Leucine motif-dependent tyrosine autophosphorylation of type III receptor tyrosine kinases

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Abstract

Activation loop tyrosine autophosphorylation is an essential requirement for full kinase activation of receptor tyrosine kinases (RTKs). However, mechanisms involved are not fully understood. In general, kinase domains of RTKs are folded into two main sub-domains, N- and C-terminal lobes. The C-terminal lobes of VEGFR-2 are folded into seven α-helices (αD-αl). In the studies presented here we demonstrate that leucine residues of helix I (αl) regulate tyrosine autophosphorylation and phosphotransferase activity of VEGFR-2. The presence of three leucines 1158, 1161 and 1162 are essential for tyrosine autophosphorylation and kinase activation of VEGFR-2 and are involved in helix-helix packing via hydrophobic interactions. The presence of leucine 1158 is critical for kinase activation of VEGFR-2 and appears to interact with αE, αF, αH and β7. The analogous residue, leucine 957 on PDGFR-β and leucine 910 on CSF-1R also found to be critical for tyrosine autophosphorylation of these receptors. Leucines 1161 and 1162 are also involved in helix-helix packing but they play but less critical role in VEGFR-2 activation. Thus, we conclude that Leucine motif-mediated helix-helix interactions are critical for kinase regulation of type III RTKs. This mechanism is likely to be shared with other kinases and might provide a basis for the design of a novel class of tyrosine kinase inhibitors.

Introduction

Receptor tyrosine kinases (RTKs) are a large family of enzymes, many of which mediate vital cellular functions of living organisms. The fine-tuning of RTKs function is essential for their normal physiological roles and their aberrant function contributes to human diseases ranging from cancer to diabetes (1-4). RTKs consist of an extracellular region that serves a ligand binding site, a transmembrane domain, and a cytoplasmic region, which possesses intrinsic tyrosine kinase activity. In the inactive state, the activation loop is thought to occupy the active site preventing substrate access and ATP binding (5,6). Ligand-mediated RTK activation leads to RTK dimerization. Dimerization is believed to facilitate transphosphorylation of one or two tyrosines within the activation loop. Based on the crystal structure of Insulin receptor (7), FGF receptor (8) it is proposed that activation loop tyrosine...
autophosphorylation removes activation loop away from the active site and create appropriate conformation for optimal substrate and ATP binding (6-8). The catalytic kinase domain of RTKs ranges from 250-300 amino acid residues and contains highly conserved amino acid sequences. The typical kinase domain of RTKs is folded into two main sub-lobes, N- and C-terminal lobs. Catalysis occurs in a cleft between the two domains. Residues in the N-terminal lob are mainly in β-sheets and are involved in ATP binding. Residues in the C-terminal lob however, are primarily in α-helical conformation and are important for catalysis and for protein substrate binding (6,9).

Vascular endothelial growth factor receptor-2 (VEGFR-2) is a type III RTK and its activation is critical for normal vasculogenesis, pathological angiogenesis and neural development (10-14). Activation of VEGFR-2 stimulates a number of key signal transduction pathways such as phosphoinositide 3-OH kinase which involved in endothelial cells survival and proliferation (PI3-kinase) (15,16), Phospholipase-Cγ1 (PLCγ1) which stimulates endothelial cells tubulogenesis (17), Src kinases (19,20) and Cbl-E3 ligase (20). VEGFR-2 also associates with a number of adaptor proteins including, VRAP (21) and Shb (22). The crystal structure analysis of VEGFR-2 has revealed that C-terminal loop in the kinase domain folds into 7 α-helices (αD-αI) and two anti-parallel β-sheets (23). Although the key structural features of kinase domain have been deduced from the crystallographic structures, the biochemical data corroborating those observations is largely unavailable. Only the roles of key motifs such as GXGXXG, HRDLA, DFG and activation loop autophosphorylation sites are fairly well-characterized (6).

Furthermore although, activation loop tyrosine autophosphorylation is the most common mechanism used by RTKs for activation, there are a number of other mechanisms for regulation of kinase activation of protein kinases. For instance, the C-terminal Src kinase (Csk) and EGFR do not require activation loop tyrosine autophosphorylation. Instead, Csk activity is regulated by its SH2 and SH3 domains (24) and EGFR activity is regulated by intermolecular interactions between the kinase domain and its carboxyl tail (25).

We recently have observed that a large deletion from the carboxyl terminus of VEGFR-2 which also removes several amino acids from helix-I (αI) region prevents full tyrosine phosphorylation of VEGFR-2 (26). We hypothesized that helix-I may play a global role in RTK activation, substrate recognition and phosphorylation. In this study we show that leucines1158, 1161 and 1162 of helix-I via hydrophobic interactions with αE, αF and αH participates in maintaining an appropriate conformation of VEGFR-2 necessary for its tyrosine autophosphorylation and kinase activation. Analogous leucine residues in PDGFR-β and CSF-1R were also found important for their activation. We propose that that a highly conserved leucine motif plays a critical role in which activation loop segment is held into active conformation in type III RTKs. Since, leucine motif is conserved in both receptor tyrosine kinases and non-receptor tyrosine kinases we propose that this mechanism is likely to be shared with other kinases and might provide a basis for design of a novel class of tyrosine kinase inhibitors.

