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Mechanism of Dimerization of a Recombinant Mature Vascular Endothelial Growth Factor C

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Supporting Information

ABSTRACT: The vascular endothelial growth factors (VEGFs) and their tyrosine kinase receptors play a pivotal role in angiogenesis and lymphangiogenesis during development and in pathologies such as tumor growth. The VEGFs function as disulfide-linked antiparallel homodimers. The lymphangiogenic factors, VEGF-C and VEGF-D, exist as monomers and dimers, and dimerization is regulated by a unique unpaired cysteine. In this study, we have characterized the redox state of this unpaired cysteine in a recombinant mature monomeric and dimeric VEGF-C by mass spectrometry. Our findings indicate that the unpaired cysteine regulates dimerization via thiol−disulfide exchange involving the interdimer disulfide bond.

The blood vasculature forms during early embryonic development in a process called vasculogenesis. The vascular plexus is then remodeled in a process termed angiogenesis, while the sprouting of lymphatic vessels from lymph sacs is a process termed lymphangiogenesis. Angiogenesis and lymphangiogenesis also occur in the mature mammal in tissues subjected to ischemia, inflammation, wound healing, and tumor growth. These processes are tightly regulated in space and time by interconnected signaling pathways. Central to these processes are the vascular endothelial growth factors (VEGFs) and their tyrosine kinase receptors (reviewed in refs 1 and 2).

There are five VEGFs in mammals: VEGF, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). They belong to the platelet-derived growth factor/VEGF superfamily of secreted dimeric glycoprotein growth factors. The VEGFs bind with differing specificities to three transmembrane tyrosine kinase receptors found mostly on endothelial cells [vascular endothelial growth factor receptors 1−3 (VEGFR-1−3, respectively)]. Neuropilins 1 and 2 function as coreceptors for the VEGFRs. Binding of VEGF to VEGFR-2 triggers proliferation, survival, sprouting, and migration of endothelial cells and is the major mediator of angiogenesis. VEGF-B, PlGF, and VEGFR-1 are involved in angiogenesis in ischemia, inflammation, wound healing, and tumor growth but do not appear to be involved in angiogenesis during development. VEGF-C and VEGF-D activation of VEGFR-3 is involved in lymphangiogenesis during development[3,4] and tumorigenesis and metastasis in the mature mammal.

Expression of VEGF-C and VEGF-D has been found to correlate with vascular invasion, lymph involvement, and metastasis in murine and human tumors (reviewed in ref 5). Secretion of VEGF-C by tumor cells widens the collecting lymphatic vessels, which drains fluid from the immediate tumor environment and also likely facilitates the dissemination of tumor cells.[6] Lymphatic metastasis in different murine tumor models is stimulated by VEGF-C and VEGF-D and blocked by a soluble decoy VEGFR-3 (reviewed in ref 1).

Productive binding of VEGFs to VEGFRs requires that the growth factors be dimeric. The VEGF dimer stimulates dimerization of the VEGFR and autophosphorylation, which leads to recruitment of other signaling molecules and changes in cell function. Monomeric VEGFs do not stimulate receptor dimerization or autophosphorylation.[7] VEGF, VEGF-B, and PlGF appear to form only disulfide-linked antiparallel homodimers. The lymphangiogenic VEGF-C[8] and VEGF-D,[9] however, exist as monomers and dimers. The monomer−dimer equilibrium is influenced by an unpaired cysteine residue that is near the interdimer disulfide bond. This cysteine residue is found in only VEGF-C (Cys137) and VEGF-D (Cys117) and the *Caenorhabditis elegans* VEGF homologue, PVF-1 (Cys120).[7,10,11] Replacing the unpaired cysteine with alanine increases the biological activity of VEGF-C and -D *in vitro*, presumably by stabilizing the dimer,[7,11] and was required to stabilize the dimer for X-ray structural studies.[10,11] Replacing Cys137 with alanine in VEGF-C increases the arteriogenic activity in mouse tissue,[12] although replacing the equivalent residue in VEGF-D did not appreciably change the biological activity in mice.[10] VEGF-D can exist as both noncovalent[13] and disulfide-linked[14] dimers, and covalent dimer formation is enhanced by the presence of a small thiol. Incubation with millimolar concentrations of cysteine led to time- and concentration-dependent increases in disulfide-linked VEGF-D dimers.[14] In this study, we have characterized the redox state of the unpaired cysteine (Cys137) in monomeric and dimeric VEGF-C by mass spectrometry to improve our understanding of its role in VEGF-C (and VEGF-D) dimerization.

