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Mini Review

VEGF receptor protein–tyrosine kinases: Structure and regulation

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ABSTRACT

The human VEGF family consists of VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). The VEGF family of receptors consists of three protein–tyrosine kinases (VEGFR1, VEGFR2, and VEGFR3) and two non-protein kinase co-receptors (neuropilin-1 and neuropilin-2). These components participate in new blood vessel formation from angioblasts (vasculogenesis) and new blood vessel formation from pre-existing vasculature (angiogenesis). Interaction between VEGFR1 and VEGFR2 or VEGFR2 and VEGFR3 alters receptor tyrosine phosphorylation.

VEGF is one of the key regulators of angiogenesis, vasculogenesis, and developmental hematopoiesis [1]. VEGF is a mitogen and survival factor for vascular endothelial cells while also promoting vascular endothelial cell and monocyte motility. VEGF-B also promotes angiogenesis in an ill-defined manner. VEGF-C participates in lymphangiogenesis during embryogenesis and in the maintenance of differentiated lymphatic endothelium in adults. VEGF-D stimulates growth of vascular and lymphatic endothelial cells. Although initially characterized in the placenta, PlGF is expressed in a wide variety of cells, tissues, and organs. PlGF participates in angiogenesis, wound healing, and the inflammatory response.

VEGF and *VEGF-C* null mice are embryonic lethal [1]. Moreover, loss of a single *VEGF* allele in mice leads to vascular deformities and embryonic death. This heterozygous lethal phenotype is indicative of an exactingly important dose-dependent regulation of embryonic vessel development by VEGF. *VEGF-B, VEGF-D,* and *PlGF* null mice are all viable.

Receptors of the VEGF family

The VEGF receptor protein–tyrosine kinases consist of an extracellular component containing seven immunoglobulin-like domains, a single transmembrane segment, a juxtamembrane segment, an intracellular protein–tyrosine kinase domain that contains an insert of about 70 amino acid residues, and a carboxyterminal tail (Fig. 1 and Table 1). These enzymes catalyze the following reaction:

$$\mathrm{MgATP^{1-}} + \text{protein-OH} \rightarrow \text{Protein-OPO}_3^{2-} + \mathrm{MgADP} + \mathrm{H^+}$$

where -OH is a tyrosyl hydroxyl group. Moreover, there are two non-enzymatic VEGF family co-receptors (neuropilin-1 and neuropilin-2) [1,2]. See Table 2 for a list of the VEGF receptors, their ligands, and receptor functions.

Binding of growth factors to the ectodomain of their transmembrane receptors leads to receptor dimerization, protein kinase activation, *trans*-autophosphorylation, and initiation of signaling pathways [3]. VEGF binds to the second immunoglobulin domain of VEGFR1 [4] and the second and third immunoglobulin domains of VEGFR2 [5]. Although it is likely that VEGF-C and VEGF-D bind to the second or second and third immunoglobulin domains of VEGFR3, this has apparently not been addressed.

There are at least two possible mechanisms for autophosphorylation: *cis* and *trans*. In a *cis* mechanism, a receptor monomer catalyzes its own phosphorylation. In a *trans* mechanism, one receptor of a dimer serves as the enzyme while the other receptor of the dimer serves as the substrate, and vice versa. A *cis* mechanism predicts that auto-phosphorylation will be enzyme concentration-independent while a *trans* mechanism will be enzyme-concentration-dependent. Parast and colleagues found that that rate of autophosphorylation of VEGFR2 is dependent on the enzyme concentration and concluded that this process occurs in *trans* [6]. Autophosphorylation of tyrosine residues within the activation segment of the kinase domain stimulates catalytic activity while autophosphorylation of tyrosine residues at other locations generates docking sites for modular Src homology 2 (SH2) and

Abbreviations: CSF, colony stimulating factor; PAE, porcine aortic endothelial; PlGF, placental growth factor; VEGFR, vascular endothelial growth factor receptor.

