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VEGFR-1 and VEGFR-2: two non-identical twins with a unique physiognomy

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Abstract

Angiogenesis involves endothelial cell differentiation, proliferation, migration and cord formation, which lead to tubulogenesis to form vessels. One group of growth factor receptors implicated in angiogenesis is the VEGFR family of receptor tyrosine kinases. VEGFR-1 and VEGFR-2 are closely related receptor tyrosine kinases and have both common and specific ligands. VEGFR-1 is a kinase-impaired RTK whereas VEGFR-2 is a highly active kinase. Despite their differential kinase activation potentials, both VEGFR-1 and VEGFR-2 are required for normal development and angiogenesis. VEGFR-1 regulates angiogenesis by mechanisms that involve ligand-trapping, receptor homo- and heterodimerization. VEGFR-2 stimulates variety of signaling pathways and broad biological responses *in vitro*. The mechanisms that govern VEGFR-2 activation, its ability to recruit signaling proteins and to undergo downregulation are highly regulated by its carboxyl terminus. This review highlights recent insights into the mechanism of activation of VEGFR-1 and VEGFR-2, and focuses on the signaling pathways employed by VEGFR-1 and VEGFR-2 that regulate angiogenesis.

Keywords

Vascular endothelial growth factor receptor-1 (VEGFR-1); vascular endothelial growth factor receptor-2 (VEGFR-2); FLT-1; FLK-1; angiogenesis; vasculogenesis; receptor tyrosine kinases; Src kinases; Phosphoinositide 3-kinase; PLC-gamma1; c-Cbl; ubiquitination; downregulation; signal transduction; tyrosine phosphorylation; Review

2. INTRODUCTION

VEGFR-1/FLT-1 (fms-like tyrosine kinase) and VEGFR-2/KDR/FLK-1 (fetal liver kinase) are the prototype of a gene family encoding structurally related receptors including FLT-3/FLK-2 and FLT-4/VEGFR-3 and belong to the receptor tyrosine kinases (RTK) subfamily (1,2). VEGFR-1 and VEGFR-2 are primarily involved in angiogenesis (3) where FLT-3 and FLT-4 are involved in hematopoiesis and lymphogenesis (4). The diversification of the VEGFR family during evolution, through the advent of multiple ligands and receptors has created a decisive signaling network capable to control angiogenesis. Through utilization of receptor homo- and heterodimers, activated by specific and common ligands, a specific angiogenic signal is propagated leading to precise angiogenic outcome. An additional level of angiogenic signaling diversity is obtained through differential activation of distinct signaling molecules downstream of each receptor.

VEGFR-1 is a kinase-impaired RTK, and may signal in the context of a receptor heterodimer (5–7). In contrast, VEGFR-2 is highly kinase active receptor and activates a broad signaling

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cascades and induces diverse biological responses (8,46). Three different gene products including, PLGF, VEGF-A, VEGF-B are known to bind VEGFR-1. VEGF-A, VEGF-D and VEGF-C are known to bind VEGFR-2 (10,11). VEGF-C and VEGF-D also bind to VEGFR-3, an RTK that is expressed by lymphatic endothelial cells and hematopoietic progenitor cells (4,11). VEGFR-1 and VEGFR-2 are structurally similar, consisting of an extracellular ligand-binding domain with seven immunoglobulin (Ig)-like motifs, a single transmembrane domain and a juxtamembrane domain, a kinase domain split by a kinase insert, and a carboxyl terminus. Overall, there is 43.2% sequence homology between VEGFR-1 and VEGFR-2. The extracellular domain of VEGFR-1 and VEGFR-2 displays 33.3% homology and the cytoplasmic region 54.6%. The kinase domain of VEGFR-1 and VEGFR-2 represent the most conserved region with 70.1% homology. In contrast, the carboxyl terminus represents the most divergent region with only 28.1% sequence homology (Figure 1).

3. VEGFR-1 SIGNALING AND ANGIOGENESIS

Initial evidence linking VEGFR-1 to endothelial cell function and angiogenesis was provided by targeted deletion of VEGFR-1, which resulted in early embryonic lethality due to abnormal blood vessel growth (12). Although the precise role of VEGFR-1 in endothelial cell functions is still emerging, in recent years several lines of evidence have developed that suggest VEGFR-1 may play both negative and positive roles in angiogenesis. The negative role of VEGFR-1 in angiogenesis was suggested based on the observation that loss of *VEGFR-1/flt-1* in mice caused an increase in the number of endothelial progenitors leading to the vascular disorganization (12,13). Further study suggested that the negative role of VEGFR-1 in angiogenesis is associated with its ability to alter endothelial cell division (14,15). Furthermore, work from several laboratories indicates that selective activation of VEGFR-1, either by creating VEGFR-1 chimeras (5,16) or VEGF mutants that selectively bind to VEGFR-1 is not associated with proliferation of endothelial cells *in vitro* (17). In these studies ligand stimulation of VEGFR-1 induced neither cell proliferation nor apoptosis. Selective activation of VEGFR-1 also results in no cell migration or intercellular calcium release (18). Altogether, these findings indicate that in a defined condition when VEGFR-1 is activated by a ligand that only causes receptor homodimerization and results in no cross-talk with VEGFR-2, VEGFR-1 is not capable of promoting biological responses such as cell migration, cell proliferation and intercellular calcium release. These findings further argue that the ability of VEGFR-1 to propagate a productive signal and to stimulate biological responses either negative or positive is limited to its ability to heterodimerize with VEGFR-2 (5,6). In line with this notion, in a recent gene targeting study where the entire cytoplasmic region of VEGFR-1 including its kinase domain was deleted, the knockin truncated VEGFR-1 mice were developed with no aberrant vasculogenesis (19). This was in startling contrast to the VEGFR-1 knockout mice, which died in early development due to overgrowth of endothelial cells (12,13). In sum, these observations reinforced the idea that VEGFR-1 may act as a decoy receptor and its function in angiogenesis may involve its ligand biding extracellular region, acting as a VEGF-trap to modulate VEGFR-2 function (Figure 2).

