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Crystal Structure of Human Vascular Endothelial Growth Factor-B: Identification of Amino Acids Important for Receptor Binding

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The development of blood vessels (angiogenesis) is critical throughout embryogenesis and in some normal postnatal physiological processes. Pathological angiogenesis has a pivotal role in sustaining tumour growth and chronic inflammation. Vascular endothelial growth factor-B (VEGF-B) is a member of the VEGF family of growth factors that regulate blood vessel and lymphatic angiogenesis. VEGF-B is closely related to VEGF-A and placenta growth factor (PlGF), but unlike VEGF-A, which binds to two receptor tyrosine kinases VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), VEGF-B and PlGF bind to VEGFR-1 and not VEGFR-2. There is growing evidence of a role for VEGF-B in physiological and pathological blood vessel angiogenesis. VEGF-B may provide novel therapeutic strategies for the treatment of vascular disease and be a potential therapeutic target in aberrant vessel formation. To help understand at the molecular level the differential receptor binding profile of the VEGF family of growth factors we have determined the crystal structure of human VEGF-$B_{10\text{-}108}$ at 2.48 Å resolution. The overall structure is very similar to that of the previously determined cysteine-knot motif growth factors: VEGF-A, PlGF and platelet-derived growth factor-B (PDGF-B). We also present a predicted model for the association of VEGF-B with the second domain of its receptor, VEGFR-1. Based on this interaction and the present structural data of the native protein, we have identified several putative residues that could play an important role in receptor recognition and specificity.

Keywords: angiogenesis; vascular endothelial growth factor-B; X-ray crystallography; receptor-binding; cysteine-knot protein

Introduction

Angiogenesis, the process of formation of vascular sprouts from pre-existing vasculature, is the result of a tightly regulated interplay between environmental and genetic regulators that display spatially and temporally coordinated activities.[1] Studies have identified several regulators of angiogenesis as therapeutic targets that have the potential to treat diseases such as cancer and ischemia.[2] The most important and extensively studied class of angiogenic regulators are the polypeptide vascular endothelial growth factors (VEGFs),[3] which belong to the cysteine-knot superfamily. The various members of the superfamily include platelet-derived growth factor (PDGF), vascular endothelial growth factor-A (VEGF-A), VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF).[4] These bioactive proteins exemplify the development of distinct biological functionalities albeit similar topology based on a cyclic knot of cysteine residues involved in both intra- and inter-chain disulphide bonds. This cysteine connectivity spatially brings the key residues involved in receptor recognition into close proximity of each other.

The function of the VEGF family of cytokines is mediated by differential binding to three receptor tyrosine kinases: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4), which are predominantly expressed on endothelial cells (EC).[5,6] The differential binding manifests such that VEGF-B

Abbreviations used: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; PlGF, placenta growth factor; r.m.s., root-mean-square; PDB, Protein Data Bank.

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and PlGF are specific for VEGFR-1,[7,8] while VEGF-A binds to both VEGFR-1 and VEGFR-2,[9] and VEGF-C and VEGF-D bind to both VEGFR-2 and VEGFR-3.[10] VEGF-A, VEGF-B and PlGF-2 also bind to the semaphorin receptor neuropilin-1 with high affinity.[11,12] Unlike the biological function of VEGFR-2 (angiogenesis) and VEGFR-3 (lymphangiogenesis) the role of VEGFR-1 has been harder to elucidate. VEGFR-1 was proposed to be a decoy receptor as deletion of the *Vegfr-1* gene was embryonic lethal due to a failure to develop vasculature[13] but deletion of only the intracellular kinase-domain of VEGFR-1 produced healthy mice.[14] Recent studies have shown that signalling through VEGFR-1 promotes endothelial cell differentiation into vascular tubes through NO release in part by limiting VEGFR-2-mediated endothelial cell proliferation[15] and that the intermolecular cross-talk between PlGF stimulated VEGFR-1 and VEGF-A activated VEGFR-2 can amplify ischemic myocardial angiogenesis.[16]