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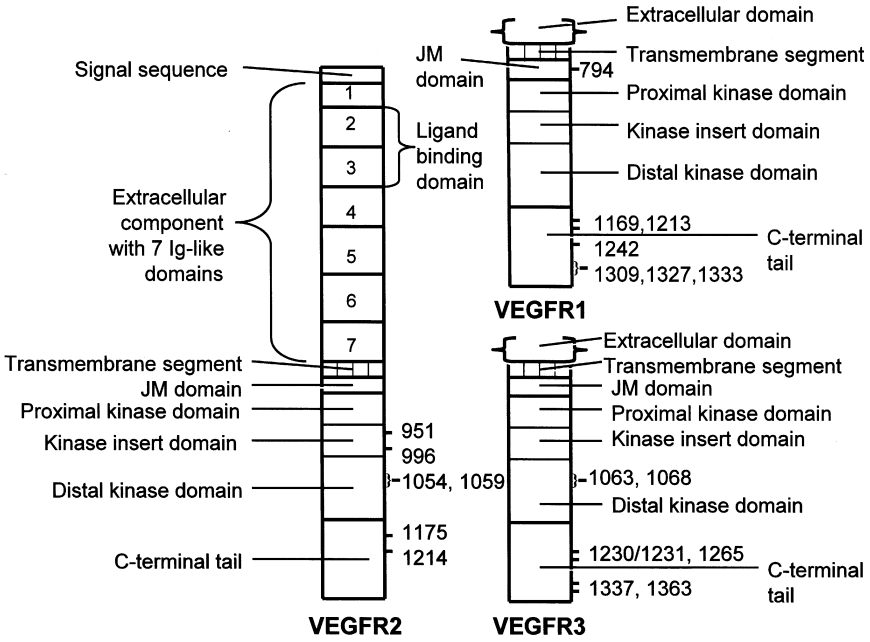

Fig. 1. Organization of the VEGF receptor protein–tyrosine kinases. Numbers on the right of each receptor correspond to human tyrosine residue phosphorylation sites. The relative lengths of the receptor components are to scale.

Table 1
Composition and important residues of the human VEGF receptors

	VEGFR1	VEGFR2	VEGFR3
Signal sequence	1–26	1–19	1–24
Extracellular domain	27–758	20–764	25–775
Ig domain 1	32–123	46–110	30–127
Ig domain 2	151–214	141–207	151–213
Ig domain 3	230–327	224–320	219–326
Ig domain 4	335–421	328–414	331–415
Ig domain 5	428–553	421–548	422–552
Ig domain 6	556–654	551–660	555–617
Ig domain 7	661–747	667–753	678–764
Transmembrane segment	759–780	765–789	776–797
Juxtamembrane segment	781–826	790–833	798–844
Protein kinase domain	827–1158	834–1162	845–1173
Glycine-rich loop	GRGAFG, 834–839	GRGAFG, 841–846	GYGAFG, 852–857
K of K/D/D	861	868	879
αC-glutamate	878	885	896
HRDLAARN catalytic loop	1020–1027	1026–1033	1035–1042
First D of K/D/D	1022	1028	1037
Second D of K/D/D	1040	1046	1055
Activation segment tyrosines	1048, 1053	1054, 1059	1063, 1068
C-terminal tail	1159–1338	1163–1356	1174–1298
No. of residues	1338	1356	1298/1369
Molecular Wt[a] (kDa)	151	152	146/153
Swiss-Prot Accession No.	P17948	P35968	P35916/Q16067

[a] Molecular weight of the unprocessed precursor.

phosphotyrosine binding (PTB) domains that recognize phosphotyrosine in sequence-specific contexts.

VEGFR1 (Flt-1, fms-like tyrosyl kinase-1, where fms refers to feline McDonough sarcoma virus) has weak, or impaired, tyrosine kinase phosphorylation activity following stimulation by VEGF [7]. VEGFR1 has higher affinity for VEGF than VEGFR2 (≈15 pM vs. 750 pM). Six residues in the C-terminal tail of VEGFR1 have been identified as phosphorylation sites (Fig. 1) [8]. Although VEGF and PlGF activate VEGFR1, the phosphorylation sites differ. For example, Autiero and colleagues found that VEGF stimulates VEGFR1 Tyr1213 phosphorylation whereas PlGF stimulates Tyr1309 phosphorylation [9]. Although VEGF and PlGF both bind to VEGFR1, these results indicate that they activate this receptor differently. Even though VEGF stimulates VEGFR1 phosphorylation, it fails to alter the gene expression profile of mouse primary capillary endothelial cells. In contrast, PlGF treatment produces changes in the expression of more than 50 genes.