Materials and Methods:
Reagents and Antibodies: Recombinant mouse PDGF-BB and recombinant human macrophage colony stimulating factor
(rhM-CSF-1) were purchased from Biosource International (Camarillo, CA) and R&D Systems (Minneapolis, MN), respectively. Mouse monoclonal anti-phosphotyrosine antibodies, 4G10 and PY-20, were purchased from Upstate Biotechnology (Lake Placid, NY) and Transduction Laboratories (Lexington, KY), respectively. Monoclonal mouse anti-VEGFR-2/FLK-1 was purchased from Chemicon International (Temecula, CA). The following antibodies were purchased from Santa Cruz Biotechnology, Inc. Pre-adsorbed goat anti-mouse IgG-HRP, pre-adsorbed goat anti-rabbit IgG-HRP, rabbit polyclonal IgG anti-c-Fms/CSF-1R(C-20), rabbit polyclonal IgG anti-PDGFR-β (958). Rabbit polyclonal anti-VEGFR-2 sera were raised against either a glutathione S-transferase-VEGFR-2 kinase insert domain fusion protein (1410) or a glutathione S-transferase-VEGFR-2 carboxy-terminus fusion protein (1412) as described elsewhere (29). Adenosine 5’-Triphosphate and Poly Glu:Tyr (4:1) were purchased from Sigma-Aldrich. Anti-phospho-VEGFR-2 (pY1054/pY1059) was purchased from Cell Signaling Technology (Beverly, MA). rProtein A Sepharose Fast Flow was purchased from Amersham Bioscences (Uppsla, Sweden).

Site-directed Mutagenesis: The cDNAs for mouse VEGFR-2, mouse PDGFR-β and human CSF-1R were used as template to generate the mutant receptors. The creation of VEGFR-2 chimera (CKR) in which their extracellular domain is replaced with that of human CSF-1R were described elsewhere (29). The mutations were made using PCR-based site-directed mutagenesis method as previously described (17,19). The reactions were carried out using Accuprime Pfx DNA Polymerase (Invitrogen). The resultant mutations were verified by sequencing and were subsequently cloned into pLXSN² or pLNCX² retroviral vectors by NotI and SalI sites.

In vitro Kinase assay and substrate phosphorylation: Equal number of PAE cells (porcine aortic endothelial cells) expressing CKR or mutant CKRs were serum-starved for overnight. Cells without stimulation were lysed. Proteins were immunoprecipitated with anti-VEGFR-2 antibody. The immunoprecipitated proteins were washed once with cold lysis buffer pH 7.4 (1% Triton-X 100, 10mM Tris-HCl, 5mM EDTA, 50mM NaCl, 50mM NaF, 2mM sodium orthovanadate, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride and 20 μg/ml aprotinin) and three times with cold PAN buffer (10mM PIPES pH 7.0, 100mM NaCl, 20mg/ml aprotinin). In vitro kinase activity was performed by incubating immunoprecipitated proteins with 20 μl of kinase buffer (10 mM MgCl₂, 1 mM dithiothreitol, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4) containing 0.1 to 1 mM ATP for 15 min at 30°C. The reaction was stopped by the addition of an equal volume of SDS sample buffer. The samples were denatured and resolved on 7.5% SDS-PAGE and subjected to Western blot analysis using anti-phosphotyrosine antibody. To measure the ability of mutant VEGFR-2s to phosphorylate a substrate, poly-Glu peptide was used as described (26). Briefly, cells were stimulated with ligand for 10 min, lysed and immunoprecipitated with anti-VEGFR-2 antibody (1410 or 1412). Substrate phosphorylation was measured as described (27). Briefly, immunoprecipitated proteins were incubated in 10 μCi of [γ-32P]ATP for 15 min at 30°C in the presence of substrate (5 μg/reaction). The reaction was stopped and samples were spotted on the p81 paper and after extensive washing, the p81 papers were subjected to scintillation
counter and their DPM value were measured.

Immunoprecipitation and Western Blot Analysis: PAE cells expressing CKR or mutant CKRs were grown in sparse condition in 10% FBS, and serum starved overnight in DMEM. Cells were left either resting or stimulated with 40 ng/ml CSF-1 at 37°C for appropriate time as indicated in figure legends. Cells were washed twice with H/S buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM Na$_3$VO$_4$) and lysed in lysis (EB) buffer (10 mM Tris-HCL, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na$_3$VO$_4$, and 20 ug/ml aprotinin). VEGFR-1 proteins were immunoprecipitated with anti-VEGFR-1 antibody and immunocomplexes were bound to Protein-A sepharose. Immunoprecipitates were resolved on a 7.5 % SDS-PAGE gel, and the proteins were transferred to Immobilon membrane. For anti-phosphotyrosine Western blot analysis, the membranes were then incubated in Block buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mg/ml BSA, 10 mg/ml ovalbumin, 0.05 % Tween-20, 0.005 % NaN$_3$, and then incubated with primary antibody diluted in Block. The membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies. Finally, the membranes were washed, and developed with ECL (Amersham Corp). In some occasions, the membranes were striped by incubating them in a buffer containing 6.25 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM β-mercaptoethanol in 50 °C for 30 min and re-probed.

