sequence and functional similarity may be misleading, since interaction sites specific to the related protein could be overlooked. This underlines the need for independent structural characterization of related VEGF family members.

As noted above, there has been significant progress in the characterization of the interactions between VEGF-R1 and the N-terminal domain of VEGF-A. However, little is known regarding the interaction of VEGF-R1 with the heparin binding domain of VEGF-A_{165} and related family members. Although it has been shown that the heparin binding domain of VEGF-A_{165} is not essential for VEGF-R1 binding, its loss decreases both VEGF-R1 binding affinity and endothelial cell mitogenic potency [38]. Furthermore, the growth-stimulatory activity of PlGF-2, which is associated with VEGF-R1 binding, is higher than that of PlGF-1, thereby suggesting a key role for the heparin binding domain [62]. Consequently, the role of the heparin binding domain must be characterized, as it may be critical to the differentiation of the VEGF family members that bind common receptors. The C-terminal region/heparin binding domain encoded by exon 7 in VEGF-A isoforms 165, 189, and 206 possesses eight additional cysteine residues that form four disulfide bonds, as illustrated in Fig. 1 [37]. Through sequence homology, exon 6 of VEGF-B_{167} is also likely to possess similar disulfide pairings, whilst this feature is not present in PlGF-2 (Fig. 2). The NMR structure of the heparin binding domain of VEGF-A_{165} has been solved

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                                          1                                    2     3  45      6
VEGF-B167 --------PVSQPDAPGHQRKVVSWIDVYTRATCQPREVVVPLTVELMGTVAKQLVPSCVTVQRCGGCCPDDGLECVPTGQHQVRMQILM
VEGF-A165 --------PMAEGGGQNHH-EVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEESNITMQIMR
PLGF-2    LPAVPPQQWALSAGNGSSEVEVVPFQEVWGRSYCRALERLVDVVSEYPSEVEHMFSPSCVSLLRCTGCCGDEDLHCVPVETANVTMQLLK

                             7 8                  9  10            11 12 13      14          15 16
VEGF-B167 IR-YPSSQLGEMSLEEHSQCECRPKKKDSAVKPDSPRPLCPRCT---QHHQRPDPRTCRCRCRRRSFLRCQGRGLELNPDTCRCRKLRR
VEGF-A165 IKPHQGQHIGEMSFLQHNKCECRPKK-DRARQEN----PCGPCSERRKHLFVQDPQTCKCSCKNTDS-RCKARQLELNERTCRCDKPRR
PLGF-2    IRSGDRPSYVELTFSQHVRCECRPLRE-KMKPERRRPKGRGKRRRENQRPTDCHLCGDAVPRR
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Fig. 2. Sequence alignment of VEGF-B_{167}, VEGF-A_{165}, and PlGF-2.
The numbers denote the sequential order of cysteines within the primary sequence.

[21]. Based on the structural flexibility exhibited within this domain and a highly localized surface charge distribution, residues thought to be critical to heparin binding have been identified. While the heparin binding domain of VEGF-A_{165} only differs modestly from that of VEGF-B_{167}, the subtle differences in charge distribution and secondary structure may be sufficient to lead to highly selective heparin fragment binding and subsequent bioavailability. In the case of PlGF-2, the lack of homology with the heparin binding domains of VEGF-A and VEGF-B suggests that there will be substantial difference in the heparin fragments that it binds.

Ligand Heterodimerization

Although there has been some recent progress toward unraveling the biological responses to VEGF receptor activation in endothelial and other cells [5, 28, 58], there is still significant uncertainty regarding the precise role of homo- and heterodimeric ligands. To date, PlGF · VEGF-A heterodimers have been the only naturally occurring heterodimers identified within the VEGF family and were first observed in rat glioma cells [19]. DiSalvo *et al.* purified what they believed to be a molecule with mitogenic activity comparable to that of VEGF-A_{165}. Subsequent analysis identified that this was a heterodimeric species made up of rat VEGF-A_{165} and PlGF-2 [19]. Heterodimeric species based on VEGF-A and PlGF isoforms (VEGF-A_{165}, VEGF-A_{121}, PlGF-1, and PlGF-2) have also been prepared *in vitro*. *E. coli*-derived inclusion bodies containing the respective monomeric subunits were solubilized and then dialyzed together to yield readily purified heterodimeric species [7, 8]. The reported mitogenic activities of these heterodimeric species were largely comparable to that of homodimeric VEGF-A_{165}.