Furthermore, recent studies revealed that VEGFR-1's role in angiogenesis is far more complex than initially thought. Clearly, VEGFR-1's ability to modulate angiogenesis is not limited to mechanism by which it acts as a VEGF-trapping receptor. Under certain circumstances upon stimulation with PLGF, a VEGFR-1 specific ligand, VEGFR-1 may heterodimerize with VEGFR-2 leading to transactivation of VEGFR-2 and angiogenesis (6). Overexpression of PLGF, in transgenic mice is also reported to promote angiogenesis (20). These findings suggest that VEGFR-1 when paired with VEGFR-2 via a mechanism of heterodimerization its activity positively regulates angiogenesis. Also, it is increasingly apparent that activation of VEGFR-1 in endothelial cells and its final biological function is subject to the microenvironment of endothelial cells. In particular, the presence or absence of the other VEGFR family such as

VEGFR-2 and neuropilines, the co-receptors for VEGF and PLGF (21). Likewise, VEGFR-1 homo- and heterodimerization may define the inhibitory or stimulatory nature of its signaling relays (5,6,16). Another factor that may determine the nature of VEGFR-1 signaling in the endothelium microenvironment is the type of ligand being utilized to activate VEGFR-1. The nature of the signal induced by PLGF, which selectively binds VEGFR-1, might be different than that induced by VEGF-A and VEGF-B. In agreement with this idea, the impaired angiogenesis associated with *plgf* null mice was not rescued by VEGF-B (22). Also, a naturally occurring VEGF-PLGF heterodimer may favor the formation of certain heterodimerization complexes among the VEGFR family involving VEGFR-1, VEGFR-2 and neuropilines, which may activate a unique signaling pathway that might not be possible by stimulation with either VEGF or PLGF alone. Finally, it is now evident that endothelial cells are morphologically and genetically different from each other (24) raising the possibility that variation in endothelial cells may ultimately determine or modify the nature of VEGFR-1 signaling. The fact that VEGFR-1 signaling in non-endothelial cells such as monocytes, trophoblasts and other cell types stimulate cell migration and proliferation resonates this possibility (24–26).

4. VEGFR-1 IS A KINASE-IMPAIRED RTK

The mechanisms by which most RTKs are activated have been well characterized. Transphosphorylation of RTK and their ability to phosphorylate target proteins are the hallmark of RTKs activation. In this context, VEGFR-1 is considered to be a kinase-impaired RTK (e.g., VEGFR-1 is poorly tyrosine phosphorylated and its ability to phosphorylate substrate is negligible). All RTKs, including VEGFR-1, contain an evolutionary conserved kinase domain containing GXGXXG, an ATP binding site, HRDLA, a motif essential for catalysis and one or two tyrosine autophosphorylation sites (1). Despite having these motifs, the ligand stimulation of VEGFR-1 results only in minor tyrosine phosphorylation of VEGFR-1 *in vivo* and *in vitro* (5,8,27). In some RTKs such as colon carcinoma kinase-4 (CCK4), ErbB3/HER3, KLG, Ror1 and DRL abnormalities in the kinase domain have been suggested to contribute to the kinase-defective phenotype of these RTKs (28–31). VEGFR-1 has no aberrant amino acid replacement in the corresponding regions although it is not known whether the kinase domain contributes for this unusual characteristic of VEGFR-1.

To date, the molecular basis of the kinase-impaired characteristic of VEGFR-1 is not understood. However, a recent study suggests that the kinase-impaired activity of VEGFR-1 can be rescued, in part, by swapping the carboxyl terminus of VEGFR-2 with VEGFR-1. The carboxyl-terminus swapped VEGFR-1 promoted ligand-dependent autophosphorylation of VEGFR-1 and induction of endothelial cell proliferation (18). It appears that this is associated with its carboxyl terminus because replacement of the JM region of VEGFR-1 with that of VEGFR-2 does not rescue its kinase-impaired characteristic (32). A detailed understanding of the molecular mechanisms underlying the carboxyl-tail regulated kinase-impaired characteristic of VEGFR-1 should wait until the crystal structure of VEGFR-1 is resolved. To date, the crystal structure of VEGFR-1 is not available, only its ligand-binding region is resolved (57).

How the carboxyl terminus of VEGFR-2 is able to rescue the kinase-impaired activity of VEGFR-1 is not fully understood. However, it is feasible that the carboxyl terminus of VEGFR-1 obstructs ligand-mediated autophosphorylation of VEGFR-1. In some RTKs, such as Tie-2, deletion of carboxyl terminus enhances its autophosphorylation and kinase activation, suggesting that the carboxyl terminus negatively regulates tyrosine autophosphorylation possibly by interacting with activation loop and keeping it in an inhibitory conformation (33). The carboxyl terminus of VEGFR-1 appears to negatively regulate its activation, although autophosphorylation of VEGFR-1 without carboxyl terminus is not enhanced (18). Further

studies are needed to fully understand the molecular basis of the kinase-impaired characteristic of VEGFR-1.