VEGF-B is expressed as two alternatively spliced isoforms, VEGF-B_{167} and VEGF-B_{186}, with non-homologous C-terminal domains.[17,18] VEGF-B exists as disulphide-linked homodimers and when co-expressed with VEGF-A as heterodimers.[19] The precise biological role of VEGF-B has remained elusive but recent evidence indicates both physiological and pathological functions (reviewed by Nash *et al.*[20]). VEGF-B is expressed highly during embryogenesis in the heart[21] and postnatally in most tissues including tumours, but most abundantly in cardiac and skeletal muscle.[22] Using gene knockout technology, VEGF-B appears to be required for normal heart function in adult mice but is not essential during development.[22,23] VEGF-B is an angiogenic cytokine as it promotes *in vivo* angiogenesis into Matrigel and neovascularization in a mouse hindlimb ischemia model *via* Akt and eNOS activation,[24] and transgenic expression of VEGF-B promotes vascular growth into Matrigel and in aortic explant assays and wound healing.[25] *Vegfb*$^{-/-}$ mice have reduced disease pathology and synovial angiogenesis in antigen-induced and collagen-induced arthritis models, implying a role for VEGF-B in sustaining chronic inflammation;[26] the cellular mediators of inflammation, monocytes and macrophages, express VEGFR-1.[27] The observation that VEGF-B correlates with the infiltrative margin of human colorectal cancer[28] and that human colorectal and pancreatic cancers express VEGFR-1 and that VEGF-B promotes migration and enhanced Matrigel invasion in these cells,[29,30] implies a role for VEGF-B in tumour development.

Here, we present the crystal structure of the receptor-binding domain of human VEGF-B (residues 10–108) at 2.48 Å resolution as well as results from a predicted model of VEGF-B in complex with the second domain of human VEGFR-1. VEGF-B, like the other growth factors from the VEGF-A family, associates with VEGFR-1 using two symmetrical binding sites located at the opposite ends of the homodimer. The VEGFR-1 binding site consists of residues presented from both the subunits of the VEGF-B dimer.

Results and Discussion

Quality of the structure

Human VEGF-$B_{10\text{-}108}$ crystallises as a homodimer in the hexagonal space group, $P6_4$. The structure has been determined at 2.48 Å resolution with one homodimer in the asymmetric unit. Details of the crystallographic statistics are given in Table 1. The overall topology of the protein is very similar to that of the other members of the cysteine-knot superfamily of growth factors. The first three amino-terminal residues of both chains (A and B) are not visible in the electron density map and hence were excluded from crystallographic refinement. Both chains contain residues 13–107. The final refined structure has an R_{cryst} of 28.6% and an R_{free} of 31.0%. Residues 38, 39, 42, 45, 88, 106 and 107 from chain A and residues 30, 37, 38, 39, 40, 45, 46, 84, 106 and 107 from chain B of the homodimer have been modelled as either alanine or glycine because of poor electron density. Analysis of the Ramachandran plot using the program PROCHECK[31] indicated that 81.1% of the residues lie in the most favourable region of the ϕ-ψ plot and 18.9% lie in the additional allowed regions. One peptide bond in the structure adopts a *cis*-confirmation: that connecting Val48 and Pro49 in both monomers. Difference density for one molecule of MPD (from the crystallisation medium) was observed during model building. The MPD molecule makes hydrogen-bonding interactions with Gln79 and Glu92 of chain B. The

Table 1. Crystallographic statistics

Unit cell dimensions ($P6_4$, 1 homodimer/a.u.)(Å)	$a=b=120.81$, $c=39.83$
Resolution (Å)	40–2.48
Reflections measured	110,109
Unique reflections	12,054
R_{sym} (%)[a]	9.6 (63.1)
$I/\sigma I$ (outermost shell)	12.9 (2.4)
Completeness (outermost shell) (%)	97.4 (99.3)
R_{cryst} (%)[b]	28.6
R_{free} (%)[c]	31.0
r.m.s. deviation in bond lengths (Å)	0.008
r.m.s. deviation in bond angles (deg.)	1.5
Average *B*-factor for protein atoms (Å^2)	
Main-chain atoms	40.1
Side-chain atoms	40.3
Solvent molecules	39.7
MPD molecule	36.9

[a] $R_{sym} = \Sigma(|I_j - \langle I \rangle|)/\Sigma\langle I \rangle$ where I_j is the observed intensity of reflection j, and $\langle I \rangle$ is the average intensity of multiple observations.

[b] $R_{cryst} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

[c] R_{free} is equal to R_{cryst} for a randomly selected ~6% subset of reflections not used in refinement.