Molecular Modeling: The three-dimensional model of VEGFR-2 was built using homology modeling technique with the Mol-Mol program (28) based on the 2.4 Å resolution X-ray structure of human VEGFR-2. All the atom coordinates were from human VEGFR-2 (23). The side chain interactions were calculated by Xtal contact of CCP4 program.

Results

Regulation of ligand-dependent tyrosine phosphorylation of VEGFR-2 by leucine residues

The amino acid residues in the C-terminal loop of the kinase domain of VEGFR-2 folds into 2 β-sheets and 7α-helices. The αI consists of 12 amino acid residues (1152-1166 in human VEGFR-2) (23). These residues in mouse VEGFR-2 corresponds to residues from 1150 to 1164 (Figure 1). Deletion of the carboxyl terminus of VEGFR-2 from leucine 1158 abrogates the ligand-dependent autophosphorylation of VEGFR-2. However, deletion of the carboxyl terminus after residue 1211 preserves its ligand-dependent tyrosine autophosphorylation (26). This suggests that autophosphorylation of VEGFR-2 is regulated by the presence of 53 amino acid residues (1158-1211) within this region. To address the mechanism involved in tyrosine autophosphorylation of VEGFR-2, we have created a panel of mutant and truncated mouse VEGFR-2s (Figure 1) and expressed them in porcine aortic endothelial (PAE) cells. To avoid cross talk with endogenous VEGF receptors we have utilized a chimeric VEGFR-2 system in which the extracellular domain of VEGFR-2 is replaced with that of human CSF-1R herein called CKR (29). As shown in Figure 2A, deletion of the carboxyl terminus of VEGFR-2 from amino acid residue 1172 (ΔCKR/194) preserves ligand-dependent tyrosine
autophosphorylation as judged by its ability to undergo tyrosine phosphorylation, using a general anti-phosphotyrosine antibody and a specific anti-phospho-1052/1057 VEGFR-2 antibody (Figure 2B). Tyrosines 1052 and 1057 are activation loop tyrosines and are involved in VEGFR-2 activation (23,30,31). To test which amino acid among the 14 amino acid residues is involved in regulation of autophosphorylation of VEGFR-2, we initially mutated N1160, Q1163, N1165, Q1167 and Q1168 to alanine (A). This mutant receptor herein is referred to 5A/CKR. The mutant receptor was expressed in PAE cells and its ability to become autophosphorylated in response to ligand stimulation was measured. Figure 2D and 2E shows that 5A/CKR is able to undergo tyrosine autophosphorylation in response to ligand stimulation, suggesting that amino acids including, N1160, Q1163, N1165, Q1167, and Q1168 are not involved in the regulation of ligand-dependent tyrosine phosphorylation of VEGFR-2.

To test whether leucine residues 1158, 1161 and 1162 are associated with the regulation of tyrosine autophosphorylation of VEGFR-2, we generated three additional constructs including; a single leucine 1158 mutation (A1158/CKR), a double mutation leucine 1161 and leucine 1162 (A1162/62/CKR) and a triple leucine mutation leucine 1158, leucine 1161, leucine 1162 (A1158/61/62/CKR) and expressed them in PAE cells. Replacement of single leucine 1158 to alanine and replacement of two leucine residues 1161 and 1162 to alanine appeared to have no effect on the tyrosine phosphorylation of CKR (Figure 2D). However, a triple mutation of leucines 1158, 1161 and 1162 significantly reduced the ability of VEGFR-2 to undergo ligand-dependent tyrosine autophosphorylation. These mutations also substantially reduced the ligand-dependent phosphorylation of activation loop tyrosines 1052 and 1057 (Figure 2G and 2H). This suggests that the presence of three leucine residues collectively contribute to ligand-dependent activation loop tyrosine autophosphorylation of VEGFR-2. To test the ability of leucine mutant VEGFR2s to phosphorylate an exogenous substrate, we assessed their capacity to phosphorylate a poly-glu peptide in vitro. Figure 2J shows that the wild type CKR is able to phosphorylate exogenous substrate in ligand-dependent manner. However, the ability of leucine mutant VEGFR-2s including, A1158/CKR, A1161/1162/CKR and A1158/1161/1162/CKR to phosphorylate poly-glu peptide were severely compromised. This suggests that the presence of leucines 1158, 1161 and 1162 are critical for possible substrate recognition or for phosphotransferase capability or for both of these functions by VEGFR-2.

