Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues

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Vascular endothelial growth factor is an important physiological regulator of angiogenesis. The function of this endothelial cell selective growth factor is mediated by two homologous tyrosine kinase receptors, fms-like tyrosine kinase 1 (Flt-1) and kinase domain receptor (KDR). Although the functional consequence of vascular endothelial growth factor binding to the Flt-1 receptor is not fully understood, it is well established that mitogenic signaling is mediated by KDR. Upon sequencing several independent cDNA clones spanning the cytoplasmic region of human KDR, we identified and confirmed the identity of a functionally required valine at position 848 in the ATP binding site, rather than the previously reported glutamic acid residue, which corresponds to an inactive tyrosine kinase. The cytoplasmic domain of recombinant native KDR, expressed as a glutathione S-transferase fusion protein, can undergo autophosphorylation in the presence of ATP. In addition, the kinase activity can be substantially increased by autophosphorylation at physiologic ATP concentrations. Mutation analysis indicates that both tyrosine residues 1054 and 1059 are required for activation, which is a consequence of an increased affinity for both ATP and the peptide substrate and has no effect on $k_{cat}$, the intrinsic catalytic activity of the enzyme. KDR kinase catalyzes phosphotransfer by formation of a ternary complex with ATP and the peptide substrate. We demonstrate that tyrosine kinase antagonists can preferentially inhibit either the unactivated or activated form of the enzyme.

Angiogenesis is critical for normal embryogenesis, growth, and tissue repair. Pathological angiogenesis, however, can support tumor growth and possibly lead to metastatic spread (reviewed in Ref. 1). Furthermore, neoangiogenesis in ischemic ocular retinal diseases, such as diabetic retinopathy and possibly age-related macular degeneration, can culminate in blindness. Tumor angiogenesis, growth, and metastasis (2) and pathological ocular neovascularization (3) have been correlated with increased local expression of vascular endothelial growth factor (reviewed in Ref. 4).

Two high affinity VEGF receptors, Flt-1 (VEGFR-1) and KDR (Flk-1/VEGFR-2), have been identified (5–7). These receptors can be divided into three structural regions: seven extracellular Ig-like domains that contain the growth factor binding sites, a single polypeptide chain hydrophobic transmembrane sequence, and intracellular cytoplasmic domains that confer the tyrosine kinase activity required for signal transduction. In addition, Flt-1 pre-mRNA is alternatively spliced to produce not only the full-length membrane-spanning form but also a soluble truncated version retaining the N-terminal six Ig-like extracellular domains containing the ligand binding sites that can heterodimerize with the corresponding region of KDR and antagonize the activity of VEGF in vitro (8) and in vivo (9).

KDR binds with high affinity not only to VEGF (6, 10, 11), also denoted VEGF-A, but also to other recently identified homologous family members, such as VEGF-D (12), and to the lymphatic endothelial cell mitogen VEGF-C (12). The HIV transcription factor, tat, has also been reported to bind to KDR and stimulate receptor autophosphorylation (13). In addition, VEGF and placenta growth factor (10, 11), VEGF-B and VEGF-D homologues bind with high affinity to Flt-1 (12). Although KDR is able to mediate VEGF-stimulated vascular endothelial cell mitogenesis, the functional role of full-length Flt-1 is not yet clear because it does not appear to transduce VEGF mitogenic signaling in these cells (14). In addition, although both the KDR and Flt-1 homozygous knockouts are each embryonically lethal, mice devoid of KDR contain few, if any, vascular endothelial cells, whereas those without Flt-1 have endothelial cell-lined vessels exhibiting a somewhat disorganized structure (15). KDR, which has not been shown to be alternatively spliced, was found to be an important mediator of VEGF function in endothelial cells through activation of the intracellular tyrosine kinase (14). Neuropilin-1, recently found to be an isoform-specific VEGF receptor, does not have intrinsic tyrosine kinase activity and is not sufficient to mediate either a mitogenic or a chemotactic signal in response to VEGF binding but might enhance VEGF binding to KDR (16).

KDR can be autophosphorylated on at least four tyrosine residues located within the cytoplasmic domains of the protein (17). Two of these residues, tyrosines 1054 and 1059, are located in the activation loop of the tyrosine kinase domains. The other two tyrosine phosphorylation sites at amino acid positions 951 and 996 are located in a poorly conserved region, known as the tyrosine kinase insert loop, that is characteristic for Flt-1, fms-like tyrosine kinase; KDR, kinase domain receptor; GST, glutathione S-transferase; pEY, poly(Glu, Tyr) 4:1; AMP-PCP, adenylyl methylene phosphate; FGFR1, fibroblast growth factor receptor 1; PDGF, platelet-derived growth factor; IRK, insulin receptor kinase.
of type III receptor tyrosine kinases (18). Phosphorylation of the corresponding tyrosines in PDGF β-receptor provide docking sites for downstream signal transduction proteins (18).