The mechanisms by which most RTKs transduce signals are well-characterized (34). Several RTKs including, DRL, CCK4, KLG, Ror1 and ErbB3 all display impaired kinase activity, yet transduce signals and regulate variety of biological functions (28–31). The best-studied kinase-impaired RTK is ErbB3, one of the four members of EGFR family (58). Data from several laboratories using various systems revealed that signaling of ErbB3 requires the kinase activity of another EGFR family including ErbB1, ErbB2 and ErbB4 (35,58). DRL, another kinase-impaired RTK, although shows no kinase activity *in vivo* and *in vitro*, nevertheless its activity is required for axon guidance in Drosophila (31). The molecular mechanism of DRL signaling and whether DRL transduces signal like ErbB3 is not known. There is some circumstantial evidence suggesting that VEGFR-1 may signal though other VEGFR family kinases, in particular, through VEGFR-2. Both VEGFR-1 and VEGFR-2 are expressed by endothelial cells, suggesting that under a favorable condition such as availability of appropriate ligand, VEGFR-1 may form heterodimer complexes with other endothelial receptors. Indeed, VEGFR-1 has been shown to heterodimerize with VEGFR-2 and that heterodimerization leads to autophosphorylation, activation of VEGFR-2, and angiogenesis (6). Thus, the mechanism by which VEGFR-1 supports angiogenesis is complex and likely involves several different mechanisms including VEGF-trapping, activation by heterodimerization and homodimerization (Figure 2). VEGFR-1 has a residual kinase activity and its selective activation that results only in homodimerization leads to activation of a limited number of signaling proteins such as p42/44 MAPK (18). Thus, the unique signaling nature of VEGFR-1 and mechanisms by which it stimulates biological responses indicates that we have more to learn about RTK signaling than the current widely accepted and generalized RTK paradigm would suggests.

5. VEGFR-2 ACTIVATION IS ESSENTIAL FOR ANGIOGENESIS

The role of VEGFR-2 in angiogenesis is well established and more comprehensive reviews on the role of VEGFR-2 in angiogenesis have been recently published (3,22,37). In this review we focus on the recent advancement on VEGFR-2 activation and its signal transduction relay.

5.1. Carboxyl terminus is critical for VEGFR-2 activation and its signaling

The mechanisms by which most RTKs transduce signals have been dissected extensively at the molecular level. The basic principles of RTKs activation can be summarized as following: Ligand-mediated dimerization of receptor monomers, transphosphorylation by dimerized receptors and docking of signaling proteins to receptor phosphotyrosines. An increase in the intrinsic catalytic activity and creation of binding sites on the RTKs to recruit cytoplasmic signaling proteins are primary features of RTKs activation (2,34). The autophosphorylation sites are either involved in the regulation of kinase activity of the receptor or serve as a binding site for SH2- and PTB-containing proteins (2,38). Central to ligand-induced RTKs activation is the phosphorylation of one or two tyrosine residues within the activation loop (34). In the unstimulated form, the activation loop orients these tyrosine sites toward the activation sites of the enzyme and thereby sterically prevents binding to ATP-Mg^{2+}. It is suggested that phosphorylation of these tyrosines in the catalytic loop orients the inhibitory loop away from the active site enabling the RTK to bind ATP-$Mg^{2+}$and efficiently phosphorylate substrates 34,39). The recent crystal structure of VEGFR-2 by McTigue *et al* (40) showed that tyrosines 1054 and 1059, two highly conserved tyrosine residues located in the activation loop of VEGFR-2 are phosphorylated. An unexpected feature of the VEGFR-2 structure is the conformation of the activation loop. Although phosphorylated, the activation loop is disordered in the region that includes phosphotyrosine 1059. This is in contrast to the structures of other

tyrosine kinases such as FGFR-1 and the insulin receptor, which are well ordered, and participate in specific interaction (40).

In last few years some of the paradigms learned from studying other RTKs revealed that catalytic activity is regulated by other segments of RTKs. For instance, substitution of two tyrosine residues with phenylalanine in the juxtamembrane region of the PDGFR-β drastically reduces autophosphorylation and activation of this receptor (41). Similar observations were reported for the MuSK tyrosine kinase, a receptor tyrosine kinase for agrin, which is involved in the functioning of the neuromuscular synapse (42) and EphB2, a receptor tyrosine kinase for ephrins (43,44). Recent crystal structures of MuSK and EphB2 revealed that in these receptors the juxtamembrane domain is directly involved in the regulation of kinase activation. The data obtained from the crystal structures suggest that the juxtamembrane domain interacts with the kinase domain and represses catalytic activity (43,44). Additional biochemical studies demonstrated that these tyrosines also bind to SH2-containing proteins (43). It is suggested that these tyrosine residues in unstimulated condition associate with the activation loop and repress the kinase activation of MuSK and EphB2 receptors. However, upon ligand stimulation and phosphorylation these tyrosine residues associate with SH2-containing proteins and thus relieve autoinhibitory conformation (44). There are two tyrosine residues within the juxtamembrane of VEGFR-2 (tyrosines 799 and 820 in mouse VEGFR-2). Replacement of these tyrosines to phenylalanine in VEGFR-2 does not impair the ligand-dependent activation of VEGFR-2 (45,46). This suggests that phosphorylation of these tyrosines are not involved in kinase activation of VEGFR-2. Unlike the phosphorylation sites in the juxtamembrane region, however, phosphorylation of tyrosine 1212 (tyrosine 1214 in human VEGFR-2) in the carboxyl terminus plays an important role in autophosphorylation and kinase activation of VEGFR-2. Mutation of tyrosine 1212 to phenylalanine impairs full activation of VEGFR-2 and its ability to efficiently activate signaling proteins. Mutation of the same tyrosine site to glutamic acid, however, preserves its ability to undergo ligand-dependent autophosphorylation (47). This suggests that tyrosine 1212 regulate VEGFR-2 autophosphorylation in a manner that is similar to that of tyrosine sites in the JM region of MuSK and EphB2. Surprisingly, this mutant receptor as recently reported preserves normal development and angiogenesis in knockin mice (48). This indicates that either the low level kinase activity of tyrosine 1212 mutant VEGFR-2 is sufficient to promote angiogenesis or that phosphorylation of tyrosine 1212 is not stringently required for function of VEGFR-2 *in vivo*. It is also possible that phosphorylation of other tyrosine sites in VEGFR-2 may compensate the lack of phosphorylation of tyrosine 1212. In agreement with this possibility, the mutant PDGFR-β lacking the binding sites for PLC-γ1, PI 3 kinase, SHP2 and Ras-GAP has been shown to stimulate gene expression in fibroblast cells almost as efficiently as the wild type PDGFR-β (49). Similarly, activation of Src kinases by many laboratories has been shown to be important for PDGFR-β autophosphorylation and its biological function (41). Yet, PDGFR-β is fully functional in SYF cells, deficient for Src family kinases (50). This argues that RTK signaling induces activation of signaling proteins with an overlapping role *in vivo* and thus the role of individual docking sites may be compensated for by the presence of other docking sites.