**Leucines 1158, 1161 and 1162 differentially contribute to tyrosine phosphorylation and kinase activation of VEGFR-2**

To understand better how the lack of leucines 1158, 1161 and 1162 contribute to tyrosine phosphorylation of VEGFR-2, we tested the ability of wild type and leucine mutant VEGFR-2s to undergo autophosphorylation in vitro kinase assay ATP varying concentrations of ATP. For this purpose, PAE cells expressing wild type and mutant VEGFR-2s were serum-starved and lysed without ligand stimulation. Cell lysates were immunoprecipitated with an anti-VEGFR-2 antibody and subjected to an in vitro kinase assay using different concentrations of ATP as indicated. The ability of these
receptors to become autophosphorylated in response to ATP was measured by immunobloting with an anti-phosphotyrosine antibody. As shown in Figure 3A, ATP promoted a dose-dependent autophosphorylation of wild type VEGFR-2. The triple leucine mutant receptor, A1158/1161/1162/CKR, however, displayed a highly compromised tyrosine autophosphorylation in response to ATP stimulation (Figure 3A), suggesting that these residues contribute to both ligand- (Figure 2G) and ATP-dependent tyrosine phosphorylation of VEGFR-2. ATP-dependent autophosphorylation of the double leucine mutant VEGFR-2 (A1161/1162/CKR) was partially reduced compared to that wild type receptor (Figure 3C). Furthermore, A1158/CKR, which displayed almost normal tyrosine autophosphorylation in response to ligand stimulation (Figure 2G), also failed to undergo ATP-induced tyrosine phosphorylation (Figure 3C). In contrast, ATP-induced tyrosine phosphorylation 5A/CKR, where five residues including N1160, Q1163, N1165, Q1167, and Q1168 were replaced with alanine showed no apparent decrease in tyrosine phosphorylation (Figure 3E). These findings demonstrate that leucine 1158 a critical role in kinase activation of VEGFR-2. Leucines 1161 and 1162 appear to play less critical but important role in kinase activation of VEGFR-2. Altogether, the data demonstrate that presence of leucines 1158, 1161, and 1162 are critically important for kinase activation and for its ability to recognize and phosphorylate substrate.

**Trans-phosphorylation of leucine mutant 1158 VEGFR-2 by the wild type VEGFR-2**

Because the leucine 1158 mutation to alanine compromised the ability of VEGFR-2 to undergo tyrosine phosphorylated in vitro kinase assay, whether the lack of this residue prevents VEGFR-2 to undergo trans-phosphorylation. For this purpose we tested whether A1158/CKR could be tyrosine phosphorylated by the wild type VEGFR-2. For this purpose we immunoprecipitated A1158/CKR from serum-starved and non-stimulated PAE cells and incubated with PAE cell lysates expressing no CKR or expressing CKR and stimulated with ligand. The result showed that A1158/CKR could be phosphorylated by the wild type VEGFR-2 (Figure 4A). Cell lysates from PAE cells expressing no CKR did not phosphorylate A1158/CKR (Figure 4A). Altogether, in vivo tyrosine phosphorylation of A1158/CKR (Figure 2G) coupled with the data presented in this figure suggests that leucine 1158 selectively participate in phosphotransferase activity of VEGFR-2, perhaps by interacting with the nucleotide binding loop or activation loop segment of VEGFR-2.

**Mutation of leucines 1158, 1161 and 1162 do not alter the ligand-mediated dimerization of VEGFR-2**

Because A1158/CKR failed to undergo kinase activation and its ability to phosphorylate an exogenous substrate was severely compromised, we tested whether this mutation alters the capacity of VEGFR-2 to undergo ligand-induced dimerization. Dimerization is a common mechanism by which ligand-induced activation of RTKs is achieved. Point mutations in the kinase domain of RTKs have been shown to lead to oncogenic activation through receptor dimerization (1). To test whether leucine mutations alters VEGF-2’s ability to undergo dimerization we analyzed the ligand-stimulated dimerization of the wild type
receptor and the leucine mutant receptors. As shown in Figure 5, VEGFR-2 undergoes ligand-dependent covalent disulfide linked dimerization. Similarly, A1158/CKR and the triple leucine mutant VEGFR-2 (leucines 1153, 1161 and 1162) were able to undergo ligand-induced disulfide linked dimerization. Altogether, the data suggest that VEGFR-2 undergoes ligand-stimulated covalent disulfide linked dimerization and leucine residues although impairs the ligand-induced tyrosine autophosphorylation, have no effect on the ligand-induced dimerization of VEGFR-2.

Leucines 1158, 1161 and 1162 are highly conserved in type III RTKs

Amino acid sequence alignment of VEGFR-2 with other type III RTKs as shown in Figure 6 revealed that leucines, 1158, 1161 and 1162 are highly conserved among these RTKs. Leucine 1158, in particular is conserved in all type III RTKs with exception of PDGFR-α. Interestingly, PDGFR-α is considered to be a weaker kinase compared to the highly related receptor, PDGFR-β (32). In addition, leucine 1158 is also conserved in non-receptor tyrosine kinases like Src family kinases (33). The presence of this highly conserved residue among these RTKs is highly suggestive that this residue may play a similar role in the activation of other RTKs. Leucine 1158 of VEGFR-2, in PDGFR-β and CSF-1R corresponds to 957 and 910, respectively. To test whether, the corresponding leucine residue in PDGFR-β and CSF-1R are also involved in the regulation of their tyrosine phosphorylation, we mutated leucine 957 in PDGFR-β and leucine 910 in CSF-1R to alanine and expressed them in PAE cells. The wild type receptors and leucine mutant receptors were analyzed for their capacity to undergo tyrosine phosphorylation.