Although VEGF and PlGF bind to VEGFR1, they exert distinct biological effects suggesting that each activates VEGFR1 in a dissimilar fashion. Autiero and co-workers suggested that the mechanism responsible for these differences may be due to the ability of these ligands to induce different conformational changes in VEGFR1 [9]. However, the X-ray crystal structures of VEGF or PlGF bound to the second immunoglobulin-like domain of human VEGFR1 fail to reveal any differences in conformation [4,10]. The elucidation of the mechanism for the disparate autophosphorylation

Table 2
VEGF receptors, ligands, and functions[a]

Receptor	VEGFR1	VEGFR2	VEGFR3	Neuropilin-1[d]	Neuropilin-2[d]
Ligands	VEGF, VEGF-B, PlGF	VEGF, VEGF-C, VEGF-D, VEGF-E[b], VEGF-F[c]	VEGF-C, VEGF-D	VEGF, PlGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E[b]	VEGF, VEGF-C, VEGF-D
Functions	Vasculogenesis, angiogenesis, and monocyte/macrophage motility	Vasculogenesis, angiogenesis, vascular permeability, and endothelial cell motility	Vascular and lymphatic development and maintenance	Vascular maturation, branching, heart development	Lymphangiogenesis

[a] Information from Ref. [1,2].
[b] Non-human factor encoded by the Orf parapoxvirus.
[c] Non-human factor found in some snake venoms.
[d] VEGF and semaphorin co-receptor.

patterns of the same receptor in response to stimulation by two different ligands promises to add new insight into protein-protein signaling interactions.

Kendall and Thomas cloned cDNAs from a human vascular endothelial cell library that encoded a soluble truncated form of VEGFR1 [11]. sVEGFR1, which contains the first six of seven extracellular immunoglobulin-like domains, binds VEGF with high affinity and inhibits its mitogenic activity for vascular endothelial cells. They suggested presciently in 1993 that sVEGFR1 could prevent blood vessel growth in normally avascular tissues such as cornea [11], a hypothesis that was established by Ambati and colleagues in 2006 [12]. Moreover, excessive sVEGFR1 that is generated by human placenta and released into the circulation of the mother leads to the hypertension and proteinuria of preeclampsia [1].

VEGFR2 (Flk-1/KDR, Fetal liver kinase-1/Kinase insert Domain-containing Receptor) is the predominant mediator of VEGF-stimulated endothelial cell migration, proliferation, survival, and enhanced vascular permeability [1]. Although VEGFR2 has lower affinity for VEGF than VEGFR1, VEGFR2 exhibits robust protein–tyrosine kinase activity in response to its ligands [7]. Six tyrosine residues of VEGFR2 are autophosphorylated (Fig. 1) [13]. Autophosphorylation of residues 1054 and 1059 within the activation loop of VEGFR2 leads to increased kinase activity [14].

Autiero and colleagues studied the interaction of VEGFR1 and VEGFR2 in immortalized mouse capillary endothelial cells [9]. They reported that PlGF (which stimulates VEGFR1 only) fails to increase the phosphorylation of VEGFR2 whereas VEGF-E (a viral factor that stimulates VEGFR2 only) produces a 4-fold increase in VEGFR2 phosphorylation when compared with unstimulated samples. However, a combination of PlGF and VEGF-E produces a 13-fold increase in VEGFR2 phosphorylation. These workers suggested that VEGFR2 is transphosphorylated by VEGFR1 through an intermolecular reaction between VEGFR1 and VEGFR2 homodimer pairs. Transactivation by homodimer pairs represents a novel interpretation in receptor protein–tyrosine kinase research where it is generally assumed that transactivation occurs between heterodimers.