Further analysis of the carboxyl terminus of VEGFR-2 revealed that this region is engaged in VEGFR-2 activation in multiple ways. First, carboxyl terminus of VEGFR-2 contains several tyrosine phosphorylation sites including tyrosine 1212, 1173 and 1221. Tyrosines 1212 and 1221 are phosphorylated but the identity of signaling proteins that are recruited to these sites is not known (47 and our unpublished data). As discussed earlier, the presence of tyrosine 1212 is required for full autophosphorylation of VEGFR-2 (47). Tyrosine 1173 is phosphorylated *in vivo* and recruits p85 of PI3 kinase (45), PLCγ1 (9,51,52) and c-Cbl (our unpublished data). In addition to possessing multiple tyrosine phosphorylation sites, the carboxyl terminus of VEGFR-2 also contains putative serine phosphorylation sites. Mutation of serines 1188 and 1191 in the carboxyl terminus of VEGFR-2 is shown to impair the ligand-dependent down

regulation of VEGFR-2 (53). This might explain why deletion of carboxyl terminus impairs the VEGFR's ability to undergo ligand-dependent downregulation (54).

Finally, deletion of the entire carboxyl terminus of VEGFR-2 is shown to impair its ligand-dependent autophosphorylation. It appears that this role of carboxyl terminus is independent of tyrosine 1212 and may involve residues other than tyrosines (54). Altogether, the current literature provides a frame work for understanding of regulation of VEGFR-2 activation and further demonstrates that the carboxyl terminus plays various important roles in VEGFR-2 functions ranging from its ability to regulate autophosphorylation and recruitment of signaling proteins to ligand-dependent downregulation. Future work to characterize the function of carboxyl terminus of VEGFR-2 will require a detailed analysis including, identification of tyrosine and serine phosphorylation sites and signaling proteins that might interact with these sites need to be evaluated. Finally, resolving the crystal structure of VEGFR-2 with its carboxyl terminus intact may provide further insight into regulation of this important protein.

5.2. Phosphoinositide 3-kinase, VEGFR-2 and angiogenesis

In the last few years, the phosphoinositide 3-kinase (PI3K) signal transduction pathway has emerged as one of the main signal routes that VEGFR-2 employs to stimulate endothelial cell survival and proliferation. PI 3 kinase is a lipid kinase that converts the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3). Proteins with pleckstrin-homology (PH) domains, such as protein kinase B (PKB/Akt), phosphoinositide-dependent kinase-1 (PDK-1) and PDK-2, bind to PIP_3. Akt is activated by PIP3, PDK1 and PDK2 leading to phosphorylation of a host of other proteins that affect cell proliferation, cell cycle progression and cell survival (55,56).

Various experiments using several *in vivo* and *in vitro* systems have demonstrated that activation of PI3 kinase by VEGFR-2 promotes endothelial cell survival, proliferation and angiogenesis (Figure 3). First, Wortmannnin and LY294002, the PI3 kinase specific inhibitors and a dominant negative form of Akt, a downstream target of PI3 kinase all are able to inhibit VEGF-stimulated endothelial cell survival (59). Second, inhibition of PI3 kinase by overexpression of a dominant negative form of p85 of PI3 kinase inhibits endothelial cell proliferation and induction of c-fos (60). Similarly, overexpression of PTEN, a lipid phosphatase that reverses PI3 kinase function by dephosphorylating its phospholipid products, inhibits VEGF-mediated endothelial cell proliferation. Overexpression of phosphatase inactive form of PTEN enhances VEGF-mediated endothelial cell proliferation (61). Third, expression of a viral form of PI3 kinase and its target Akt promotes angiogenesis and VEGF expression in chicken embryo (62). The most recent study on PI3 kinase also suggests that it may regulate angiogenesis by regulating expression of Tie-2 receptor (63).

A mechanistic explanation on how PI3 kinase is activated by VEGFR-2 is demonstrated by a chimeric VEGFR-2 system in which the PI3 kinase binding sites were mutated and its role in VEGFR-2-mediated endothelial cell proliferation was tested. The tyrosine mutant chimeric VEGFR-2 (where tyrosines 799 and 1173 of mouse VEGFR-2 were mutated) abolished the ability of VEGFR-2 to stimulate PI3 kinase and cell proliferation. The mutant receptor is able to activate MAPK, suggesting that MAPK activation is neither regulated by PI3 kinase nor is its activation associated with endothelial cell proliferation (45). Also, PI3 kinase-dependent proliferation of endothelial cells is linked to activation of S6 kinase. This is supported by the observation that a mutant chimeric receptor that fails to activate PI3 kinase also fails to activate S6 kinase (45). Consistent with this observation, selective inhibition of S6 kinase inhibits VEGF-induced endothelial cell proliferation and *in vivo* angiogenesis (64,65). In agreement with a critical role of PI3 kinase activation in endothelial cell growth and survival a recent study demonstrated that tumstatin, a naturally occurring angiogenesis inhibitor promotes apoptosis of endothelial cells by inhibiting the PI3 kinase pathway (66).