Leucine 957 regulates both ligand and ATP stimulated tyrosine phosphorylation of PDGFR-β

To test the contribution of conserved leucine 957 to tyrosine phosphorylation of PDGFR-β, PAE cells individually expressing PDGFR-β and Ala957/PDGFR-β were stimulated with PDGF-BB and analyzed for their ability to undergo ligand-dependent tyrosine phosphorylation. As shown in Figure 7A, ligand stimulation of the wild type PDGFR-β resulted in robust tyrosine phosphorylation, Ala957/ PDGFR-β, however, failed to undergo tyrosine phosphorylation. This observation was rather unexpected, because similar mutation in VEGFR-2 impaired only ATP-dependent but not ligand-dependent tyrosine phosphorylation (Figures 2G and 3C). It also appears that the ability of mutant PDGFR-β to be fully processed and localized in the cell membrane is also altered as seen by the accumulation of the premature form of the Ala957 PDGFR-β (Figure 7B). To test whether leucine 957 mutant can be tyrosine phosphorylated in response to ATP treatment, we immunoprecipitated PDGFR-β and Ala957/ PDGFR-β from serum-starved and non-stimulated PAE cells and subjected to in vitro kinase assay using different concentrations of ATP as described in materials and methods. The data shows that the ability of A957/PDGFR-β to become tyrosine phosphorylated in response to ATP is severely compromised (Figure 7C). Collectively, the data suggest that in PDGFR-β unlike VEGFR-2 the conserved leucine (1158 in VEGFR-2, 957 in PDGFR-β) is required for both ligand and ATP stimulated tyrosine phosphorylation of PDGFR-β.
Leucine 910 regulates both ligand and ATP stimulated tyrosine phosphorylation of CSF-1R

In addition to VEGFR-2 and PDGFR-β, we wished to evaluate mutation of conserved leucine to alanine in another type III RTK. To this end, we mutated leucine 910 on human CSF-1R/c-fms to alanine and assessed its impact in tyrosine phosphorylation of CSF-1R. Figure 8A shows that like PDGFR, the ligand-dependent tyrosine phosphorylation of Ala910/CSF-1R is markedly inhibited. In addition, we evaluated ATP-stimulated tyrosine phosphorylation of CSF-1R. As shown in Figure 8B, ATP-stimulated tyrosine phosphorylation of Ala910/CSF-1R is almost totally abolished. In sum, data indicate that the conserved leucine residue in question plays a major role in the activation of RTKs. It also appears that this highly conserved residue plays a differential role in RTKs activation. In VEGFR-2 it alters ATP-stimulated tyrosine phosphorylation and phosphotransferase activity toward exogenous substrate. In PDGFR and CSF-1R, mutation of this residue impairs tyrosine phosphorylation in response to both ATP and ligand stimulation.

Leucines 1158, 1161 and 1162 are involved in helix-helix interactions

In order to understand the role of these leucine residues in VEGFR-2 activation, a three dimensional model of VEGFR-2 was generated based on the X-ray crystal structure of human VEGFR-2 (23). Alignment of human and mouse VEGFR-2 indicated that the kinase domain is highly conserved and the leucine 1158 region is invariable between the two species. The model (Figure 9A) shows that leucines, 1158, 1161 and 1162 are part of α-helical (helix I) conformation and are buried in VEGFR-2 structure.

Leucines 1158, 1161 and 1162 make hydrophobic interactions with a number of residues and are involved in helix-helix interactions including αE, αF and αH (Figure 9B). Whether these leucine residues also interact with the activation and the catalytic loop is not clear, because in the original crystal structure the activation loop of VEGFR-2 is highly disordered (20). Leucine 1158 and 1161 also make hydrophobic interactions with residues of β-sheet (β7), which separates the catalytic loop from the activation loop. Leucine 1162 mainly associates with the residues of αE whereas leucine 1161 mostly associates with the residues of αH. Among all the three leucines, leucine 1158 has the most contacts. It associates with the residues of αE, αF, αH and β-sheet (β7). This might explain why mutation of this site in VEGFR-2, CSF-1R and PDGFR-β interferes with autophosphorylation of these receptors. Furthermore, it suggests that mutation of leucine1158 and its analogous sites in CSF-1R and PDGFR-β disrupts hydrophobic interactions between leucine 1158 and amino acid residues of αE, αF, αH and β7.

Discussion

In the present study, we have focused on the molecular mechanisms of VEGFR-2 activation. Our study demonstrates that the highly conserved leucine residues of αI, the last helix of the kinase domain of VEGFR-2, are involved in helix-helix packing and play a critical role in VEGFR-2 activation. Regulation of VEGFR-2 activation plays a key role in the induction of normal and pathological angiogenesis as well as neural development (10-13,34). Despite its well-known physiological importance, the molecular mechanism that governs VEGFR-2 activation is not fully understood.
VEGFR-2 is a type III RTK and it is well-recognized that activation of RTKs are regulated by tyrosine phosphorylation of activation loop tyrosine sites (7), but it is less clear precisely how tyrosine phosphorylation of activation loop is regulated. In particular, the influence of the other sub-domains on tyrosine phosphorylation and activation of RTKs is not known. Our study demonstrates that leucines 1158, 1161 and 1162 contribute to tyrosine autophosphorylation and kinase activation of VEGFR-2. The lack of leucine 1158 alone impaired the kinase activation of VEGFR-2 as defined by its ability to undergo tyrosine autophosphorylation in an in vitro kinase assay and to phosphorylate exogenous substrate. In vivo ligand-induced tyrosine phosphorylation of VEGFR-2 including phosphorylation of activation loop tyrosines 1052 and 1057, however, were not impaired by the lack of leucine 1158.