VEGFR3 plays a key role in remodeling the primary capillary plexus in the embryo and contributes to angiogenesis and lymphangiogenesis in the adult [1]. This receptor occurs in embryonic vascular endothelial cells where its production decreases during development and is subsequently restricted to lymphatic vessels after their formation [15]. Alternative splicing of VEGFR3 pre-mRNA in humans generates two isoforms of VEGFR3 that differ in their C-terminal tails [16]. VEGFR3 undergoes a proteolytic cleavage in the sixth immunoglobulin-like domain; the two components of the original chain remain linked by a disulfide bond. Dixelius and co-workers identified five tyrosine residues in the C-terminal tail of human VEGFR3 as autophosphorylation sites (Fig. 1) [17]. These investigators found that, following VEGF-C treatment (but not VEGF treatment) of cells, VEGFR2 co-immunoprecipitated with VEGFR3. Moreover, VEGFR3 residues 1337 and 1363 were not autophosphorylated in the VEGFR2-VEGFR3 immunocomplex but were phosphorylated in the VEGFR3 homodimer. These results suggested that the interaction of the two receptors influences the pattern of transphosphorylation and signal transduction.

Structure and inferred mechanism of the protein kinase core of the VEGF receptors

The VEGFR2 protein–tyrosine kinase core has the characteristic bilobed architecture observed in all protein kinases (Fig. 2). The active site is located in the cleft between the two lobes and consists of residues contributed by both lobes. There are two general types of conformational changes associated with protein kinases [20].

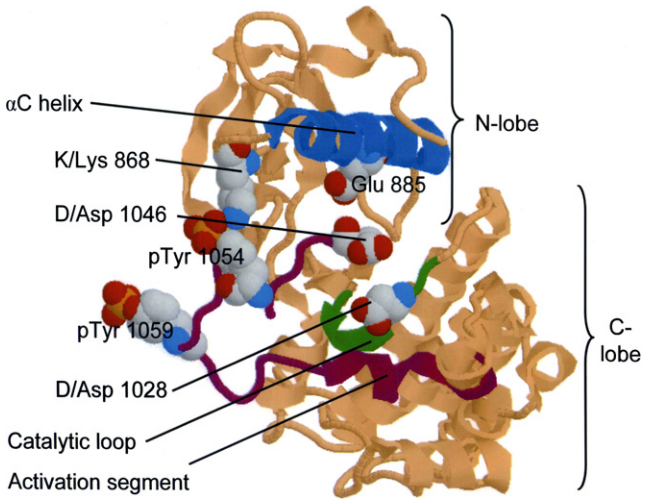

Fig. 2. Structure of the protein kinase catalytic core of VEGFR2. The αC helix is colored blue, the catalytic loop is green, and the activation segment is magenta. The glycine-rich loop is hidden by Lys868. Prepared from protein data base file 2OH4 [18] using Protein Explorer [19].

The first involves the interconversion of inactive and active states. Inactivation-activation involves changes in the position of the αC helix in the N-lobe and the conformation of the activation segment in the C-lobe. The second type of conformational change occurs in the active state as the two lobes move relative to each other to open and close the cleft as the enzyme goes through its catalytic cycle: ATP and protein substrate bind to the open conformation, catalysis occurs in the closed conformation, and ADP and phosphorylated substrate are released during progression to the open state that completes the cycle. The three-dimensional structures of the protein kinase cores of VEGFR1 and VEGFR3 have not yet been solved crystallographically, but they are expected to conform to the canonical structures of other protein kinases.