5.3. Phospholipase C-gamma1 activation and angiogenesis

Phospholipase Cγ1 (PLCγ1) hydrolyzes phosphoinositides to generate the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases intracellular Ca^{2+} levels and DAG activates protein kinase C (PKC) leading to variety of cellular responses (67). Several lines of evidences indicate that activation of PLC-γ1 plays a pivotal role in angiogenesis and VEGFR-2 signal transduction (Figure 3). First, a genetic study shows that lack of PLC-γ1 in mice results in significantly diminished vasculogenesis and erythropoiesis (68). Second, in transgenic zebrafish the PLC-γ1 deficiency shows defects in arterial formation. The PLC-γ1 deficient embryos also failed to respond to exogenous VEGF, highlighting the importance of PLC-γ1 for VEGFR-2-mediated signal transduction relay (69). However, the exact role of PLC-γ1 in angiogenesis is not clear. VEGFR-2-mediated activation of PLC-γ1 in a certain endothelial cellular background is suggested to stimulate cell proliferation and in other endothelial cells to stimulate differentiation and tubulogenesis (9,5). Phosphorylation of tyrosines 1006 and 1173 (Y1006 and 1173 correspond to Y1008 and 1175 in human VEGFR-2, respectively) are important for recruitment of PLC-γ1 by VEGFR-2 (9,51). The tyrosine mutant VEGFR-2 that fails to activate PLC-γ1 also fails to stimulate tubulogenesis *in vitro* (9). Recent knockin approach in mice showed that mutation of tyrosine 1173 of VEGFR-2 abrogates its ability to promote angiogenesis (70). Although this finding suggests an essential role for tyrosine 1173 in the regulation of angiogenesis, it is not clear whether the defect associated is exclusively linked with the inability of the tyrosine 1173 mutant VEGFR-2 to activate PLC-γ1. Tyrosine 1173 of VEGFR-2 appears to act a multi-docking site for recruitment of signaling proteins by VEGFR-2 including, PLC-γ1 (51), p85 of PI3 kinase (45), Shb (52) and c-Cbl (our unpublished data).

5.4. Src kinases and Angiogenesis

Activation of Src kinases has been implicated in the regulation of a variety of biological responses including cell proliferation, migration, differentiation and cell survival (71). Activation of many RTKs leads to stimulation of Src family kinase activity via tyrosine phosphorylated docking sites on RTKs and the SH2 domain of Src kinases (72). Selective activation of VEGFR-2 stimulates Src phosphorylation in endothelial cells (47). However, the precise mechanism involved is not established. In particular, Src binding sites on VEGFR-2 and biological importance of Src in VEGFR-2-mediated signal transduction is not fully understood. Recent study demonstrated that Src kinase activity is required to protect endothelial cells from apoptosis and it appears that Src elicits its anti-apoptatic function via Raf-1 activation (73).

It has also been shown that the ability of VEGF to induce vascular permeability requires Src kinases. Curiously, angiogenesis is normal in mice deficient for Src and Yes (74). Src activation also is implicated in GPCR (such as nucleotide receptor P2Y2)-mediated transactivation of VEGFR-2 but not in ligand-mediated VEGFR-2 activation (47,75). Indeed, ligand-dependent autophosphorylation of VEGFR-2 is not compromised in SYF cells, deficient in Src, Yes and Fyn and also overexpression of a dominant negative form of Src in endothelial cells has no apparent effect on the ligand-stimulated VEGFR-2 autophosphorylation (47). In addition to role of Src in VEGFR-2-mediated signal transduction, Src also plays a key role in angiogenesis by promoting expression of VEGF in response to hypoxic condition (76). Further studies are required to delineate exact role of Src in VEGFR-2 signal transduction, in particular, its association with VEGFR-2 and tyrosine phosphorylation site on VEGFR-2 that might be involved in recruitment of Src to VEGFR-2.

5.5. Protein kinase C, c-Cbl and VEGFR-2 downregulation

In addition to activating positive signaling pathways, RTKs activation also induces negative feedback signaling pathways that attenuate or antagonize positive signaling. One mechanism

that attenuates RTKs signaling is a rapid depletion of the cellular receptor pool, a phenomenon termed downregulation (77). Recent study indicates that VEGFR-2 undergoes ligand-dependent downregulation and degradation in a novel mechanism involving non-classical PKCs. Singh *et al.*, (53) demonstrated that the carboxyl terminus is required for PKC-dependent downregulation of VEGFR-2 (Figure 4). Surprisingly, the ligand-dependent downregulation and degradation of VEGFR-2 proceeds without involvement of its ectodomain and engagement of γ-secretase activity. Mutation of serine sites at 1188 and 1191 compromised the ability of VEGFR-2 to undergo efficient ligand-dependent downregulation. These findings suggest that serines 1188 and 1191 of VEGFR-2 are phosphorylated either directly by PKC or by serine kinases whose activity is regulated by PKCs. Upon phosphorylation these sites may recruit an E3 ligase, leading to ubiquitinylation and degradation of VEGFR-2. The identity of the E3 ligase responsible for ubiquitinylation of VEGFR-2 is not known. It appears, however, that c-Cbl is not the E3 ligase that involved in ubiquitinylation and downregulation of VEGFR-2 although it is phosphorylated upon VEGFR-2 stimulation with ligand (53). Since c-Cbl is activated by VEGFR-2 it is interesting to know what role it plays in angiogenesis.