The lack of tyrosines 1161 and 1162 also appeared not to impair in vivo ligand-induced tyrosine phosphorylation, but their absence partially impaired kinase activation and diminished phosphorylation of exogenous substrate by VEGFR-2. The data clearly indicate that these leucines participate in the maintaining the active conformation of RTKs. The observation that assay conditions (e.g., in vivo versus in vitro) influences tyrosine phosphorylation of these leucine mutant VEGFR-2s although surprising, but it may suggest that the lack of these leucine residues predisposes RTK to a less kinase active conformation and perhaps the leucine mutant VEGFR-2 proteins are partially misfolded. The analogous leucine sites in PDGFR-β (leucine 957) and CSF-1R (leucine 910) also play critical role in the tyrosine phosphorylation and activation of these RTKs. The crystal structure of PDGFR-β and CSF-1R are not currently available, the crystal structure of human VEGFR-2 has been resolved (23). It appears that leucines, 1158, 1161 and 1162 are part of α-helical (helix I) conformation and are buried in VEGFR-2 structure. Leucines1158, 1161 and 1162 make hydrophobic interactions with a number of residues and are involved in helix-helix interactions. The interdependence tyrosine phosphorylation of RTKs and leucines 1158 suggest that leucine 1158 contributes to the stability of the active conformation of kinase domain and therefore plays a critical role in which the active conformation is held and mutation of this residue may destabilize the active conformation. A number of point mutations in the kinase domain of RTKs including FGFR3, MET and RET have been identified in which they give rise to gain-of-function in these RTKs (1). The mechanism by which leucine mutations inactivates RTKs is novel as appears to abrogate both RTK kinase activation and substrate phosphorylation.

Although in this study we have only evaluated the role of leucine 1158 in VEGFR-2, and the analogous site in PDGFR-β and CSF-1R activation, because this residue is highly conserved in several key tyrosine kinase superfamily including, insulin receptor (35), Src family kinases (36), FGFR family and HGFR family (37) we envision this residue to play a similar role in the tyrosine phosphorylation of these kinases. Understanding the precise mechanisms involved in regulating enzymatic activity of RTKs is important, as it may provide important insights that may facilitate the design of new class of pharmacological agents to inhibit RTKs activation. In light of the importance of VEGFR-2 activation in normal and
pathological angiogenesis, VEGFR-2 provides an attractive target.

Understanding the regulation of VEGFR-2 enzymatic activity and the mechanism by which it signals is of great interest not only for the development of new and more effective antagonists, but also because this understanding should provide clues into mechanisms of regulation of angiogenesis.

Figures (1-9)

Figure 1: Schematic presentation of truncated and leucine mutant VEGFR-2s. 15 amino acid residues within the boundary of kinase domain and carboxyl terminus of mouse VEGFR-2 is shown. Based on the crystal structure, N1165 possibly corresponds to the last residue of the kinase domain. Amino acids (1158-1165) are folded in α-helix conformation and correspond to helix I (23). Alinine1166 (A1166) corresponds to the first residue of carboxyl terminus of VEGFR-2. ΔCKR/194 construct was created by deleting 194 amino acids from carboxyl terminal of mouse VEGFR-2 retaining only seven amino acids in the carboxyl terminal (amino acids 1166-1172 in mouse VEGFR-2). 5A/CKR construct was created by mutating N1160, Q1163, N1165, Q1167 and Q1168 to A (A: alanine). A1158/1161/1162/CKR construct was created by mutating L1158, L1161, and L1162 to A. A1161/1162/CKR construct was created by mutating L1161 and L1162 to A. A1158/CKR construct was made by mutating L1158 to A. The extracellular domain, the transmembrane domain (TM) and the kinase domain (KD) also are shown.

Figure 2: Effect of mutation of leucines 1158, 1162 and 1163 to ligand-dependent tyrosine autophosphorylation of VEGFR-2 and its ability to phosphorylate exogenous substrate. Equal number of serum-starved PAE cells expressing wild type chimeric VEGFR-2 (CKR), carboxyl terminal deleted, ΔCKR/194 and 5A/CKR were either not stimulated or stimulated for 10 or 30 minutes with CSF-1 (40 η/ml). Cells were lysed and total cell lysates were subjected to Western blot analysis using anti-phospho-tyrosine antibody (A and D) or a phospho-specific 1052/1057 VEGFR-2 antibody (B and E). The same cell lysates were subjected to Western blot analysis using anti-VEGFR-2 for protein loading as a control (C and F). In a similar manner, equal number of serum-starved PAE cells expressing wild type chimeric VEGFR-2 (CKR), A1158/CKR, A1161/1162/CKR and A1158/1161/1162 were either not stimulated or stimulated for 10 or 30 minutes with CSF-1. Cells were lysed and total cell lysates were subjected to Western blot analysis using anti-phospho-tyrosine antibody (G) or a phospho-specific 1052/1057 VEGFR-2 antibody (H). The same cell lysates were subjected to Western blot analysis using anti-VEGFR-2 for protein loading as a control (I). Serum-starved PAE cells expressing wild type chimeric VEGFR-2 (CKR), A1158/CKR (1158), A1161/1162/CKR (2Ala) and A1158/1161/1162/CKR (3Ala) were either not stimulated or stimulated for 10 minutes with CSF-1. Cells were lysed, and proteins were immunoprecipitated with anti-VEGFR-2 antibody. The immunoprecipitated proteins were extensively washed and were subjected to an in vitro kinase assay as described in materials and methods and phosphorylation of exogenous substrate was measured (J).
Figure 3: Effect of mutation of leucines 1158, 1162 and 1163 on ATP-induced tyrosine autophosphorylation of VEGFR-2. Equal number of serum-starved PAE cells expressing wild type chimeric VEGFR-2 (CKR), A1158/1161/1162, A1158/CKR and A1161/1162/CKR were lysed without stimulation. CKR proteins were immunoprecipitated with anti-VEGFR-2 antibody and divided into two groups. One group was subjected to in vitro kinase assay using ATP (A, C and E) the second group was subjected to Western blot analysis using anti-VEGFR-2 antibody as a control for protein levels (B, D and F). In vitro kinase assay was performed as described in materials and methods. Briefly, the immunoprecipitated proteins were incubated with different concentrations of ATP. After 15 minutes incubation, the reaction was terminated by adding 2X sample buffer. The samples were boiled and subjected to Western blot analysis using anti-phosphotyrosine antibody (A, C and E).