A ΔNΔC-VEGF-C protein construct consisting of an N-terminal FLAG tag followed by residues 112−227 that mimics a mature form of human VEGF-C[8] was produced in 293-EBNA cells (Supporting Information). The dimer was separated from monomeric VEGF-C by Superdex 200 gel filtration chromatography. The proteins were resolved on a NuPAGE Novex 12% Bis-Tris gel under nonreducing conditions and stained with

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Coomassie Brilliant Blue (Figure 1A). The protein bands were excised from the gel, destained, dried, and alkylated with 50 mM iodoacetamide in 25 mM ammonium bicarbonate for 30 min at 25 °C in the dark to prevent possible thiol−disulfide exchange in the protein. The gel slices were washed with 25 mM ammonium bicarbonate/50% (v/v) acetonitrile buffer, dried, and digested with 12.5 μg/mL trypsin in 25 mM ammonium bicarbonate at 30 °C overnight. The digestion was stopped by adding formic acid to a final concentration of 5% (v/v), and the peptides were eluted from the gel slices, resolved by high-performance liquid chromatography, and analyzed by mass spectrometry as described previously.[15] Positive ions were generated by electrospray and analyzed in an LTQ FT Ultra (Thermo Electron) mass spectrometer operated in data-dependent MS/MS acquisition mode. Data from mass spectrometry were searched using Mascot (version 2.3, Matrix Science) against the Human Uniprot database (2013_02). Search parameters were as follows: precursor tolerance of 10 ppm and product ion tolerances of ±0.4 Da. Cys-carboxyamidomethyl, oxidized Met, and pyro-Glu/Gln were selected as variable modifications with full tryptic cleavage of up to three missed cleavages. To calculate the ion abundance of peptides, extracted ion chromatograms were generated using XCalibur Qual Browser (version 2.0.7, Thermo). The area was calculated using the automated peak detection function built into the software.

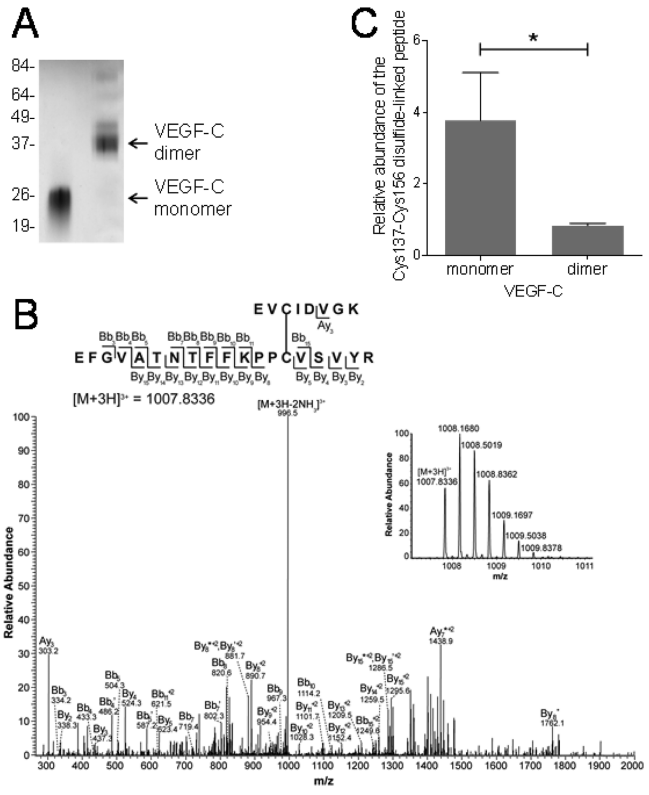

Figure 1. Cys137 forms a disulfide bond with Cys156 in the VEGF-C monomer. (A) The VEGF-C monomer and dimer were resolved via sodium dodecyl sulfate−polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. The main band of the protein was excised and analyzed by mass spectrometry. The positions of molecular weight markers are shown at the left. (B) Tandem mass spectrum of the peptide linked by a disulfide bond between Cys137 and Cys156 in the VEGF-C monomer. The accurate mass spectrum of the peptide is shown in the inset (observed $[M + 3H]^{3+} = m/z$ 1007.8336; expected $[M + 3H]^{3+} = m/z$ 1007.8331). Ions with a neutral loss of small molecules are labeled with an asterisk (loss of ammonia) and a prime (loss of water). (C) Relative abundance of the Cys137−Cys156 disulfide-linked peptide in the VEGF-C monomer and dimer with reference to the control peptide, SIDNEWR. The bars and errors are the mean and standard error of three independent experiments. The asterisk indicates a p value of <0.05 using a Student's paired t test.