Heterodimers of VEGF-A_{165} and VEGF-B_{167} have also been observed following coexpression in 293EBNA cells [53]. In addition, in our own studies purifying VEGF-B from transfected CHO cells, problems were encountered with dimerization to endogenous CHO-derived VEGF-A. A Northern blot analysis that demonstrated a largely overlapping expression profile for these two ligands suggests that the heterodimers may also appear *in vivo*, although this has not yet been formally demonstrated. The possibility of VEGF-B heterodimerization with PlGF isoforms must also be considered in this context, although there are currently no reports describing the formation of such a molecule.

Overexpression of VEGF Family Members for Structure Function Analyses

There is significant interest in the therapeutic use of the VEGF family members in a variety of pathological processes associated with tissue ischemia. The clinical development of protein based therapeutics will require the production of substantial amounts of purified protein [10]. In addition, the availability of VEGF family members as either homodimeric or heterodimeric species will allow specific interactions between these ligands and their receptors to be studied in molecular detail through NMR and X-ray crystallographic techniques. As a result of advances in recombinant protein expression technologies, there are a number of potential strategies now available for large-scale protein production, including expression in yeast, mammalian cells, insect cells, and bacterial systems. The choice of expression systems must be considered in the context of the physical and biological characteristics of the protein to be expressed. Crucial issues that must be taken into account include the significance of protein glycosylation on the biological function, and the ability of the expression system to produce a protein that is, or can subsequently be, folded correctly.

Although glycosylation appears to be common to many VEGF family members, it is unlikely that it is critical for activity *in vivo* [74]. Consequently, the inability of *E. coli* to glycosylate proteins does not preclude its use as an expression system for VEGF family members. While most expression systems are capable of folding proteins with complex disulfide bond pairings, the isomerases located within the *E. coli* periplasm are generally less able to do this [61]. This can contribute to the partitioning of the protein into insoluble inclusion bodies. If *E. coli* is the expression system of choice, refolding strategies that yield biologically active material from the inclusion bodies are essential. VEGF family members are characterized by a complex set of inter- and intramolecular disulfide pairings, and as a result, it is not surprising that they are most often expressed in *E. coli* as inclusion bodies. Although the need to refold material from an aggregate state may not be considered ideal, there has been remarkable success with VEGF family members using relatively straightforward refolding protocols.

The purity of the target protein prior to refolding can significantly affect the success of any refolding strategy adopted. Commonly, a prefolding chromatographic purification step is included to remove any contaminating protein material that may interfere with the folding process [16, 43]. Several protocols for VEGF family members include chromatographic purification steps prior to refolding (Table 1). For example, the expression of VEGF-B_{167} yields a C-terminally truncated form of the protein that appears to possess the conserved cysteines required for dimerization [63]. Failure to remove the clipped form yields a combination of heterodimeric and homodimeric species made up of both the full-length and clipped forms.

The incorporation of an oxidation step appears to be a critical requirement in the refolding of VEGF family members, presumably due to the large number of disulfide bonds present [2, 76]. Methods used to induce disulfide

Table 1. Bacterial expression and refolding strategies for selected VEGF family members. (n.d.: not described).

Protein	Expression system	Vector	Tag	Pre-refolding treatment	Refolding procedure	Final purification	Reference
VEGF-A_{11-109}	SB558	pQE30	His_6	Metal chelate affinity	Dialysis (Tris/cysteine, pH 8.4)	Anion exchange	[20]
VEGF-A_{121}	BL21(DE3)	His-pET	His_6	–	Sequential dilution to 0.3 M urea	Metal chelate affinity	[65]
VEGF-A_{121} VEGF-A_{165}	n.d.	n.d.	n.d.	–	Dialysis (0.4 M NaCl, Tris, pH 8.0)	1. Cation exchange 2. RP-HPLC	[38]
VEGF-A_{121} VEGF-A_{165}	JM109	pCYTEXP3	–	1. S-sulfonation 2. Size exclusion 3. Dialysis (Tris, pH 8.0)	Dialysis (2 M urea, reduced/oxidized glutathione, Tris, pH 8.0)	1. Dialysis (Tris/Mes, pH 5.0) 2. Cation exchange	[7]
VEGF-A_{165}	BL21(DE3)	pET3d	–	1. Cation exchange 2. Size exclusion	Sequential dilution to 0.3 M urea	–	[65]
VEGF-B_{167}	BL21(DE3)	pET15b	His_6	1. Metal chelate affinity 2. Cation exchange	Dialysis (0.5 M GdCl, cysteine/cystine, pH 8.5)	1. Dialysis (0.1 M acetic acid) 2. HILIC 3. RP-HPLC	[63]
VEGF-A_{121} · PlGF-1 VEGF-A_{165} · PlGF-1 VEGF-A_{165} · PlGF-2	JM109	pCYTEXP3	–	1. S-sulfonation 2. Size exclusion 3. Dialysis (Tris, pH 8.0)	Dialysis (3 M urea, reduced/oxidized glutathione, Tris, pH 8.0)	1. Dialysis (Tris/Mes, pH 5.0) 2. Cation exchange	[7]
PlGF-1 PlGF-2	JM109	pCYTEXP3	–	1. S-sulfonation 2. Size exclusion 3. Dialysis (Tris, pH 8.0)	Dialysis (4 M urea, reduced/oxidized glutathione, Tris, pH 8.0)	1. Dialysis (Tris/Mes, pH 5.0) 2. Cation exchange	[7]