Figure 4. Tyrosine phosphorylation of A1158/CKR by wild type CKR: Serum-starved PAE cells expressing A1158/CKR were lysed without stimulation with ligand and proteins were immunoprecipitated with anti-VEGFR-2 antibody and the immunoprecipitated 1158/CKR proteins were divided into seven groups (lanes 1-7). Lane one left untreated, lane 2 incubated with serum-starved PAE cell lysates for 15 minutes, lane 3 incubated with lysates from PAE cells expressing CKR without stimulation with CSF-1, lane 4 incubated with PAE cells expressing CKR stimulated with CSF-1 for 10 minutes. Lanes 5, 6 and 7 were pre-incubated with ATP (1mM) for 15 minutes then incubated either with PAE cell lysates (lane 5), incubated with cell lysates from PAE cells expressing CKR (lane 6), or incubated with cell lysates from PAE cells expressing CKR stimulated with CSF-1 (lane 7). Reactions were stopped by adding 2X sample buffer, boiled for 5 minutes and subjected to Western blot analysis using anti-phosphotyrosine antibody (A). The same membrane was stripped and re-blotted with anti-VEGFR-2 antibody for protein level (B).

Figure 5. Effect of mutation of leucines 1158, 1162 and 1163 on ligand-induced dimerization of VEGFR-2. Equal number of serum-starved PAE cells expressing wild type chimeric VEGFR-2 (CKR), A1158/CKR and A1158/1161/1162 (3Ala/CKR) were either not stimulated or stimulated for 10 minutes with CSF-1 (40 ng/ml). Cells were lysed and total cell lysates were immunoprecipitated with anti-VEGFR-2 antibody. The immunoprecipitated proteins were resolved in gradient 4-15% non-reducing SDS-PAGE and proteins were transferred to PVDF membrane and finally subjected to Western blot analysis using an anti-VEGFR-2 antibody. The positions of monomeric and dimeric VEGFR-2s are shown. It is also evident that expression of the mutant VEGFR-2s are slightly higher than the wild type VEGFR-2. This may explain why there is more dimmerized mutant of VEGFR-2s compared to that of the wild type VEGFR-2.

Figure 6. Structure-based amino acid sequence alignment of the kinase domain of VEGFR-2 with the other type III RTKs: The secondary structure of VEGFR-2 indicates that C-terminal loop of the kinase domain is folded into 7 α-helices and 2 β-sheets (23). Residues from 1150 to 1164 constitute αl. The αl is the last helix and it separates the kinase domain from the carboxyl terminus of VEGFR-2. The position of activation loop tyrosine autophosphorylation sites Y1052 and Y1054 are also shown. Partial sequence alignment of
kinase domain and activation loop regions of human VEGFR-1 and various receptor tyrosine kinases of type III family are shown.

**Figure 7. Effect of mutation of leucine 957 on tyrosine phosphorylation of PDGFR-β:**
Equal number of serum-starved PAE cells expressing wild type PDGFR-β and A957/PDGFR-β were either not stimulated (0) or stimulated for 10 or 30 minutes with PDGF-BB (40 μ/ml). Cells were lysed and total cell lysates were subjected to Western blot analysis using anti-phospho-tyrosine antibody (A). The same cell lysates were subjected to Western blot analysis using anti-PDGFR-β antibody for protein loading as a control (B). Equal number of serum-starved PAE cells expressing wild type PDGFR-β and A957/PDGFR-β were lysed without ligand stimulation. CSF-1R proteins were immunoprecipitated with anti-PDGFR-β antibody and divided into two groups. One group was subjected to in vitro kinase assay using ATP (C), the second group was subjected to Western blot analysis using anti-PDGFR-β antibody as a control for protein levels (D). In vitro kinase assay was performed as described in Figure 4.

**Figure 8. Effect of mutation of leucine 910 on tyrosine phosphorylation of CSF-1R:**
Equal number of serum-starved PAE cells expressing wild type CSF-1R and A910/CSF-1R were either not stimulated (0) or stimulated for 10 or 30 minutes with CSF-1 (40 μ/ml). Cells were lysed and total cell lysates were subjected to Western blot analysis using anti-phospho-tyrosine antibody (A). The same cell lysates were subjected to Western blot analysis using anti-CSF-1R for protein loading as a control (B). Equal number of serum-starved PAE cells expressing wild type CSF-1R and A910/CSF-1R were lysed without ligand stimulation. CSF-1R proteins were immunoprecipitated with anti-CSF-1R antibody and divided into two groups. One group was subjected to in vitro kinase assay using ATP (C), the second group was subjected to Western blot analysis using anti-CSF-1R antibody as a control for protein levels (D). In vitro kinase assay was performed as described in Figure 4.