6. CONCLUSIONS

Abnormal angiogenesis is associated with pathology of many diseases including, cancer, age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), and retinopathy of prematurity (ROP). What is clear is that the more we learn about molecular mechanisms of VEGFR-1 and VEGFR-2 regulation and their signal transduction, the more we continue to learn about angiogenesis and molecules that regulate it. The emerging scenario in VEGFR-1 and VEGFR-2 activation is that the complexity of blood vessel development necessitated more stringent control over VEGFR-1. In one had VEGFR-1 evolved to have higher affinity to bind various ligands, in the other had VEGFR-1 is lost its potency in enzymatic activity, creating a unique mechanism to control its function. VEGFR-2 plays a broader role in angiogenesis. Its activity regulates endothelial cell growth, differentiation, migration and tubulogenesis. The carboxyl terminus of VEGFR-2 plays a central role in its activation, its ability to recruit signaling proteins and its ligand-induced downregulation.

Finally it should be noted that activation and signaling of VEGF receptors appear to be regulated not only by each other in both negative and positive manners but also by other receptors including, integrins (77,78), cadherins (79,80) and neuropilines (21). In addition, given the fact that multiple VEGF ligands with different preference and capability bind to VEGFRs and activate them in homo- and heterodimeric manner present a great challenge to study their selective biochemical and cellular signaling in endothelial cell background. Although, characterization of specific function of VEGFR-1 and VEGFR-2 and their signal transduction relays has been frustrating task, but nevertheless was illuminating. Continued research into the function of VEGFRs as well as techniques involving creating chimeric VEGFRs in which the extracellular domain of each VEGFR is replaced with that of CSF-1R (5), mutant VEGFs that selectively bind to VEGFRs (17) continue to provide insight into their specific roles in angiogenesis. Evaluation of targeted mouse knockouts (12,14) and knockin (70) and the use of RNA interference may provide further insight into function of this important family of receptors.

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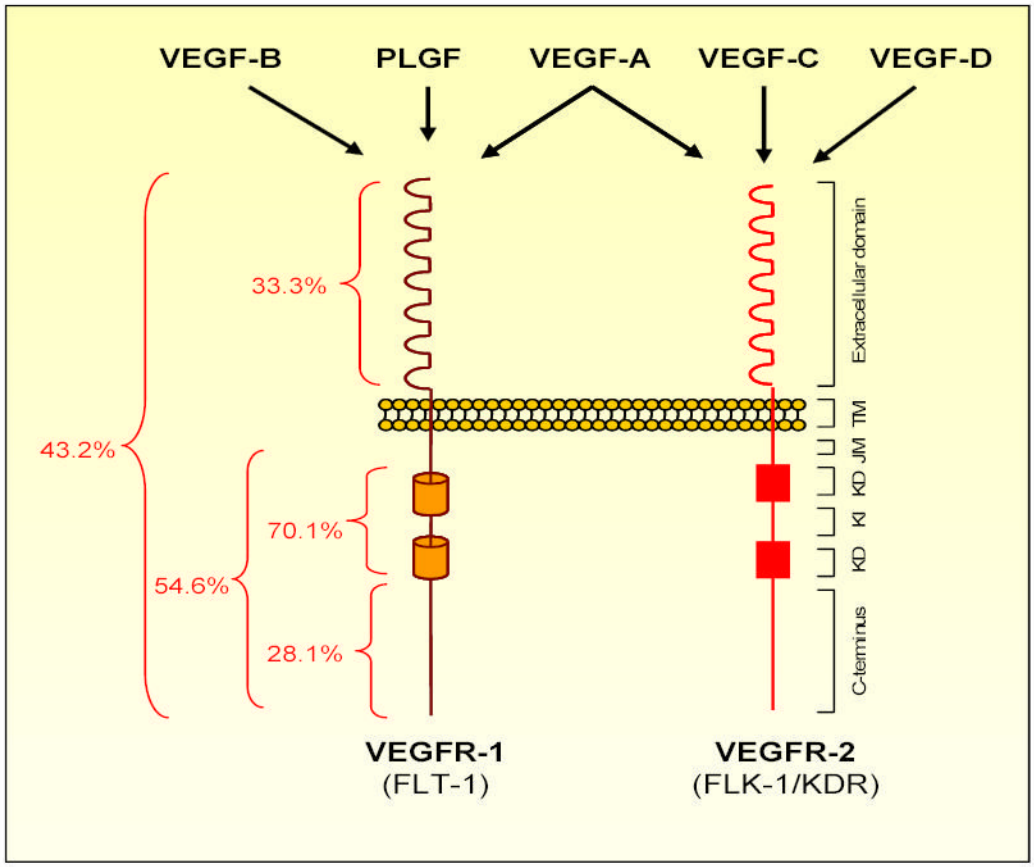

Figure 1.

Schematic representation of VEGFR-1, VEGFR-2 and their corresponding ligands: Multiple VEGF ligands bind to VEGFR-1 and VEGFR-2. Placenta growth factor (PLGF) and VEGF-B bind to VEGFR-1 but not to VEGFR-2. VEGF-C and VEGF-D bind VEGFR-2 but not to VEGFR-1. VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGFR-1 and VEGFR-2 are structurally similar, consisting of an extracellular ligand-binding domain with seven immunoglobulin (Ig)-like motifs, a single transmembrane domain (TM) and juxtamembrane (JM) domain, a kinase domain (KD) split by a kinase insert (KI), and a carboxyl terminus. The sequence homology between VEGFR-1 and VEGFR-2 also is shown. The kinase domain of VEGFR-1 and VEGFR-2 represent the most conserved region with 70.1% homology. The carboxyl terminus represents the most divergent region with only 28.1% sequence homology.