bond formation include air oxidation [47] or the use of oxido shuffling reagents [20, 38, 63, 65, 68], which act by continually breaking and reforming disulfide bonds until the most energetically favored global fold (or ensemble of folds) is obtained.

A common feature of all refolding systems used for VEGF family members is the addition of small molecules that act to enhance the yield of the renatured protein [61]. Although the precise mechanism of action is not fully understood, it is likely that these additives influence both the solubility and stability of the protein in the unfolded, intermediate, and fully folded forms [61]. For example, nondenaturing concentrations of chaotropic agents such as guanidinium hydrochloride (GdCl) or urea may act by destabilizing aggregate intermediates held together by hydrophobic interactions [60]. L-arginine has also been widely used in recent times [61], and while a precise role is not fully understood, its beneficial effects appear to lie in an ability to increase the solubility of folding intermediates [42]. The incorporation of small molecule additives has been used to successfully refold VEGF family members [7, 63, 65]. For instance, the refolding of VEGF-B_{167} in the absence of additives such as L-arginine or GdCl yields a protein that is predominantly aggregated, whilst high concentrations of NaCl have also been used in refolding buffers for VEGF-A isoforms [38]. The incorporation of many other potential additives including detergents remains to be explored [42].

The protein concentration prior to refolding has also been noted as an important factor for successful protein refolding [61]. Whilst significant aggregation occurs at protein concentrations greater than 100 μg/ml for VEGF-B_{167}, concentrations far greater than this have been used successfully for other VEGF family members [20]. Since dialysis at low protein concentrations may be prohibitively expensive, alternative approaches that yield a soluble and active protein would be highly advantageous for commercial production. There are several approaches to improving the efficiency of producing soluble, refolded protein that is directed toward the periplasm. These include the coexpression of various *E. coli* chaperone genes that minimize off-pathway aggregation reactions, and the incorporation of folding catalysts to accelerate specific rate-limiting steps in the folding pathway [71]. An alternative approach involves the production of soluble refolded protein in the cytoplasm. Strains of *E. coli* containing a thioredoxin reductase mutation have been used to allow foreign proteins with disulfide bonds to be expressed and folded correctly within the cytoplasm [17]. Due to the less reducing environment within the cytoplasm under these conditions, cysteines are capable of oxidizing at a sufficiently slow rate, thereby allowing the correctly folded conformation to form.

While many of the experimental techniques used to refold VEGF family members are drawn from general procedures, there are critical steps in the refolding process that are common to all VEGF family members. As illustrated for VEGF-B_{167}, the inherent difficulty in producing active material is not necessarily associated with the refolding procedure itself, but may lie in the purification of refolded material.

CONCLUSION

The functional significance of the distinct receptor binding properties exhibited by VEGF family members remains unclear and is further complicated by their ability to heterodimerize. Whether heterodimerization plays a biological role *in vivo* remains to be resolved. Understanding the significance of the various dimerization pathways available to members of this family in terms of structure and function is critical if we are to gain a deeper understanding of their role in both normal physiological and pathological processes.

To date, structural studies examining members of the VEGF subfamily have concentrated on variants of VEGF-A, both free and bound to either receptor domains or peptide antagonists [20, 48, 49, 77, 78]. Conclusions regarding VEGF family receptor binding have to a large extent been extrapolated from these studies. Novel refolding and purification strategies can also be used to further develop and refine structure-function relationships exhibited by these members in order to unravel the complex nature of receptor binding within the VEGF family of cytokines. Ultimately, the biological role of novel homo- and heterodimeric species will need to be determined with *in-vitro* or *in-vivo* models designed to demonstrate angiogenic or other activities.

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