**Figure 9. Leucines 1158, 1161 and 1162 are involved in helix-helix interactions:** The surface representation of αI of VEGFR-2 is shown. The αI is shown in red ribbon and the side chains of leucines 1158, 1161, 1162 are shown in yellow (A). The leucine residues are all buried in the structure of VEGFR-2 and are involved in the helix-helix packing via hydrophobic interactions. Stereo view of ribbon diagram of VEGFR-2 is shown (B). Helix I (αI) is shown in red, helix-E (αE), helix-F (αF) and helix-H (αH) are shown in blue. The side chains of leucines 1158, 1161, 1162 are shown in green and the side chain of residues of αE, αF and αH are shown in yellow. Leucine (Leu1158) associates with amino acid residues of αE, αF, αH and β7. Leucine (Leu 1161) associates largely with αE. Leucine (Leu 1162) associates with αE and β7.

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Figure 1

Chimeric VEGFR-2 (CKR)

Extracellular domain

Chimeric VEGFR-2 (CKR)

ΔCKR/194

5A/CKR

A1158/61/62/CKR

A1161/62/CKR

A1158/CKR

Carboxyl terminus

α

TM

L 1158
G 1159
N 1160
L 1161
L 1162
Q 1163
A 1164
N 1165
A 1166
Q 1167
Q 1168
D 1169
G 1170
K 1171
D 1172

L 1160
L 1163
A 1165
A 1167
A 1168
D 1169
D 1170
K 1171
D 1172

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| Time (min): | CKR | ΔCKR/194 |
|------------|-----|---------|
| 0          |     |         |
| 10         |     |         |
| 30         |     |         |

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Figure 2

| Time: | CKR | A1158 | A1181/62 | A1158/61/62 |
|-------|-----|-------|----------|-------------|
| 0     |     |       |          |             |
| 10    |     |       |          |             |
| 30    |     |       |          |             |

Blot: Anti-Phosphotyrosine

Blot: Anti-Phospho-1052/1057

Blot: Anti-VEGFR-2

Substrate phosphorylation (AU)

| CSF-1 | CKR | 1158 | 2Ala | 3Ala |
|-------|-----|------|------|------|
|       |     |      |      |      |
|       |     |      |      |      |

By guest on March 25, 2020
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Figure 3

A

B

Blot: Anti-phosphotyrosine

Blot: Anti-VEGFR-2

C

D

E

F

G

Ipt: Anti-VEGFR-2

Ipt: Anti-VEGFR-2

Autophosphorylation (AU)

5

ATP (mM):
0 0.1 0.5 1

ATP (mM):
0 0.1 0.5 1

ATP (mM):
0 0.1 0.5 1

ATP (mM):
0 0.1 0.5 1

ATP (mM):
0 0.1 0.5 1
**Figure 4**

| A1158/CKR/PAE | Ipt: Anti-VEGFR-2 |
|---------------|-------------------|
| PAE (TCL)     | - + - - + - - - |
| CKR - CSF-1 (TCL) | - - + - - + - |
| CKR + CSF-1 (TCL) | - - - + - - + |
| ATP (mM):     | 0 0 0 0 1 1 1 1 |

Lane: 1 2 3 4 5 6 7

**Blot: Anti-Phosphotyrosine**

**Blot: Anti-VEGFR-2**
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Figure 5

|          | CKR | A1158/CKR | 3Ala/CKR |
|----------|-----|-----------|----------|
| CSF-1:   | -   | +         | -        |
|          | +   | -         | +        |

- Dimeric CKR
- Monomeric CKR
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Figure 7

| PDGF-BB: (min) 10ng/ml | PDGFR-β/PAE | A957/PDGFR-βPAE |
|-------------------------|-------------|------------------|
|                         | 0 10 30     | 0 10 30          |

**A**

Blot: Anti-Phosphotyrosine

**B**

Blot: Anti-PDGFR-β

| ATP(mM) | PDGFR-β/PAE | A957/PDGFR-βPAE |
|---------|-------------|------------------|
|         | 0 0.1 0.5 1 | 0 0.1 0.5 1      |

**C**

Blot: Anti-Phosphotyrosine

**D**

Blot: Anti-PDGFR-β
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Figure 8

| CSF-1(min): 40ng/ml | CSF-1R/PAE | A910/CSF-1R/PAE |
|---------------------|------------|------------------|
|                     | 0   10  30 | 0   10  30       |

**A**
Blot: Anti-Phosphotyrosine

**B**
Blot: Anti-CSF-1R

**C**
Blot: Anti-Phosphotyrosine

**D**
Blot: Anti-CSF-1R

ATP(mM):

| CSF-1R/PAE | A910/CSF-1R/PAE |
|------------|-----------------|
| 0   0.1 0.5 1 | 0   0.1 0.5 1   |

Ipt: Anti-CSF-1R

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Figure 8

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Rosana D. Meyer, Xiaofeng Qian, Hwai-Chen Guo and Nader Rahimi
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