The smaller N-terminal lobe has a predominantly antiparallel β-sheet structure. A glycine-rich (GXGXXG) ATP-phosphate binding loop occurs in each of the VEGF receptors (Table 1). The larger C-terminal lobe, which is predominantly α-helical in nature, contains the catalytic loop and the activation segment. Hanks and colleagues identified 12 subdomains with conserved amino acid signatures that make up protein kinases [21]. Of these, the following three amino acids, which define a K/D/D (Lys-Asp-Asp) motif, illustrate the inferred catalytic properties of the VEGFR2 kinase. In the activated enzyme (Fig. 3), Lys868 is an invariant residue that forms ion pairs with the α- and β-phosphates of ATP and with Glu885 of the αC helix. In the inactive enzyme (Fig. 2), which lacks bound ATP, Lys868 binds instead to an activation segment phosphotyrosine and is far from Glu885. Asp1028, the catalytic base in a conserved HRD (His-Arg-Asp) sequence, orients the tyrosyl group of the substrate protein in a catalytically competent state. Asp1046 is the first residue of the activation loop in a conserved DFG (Asp-Phe-Gly) sequence found in the large lobe. This residue, which is part of a magnesium-binding loop, binds to Mg^{2+} that in turn coordinates the β and γ phosphate groups of ATP; Asp1046 also binds to the α-phosphate (Fig. 3).

Within each lobe is a polypeptide segment that can assume active and inactive orientations. In the small N-lobe, this segment is the αC helix (which is preceded by small A and B helices). The αC helix rotates and translates with respect to the rest of the lobe, making or breaking part of the catalytic site. In the active state, Glu885 of the αC helix forms a salt bridge with Lys868 of the N-lobe. The conformation of the activation segment of the large

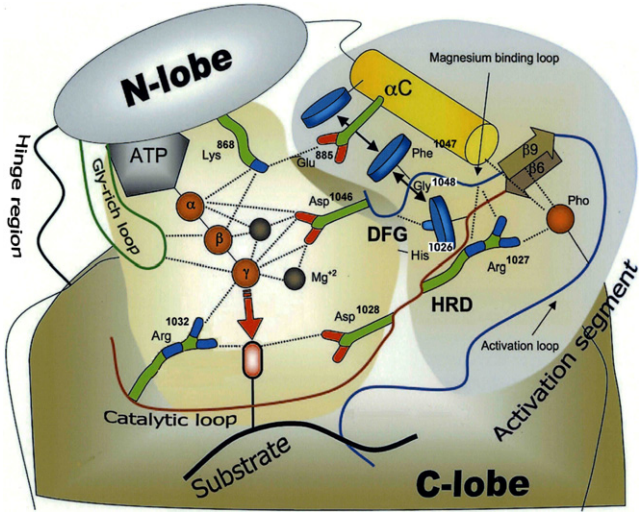

Fig. 3. Diagram of the inferred interactions between the human VEGF receptor 2 protein–tyrosine kinase catalytic core residues, ATP, and a protein substrate. Catalytically important residues that are in contact with ATP and protein substrate occur within the light khaki background. Secondary structures and residues that are involved in regulation of catalytic activity occur within the gray background. Hydrophobic interactions between the HRD motif, the DFG motif, and the αC helix are shown by black arrows while polar contacts are indicated by dashed lines. Pho refers to a phosphotyrosine within the activation segment. This figure is adapted from Ref. [20], copyright *Proceedings of the National Academy of Sciences USA*.

C-lobe differs between active and inactive enzymes. The activation segment of nearly all protein kinases begins with DFG and ends with APE (Ala-Pro-Glu). The D of DFG corresponds to Asp1046, the first residue of the activation segment. In protein kinases that are in the inactive state, the activation loop is positioned to prevent protein substrate binding. In the structure shown in Fig. 2, the activation segment in the active conformation would be extended far toward the right. Phosphorylation of the activation segment in protein kinases generally stabilizes it in its active conformation; the structure of VEGFR2 shown in Fig. 2 is unusual in that the activation segment, although doubly phosphorylated, assumes an inactive conformation. Although there are a half dozen X-ray structures of VEGFR2 in the public protein data base including 2QU5, 2QU6, 1YWN, 2P2H and 2P2I, only that illustrated in Fig. 2 (2OH4) exhibits the entire activation segment; the segment in all of the others is disordered.