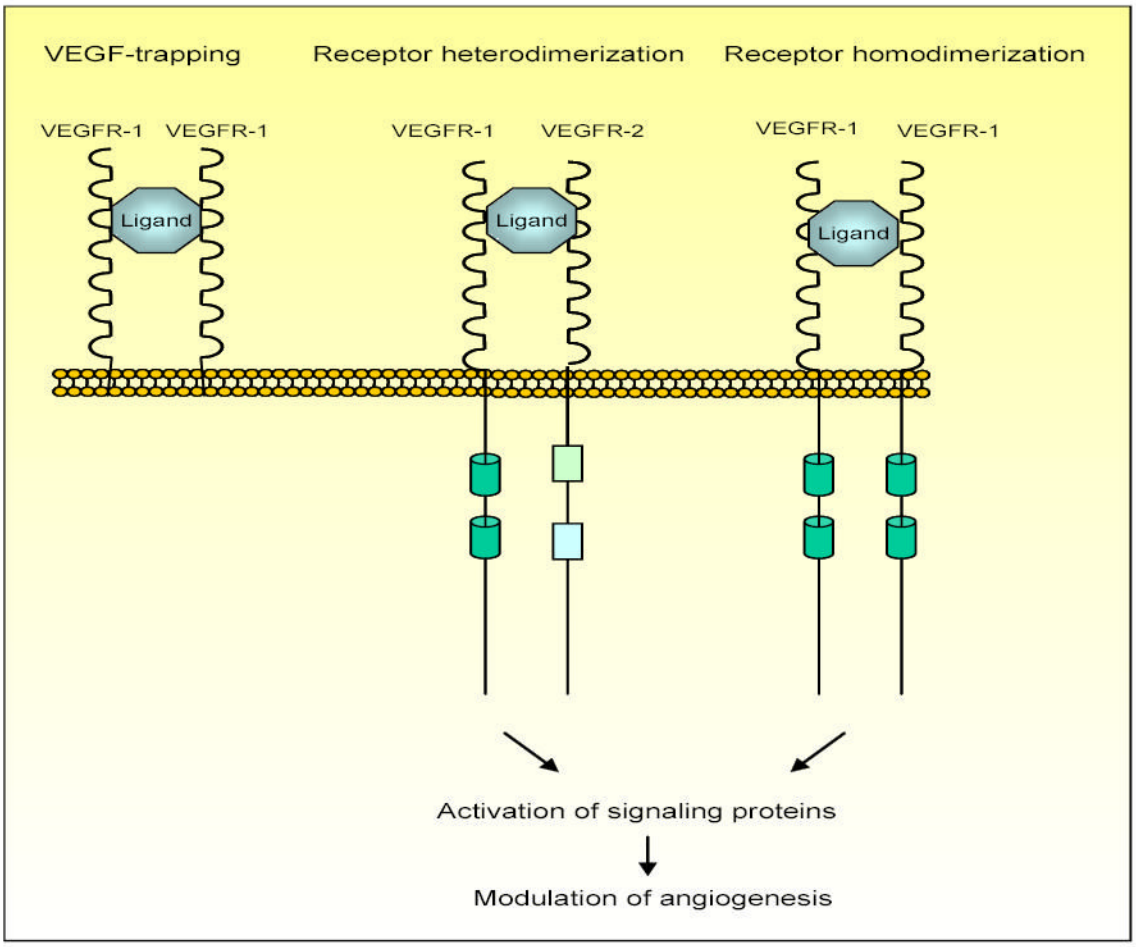

Figure 2.
Schematic representation of the mechanisms of involvement of VEGFR-1 in angiogenesis: VEGF-trapping: the mechanism by which VEGFR-1 binds to VEGF and prevents it from binding to VEGFR-2. The cytoplasmic domain of VEGFR-1 is not required for its VEGF-trapping function (19). VEGFR-1 may elicit its role in angiogenesis by heterodimerization with VEGFR-2 and VEGFR-3 (5,6,36). The third mechanism by which VEGFR-1 regulates angiogenesis is through receptor homodimerization. This type of activation of VEGFR-1 leads to activation of signaling proteins that might regulate angiogenesis in either positive or negative manner (5,18).