Cys137 was found to form a disulfide bond with Cys156 in the VEGF-C monomer and dimer (Figure 1B and Table 1 of the Supporting Information). The incidence of the disulfide bond in the monomer and dimer was calculated by reference to a control VEGF-C peptide that was reliably resolved in the analysis. The VEGF-C dimer contained ∼4-fold less disulfide-bonded peptide than monomer (Figure 1C and Table 1 of the Supporting Information). In the crystal structure of VEGF-C, the antiparallel dimer is linked by two disulfide bonds between cysteine residues 156 and 165[11] (Figure 2A). Our findings indicate that Cys156 is linked to Cys137 in the monomer and that this disulfide is displaced in the dimer to form the Cys156−Cys165 intermolecular bond. The identification of the Cys137−Cys156 disulfide bond in the dimer implies that a fraction of the dimer is linked by only one Cys156−Cys165 bond. These results suggest the following molecular events in the VEGF-C monomer−dimer transition.

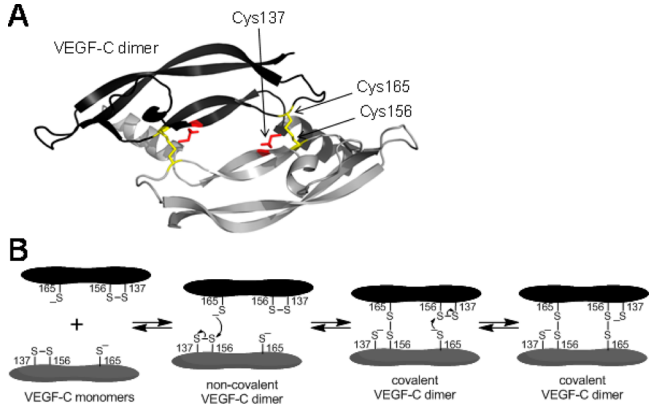

Figure 2. Schematic representation of VEGF-C dimerization. (A) Ribbon structure of the VEGF-C dimer. The dimer is linked by two disulfide bonds between cysteine residues 156 and 165 (yellow sticks) in the antiparallel structure. Cys137 was mutated to alanine to allow stabilization of the dimer for crystallization. The alanine has been replaced with the native Cys137 in the structure (red sticks) to highlight its proximity to Cys156. The structure consists of chains A (black) and B (gray) of Protein Data Bank entry 2X1W.[11] (B) Model for the molecular events involved in VEGF-C dimer formation.

Initial noncovalent association between two monomers in an antiparallel configuration positions a Cys165 thiolate anion of one monomer within a few angstoms of the Cys156 sulfur atom of the Cys137−Cys156 disulfide bond of the other monomer. The thiolate attacks the disulfide bond, resulting in a disulfide-linked antiparallel dimer linked by one disulfide bond (Figure 2B). The same event at the other Cys165 thiolate anion and the Cys137−Cys156 disulfide bond will result in a dimer liked by two disulfide bonds. Singly linked dimers can convert to monomers when the Cys137 thiolate anion attacks the Cys156 sulfur atom of the Cys156−Cys165 interdimer disulfide bond. The sulfur atom of Cys137 is only a few angstroms from the interdimer disulfide bond (Figure 2A), which supports this

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sequence of events. The same sequence of events likely also occurs in VEGF-D,[7] and possibly *C. elegans* PVF-1. It should be noted, however, that recombinant mature growth factor has been used in these studies. The mechanism of dimerization of full-length native VEGF-C *in vivo* could be quite different.

Compounds that influence the redox state of Cys137 in VEGF-C (and VEGF-D) are predicted to impact the biological activities of these growth factors. Alkylation of the Cys137 thiolate in the dimer by other thiols or compounds such as NO should influence the monomer−dimer equilibrium. The observation that a small thiol can promote VEGF-D dimerization supports this scenario.[14] Blocking dimer formation by alkylating Cys165 or cleaving the Cys137−Cys156 disulfide bond in the monomer with targeted small molecules might also be a useful therapeutic approach to inhibiting the function of VEFG-C and -D in pathological lymphangiogenesis.[16]

■ ASSOCIATED CONTENT

Supporting Information

VEGF-C production and results for the dimer Cys137−Cys156 disulfide-linked peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

J.C., J.W.H.W., and M.G. contributed to the performance and analysis of the experiments. P.J.H. conceived the study and wrote the manuscript.

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Notes

The authors declare no competing financial interests.

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