VEGFR1, an impaired protein kinase

By comparing the amino acid sequence of the activation segment of VEGFR1 with several related receptor tyrosyl kinases, Meyer and colleagues noted that VEGFR1 contains an asparagine residue (mouse Asn1050) in place of an aspartate that occurs in other kinases [22]. These investigators prepared chimeric receptors containing the extracellular domain of the human colony stimulating factor (CSF) receptor and the transmembrane and intracellular domains of murine VEGFR1 or VEGFR2 and expressed these receptors (CSF-R1 and CSF-R2) in porcine aortic endothelial (PAE) cells. They found that CSF treatment of the Asn1050Asp mutant of CSF-R1 (CSF-R1-N1050D) expressed in PAE cells promotes strong autophosphorylation compared with CSF-R1. Moreover, these workers showed that VEGF increases the extent of autophosphorylation of the non-chimeric Asn1050Asp VEGR1 mutant when compared with VEGFR1 expressed in PAE cells. These investigators reported that CSF stimulates the phosphorylation of the activation loop tyrosines (mouse Y1052 and Y1057) of CSF-R2 to a greater extent than those of the Asp1054Asn mutant of CSF-R2 expressed in PAE cells. Meyer and co-workers suggested that the activation segment asparagine serves as a negative substrate determinant that partially inhibits activation segment autophosphorylation.

Essential nature of the VEGF receptors

Fong and co-workers showed that *VEGFR1* null mice die between embryonic days 8.5 and 9.0 [23]. Endothelial cells form normally in both embryonic and extra-embryonic sites in these mice, but the cells fail to assemble into organized blood vessels. However, Hiratsuka and collaborators reported the surprising finding that mice expressing the VEGFR1 extracellular ligand-binding and transmembrane segments but lacking the tyrosine kinase (TK) and its insert domain (*VEGFR1-TK*$^{-/-}$) are viable and fertile [24]. The only defect noted in these mice was an inability of VEGF to stimulate macrophage migration. Hiratsuka and co-workers subsequently demonstrated that about half of the mice with a deletion of both the transmembrane segment and tyrosine kinase domain (*VEGFR1-TM*$^{-/-}$*-TK*$^{-/-}$) were embryonic lethal [25]. These observations indicate that the membrane-anchored ligand-binding domain is the essential part of the receptor during development. These findings are consistent with the concept that the chief function of VEGFR1 in embryos is to sequester VEGF and modulate the concentration of the free ligand near the cell surface.

Shalaby and colleagues reported that *VEGFR2* null mice die between embryonic days 8.5 and 9.5 as a result of defects in the development of hematopoietic and endothelial precursors [26]. Yolk-sac blood islands were absent at 7.5 days, organized blood vessels were not observed in the embryo or yolk sac at any stage, and hematopoietic progenitors were severely reduced. These findings indicate that VEGFR2 is essential for yolk-sac blood-island formation and vasculogenesis in the mouse embryo and are consistent with the concept that VEGFR2 is one of the earliest markers of embryonic endothelial cells.

Dumont et al. showed that *VEGFR3* null mice died by embryonic day 9.5 and exhibited defective blood vessel development [27]. Vasculogenesis and angiogenesis occurred, but large vessels became abnormally organized with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure. Thus, VEGFR3 has an essential role in the development of the embryonic cardiovascular system before the emergence of the lymphatic vessels where VEGFR3 also plays a pivotal role.

Epilogue

Inhibition of angiogenesis represents a potential therapy for disorders with non-physiologic angiogenesis including age-related macular degeneration of the eye, diabetic retinopathy, rheumatoid arthritis, and tumor growth and metastasis [1]. Targeting VEGF receptors represents one approach that has enjoyed some therapeutic success. Sunitinib (Sutent), which is an orally effective low molecular weight drug that inhibits VEGFR1, VEGFR2, VEGFR3, and other protein kinases, is approved by the US Food and Drug Administration for the treatment of metastatic renal cell carcinoma and gastrointestinal stromal tumors [28]. Sorafenib (Nexavar), another orally effective low molecular weight drug that inhibits VEGFR1, VEGFR2, VEGFR3, Raf, and other kinases [29] is approved for the treatment of renal cell carcinoma and advanced hepatocellular carcinoma. See [http://www.cancer.gov/cancertopics/factsheet/Therapy/angiogenesis-inhibitors] for a listing of anti-angiogenic clinical trials that are planned or in progress for various forms of cancer.

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