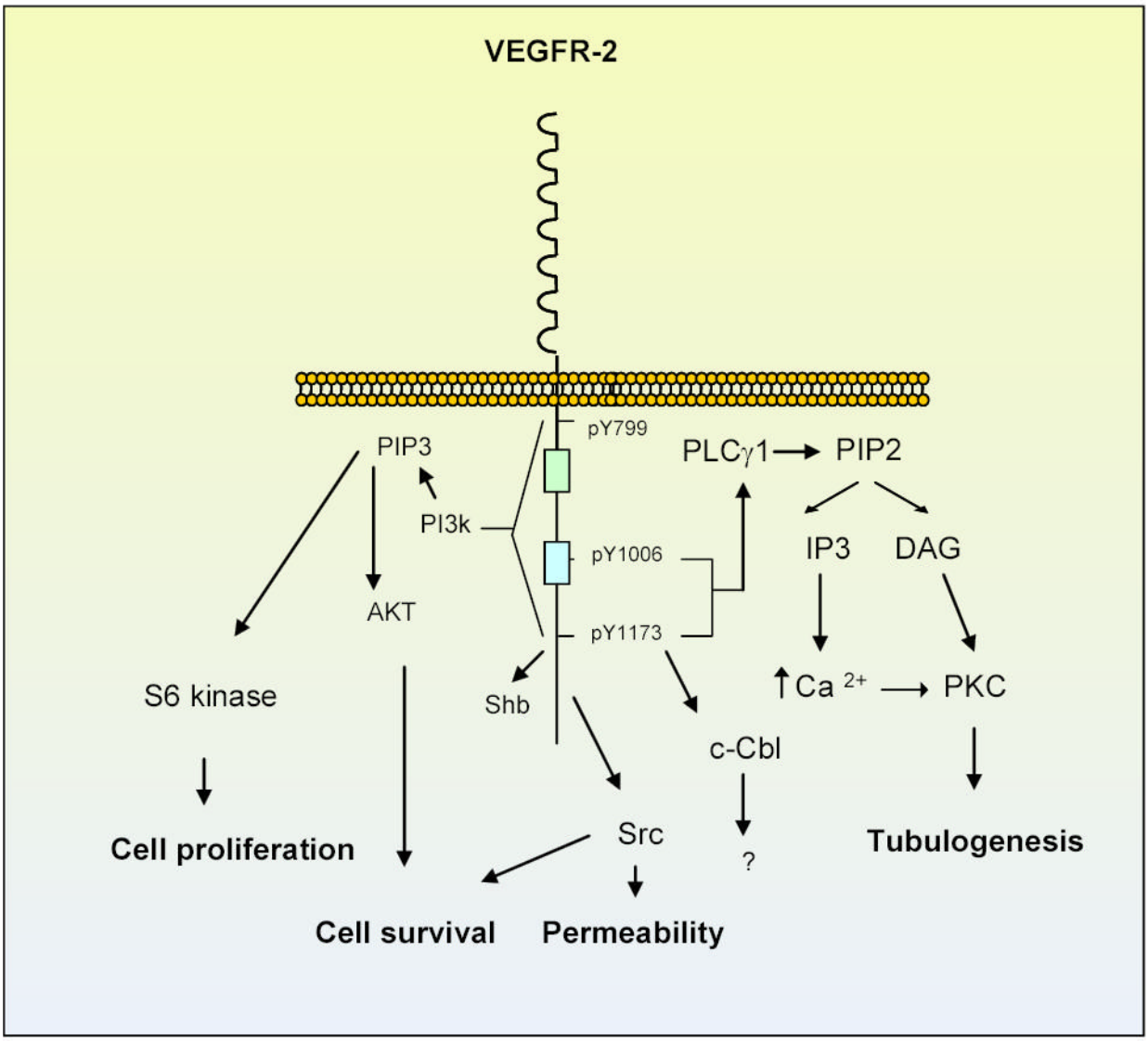

Figure 3.

Schematic representation of VEGFR-2 mediated signal transduction and their role in endothelial biology: VEGFR-2 activation induces tyrosine phosphorylation and activation PLCγ1 via tyrosines 1006 and 1173. Activation of PLCγ1 leads to tubulogenesis and proliferation of endothelial cells. Activation of PI3 kinase by VEGFR-2 is mediated by tyrosines 799 and 1173 and is involved in proliferation and survival of endothelial cells. Tyrosine 1173 of VEGFR-2 also associates with the adaptor protein, Shb and the E3 ligase, c-Cbl. Src family kinases also are activated by VEGFR-2 and involved in endothelial cell survival and permeability (see text for details).

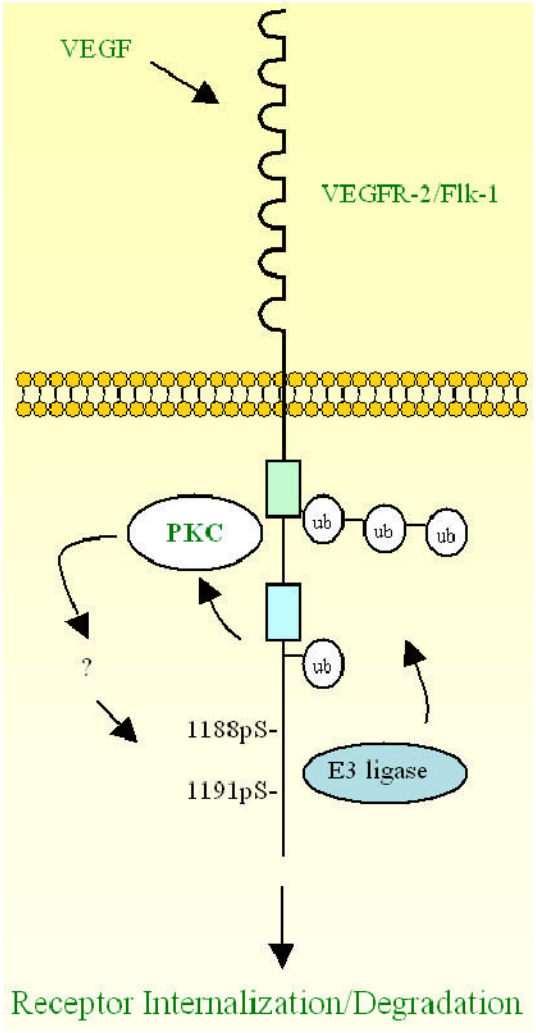

Figure 4.
Schematic representation of ligand-dependent downregulation and degradation of VEGFR-2: VEGFR-2 undergoes ligand-dependent downregulation and degradation via a mechanism that involves activation of non-classical PKCs. Activation of non-classical PKCs by VEGFR-2 results in phosphorylation of serines 1188 and 1191 of VEGFR-2. Serines 1188 and 1191 are phosphorylation either directly by PKC or by serine kinases whose activity is regulated by PKCs. Upon phosphorylation, serines 1188 and 1191 may recruit an E3 ligase, leading to ubiquitinylation and degradation of VEGFR-2 (53).