We have cloned the KDR tyrosine kinase from a human umbilical vein endothelial cell cDNA library, and we report a functionally important discrepancy in the tyrosine kinase active site with the published sequence (6). Using the active kinase, we found that phosphorylation of the activation loop tyrosine residues at positions 1054 and 1059 is required for activation of the catalytic activity of the KDR kinase. Finally, we demonstrated that antagonists of this tyrosine kinase can preferentially inhibit either the unactivated or activated form of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—**[γ-33P]ATP (3000 Ci/mmole) and the ECL kit were from Amersham Pharmacia Biotech. ATP and polyGlu, Tyr 4:1 (pEY) were from Sigma. All DNA modifying enzymes were from Promega unless otherwise stated. Staurosporine was from Calbiochem, and 3-(4-di-methylamino-benzylidene)-1,3-dihydro-indol-2-one was from Salor (Tokyo, Japan) or prepared as follows: equimolar quantities of oxindole (Aldrich, 318 mg, 2.4 mmol) and p-N,N-dimethylbenzaldehyde (Aldrich, 1.2 g, 2.4 mmol) were mixed in MeOH (5 ml). Piperidine was added (95 μl, 1 mmol) and the reaction was stirred at reflux for 3.5 h. The reaction was cooled to room temperature, and the orange solid was filtered and dried. 1H NMR and mass spectroscopy (M + 1)/265 data were consistent with the desired structure.

**Site-directed Mutagenesis**—The cDNA encoding human KDR was cloned from a human umbilical vein endothelial cell cDNA library as described (18). The 5’-end of the cytoplasmic region of KDR was modified by polymerase chain reaction to facilitate subsequent cloning steps by introduction of two silent mutations into the coding sequence to generate a Kpn I (ACC 65 1) site at residues Glu900-Tyr901-Leu902 to eliminate the endogenous Bam HI site at Asp927. The cytoplasmic region of KDR was then cloned as a GST fusion into pBluebac 4 (Invitrogen) according to the protocol of the manufacturer. Recombinant KDR-GST fusion proteins were expressed and purified by polymerase chain reaction to facilitate subsequent cloning steps by introducing the oligonucleotides 5′-GGT GGC TTT GCC GCA CAA GAG ATT GAA GCA GAT GC-3′ and 5′-GC ATC TGC TGC ATC AAT CTT TCT TGG GCC AAA GGC ACC-3′ for the V848E mutant, and 5′-CGG GAG ATT TTT AAA GAT CCA GAT TAT AGC-3′ and 5′-CT GAC GAT TCT TGG ATC TTT AAA AAT ATC GCC C-3′ for the Y1054F/Y1059F double mutant. The resultant changes were confirmed by DNA sequencing using an ABI 377 sequencer.

**Expression and Purification of the GST-fused KDR Cytoplasmic Domain in Insect Cells**—The above cDNA constructs encoding either the unactivated or activated form of KDR were transfected into insect Sf21 cells with Bac-N-BlueTM transfection kit (Stratagene), according to the protocol of the manufacturer. Recombinant KDR-GST fusion proteins were expressed and purified by polymerase chain reaction to facilitate subsequent cloning steps by introduction of two silent mutations into the coding sequence to generate a Kpn I (ACC 65 1) site at residues Glu900-Tyr901-Leu902 to eliminate the endogenous Bam HI site at Asp927. The cytoplasmic region of KDR was then cloned as a GST fusion into pBluebac 4 (Invitrogen). Using this construct as the template, site-directed mutagenesis was performed with QuikChange™ site-directed mutagenesis kit (Stratagene), according to the protocol of the manufacturer. Point mutations were introduced with the oligonucleotides 5′-GGT GGC TTT GCC GCA CAA GAG ATT GAA GCA GAT GC-3′ and 5′-GC ATC TGC TGC ATC AAT CTT TCT TGG GCC AAA GGC ACC-3′ for the V848E mutant, and 5′-GGG GCC ATG ATT TTT AAA GAT CCA GAT TAT AGC-3′ and 5′-CT GAC GAT TCT TGG ATC TTT AAA AAT ATC GCC C-3′ for the Y1054F mutant, and 5′-GGG GCC ATG ATT TTT AAA GAT CCA GAT TAT AGC-3′ and 5′-CT GAC GAT TCT TGG ATC TTT AAA AAT ATC GCC C-3′ for the Y1054F/Y1059F double mutant. The resultant changes were confirmed by DNA sequencing using an ABI 377 sequencer.

**Enzyme Kinetics**—Kinetic constants were determined by arranging a two-dimensional array across a 96-well microtiter plate: increasing ATP on the x axis and increasing pEY in the y direction. Reactions were done as described above. Kinetic analysis was done using the following equations (20).

\[ v = V_{max} \frac{[A][K]}{K_A + [A]} + [A][B] \] (Eq. 1)

\[ v = \frac{[S][V]}{K_{cat}}(1 + \frac{[S]}{K_m}) + \frac{[S]}{K_{cat}(1 + \frac{[S]}{K_m})} \] (Eq. 2)

\[ v = V_{max}(\frac{[K]}{[K]_m}) + \frac{(1 + \frac{[S]}{K_m}) + \frac{[S]}{K_{cat}(1 + \frac{[S]}{K_m})}}{1} \] (Eq. 3)

These equations describe the ternary complex, competitive inhibition, and noncompetitive inhibition, respectively, where \( v \) is the measured velocity, \( K_m \) and \( K_m \) are the Michaelis constants for substrates A and B, respectively, and \( K_i \) and \( K_i \) are the dissociation constants for substrate A and B from EA and EB, respectively. Kinetic constants were determined from a nonlinear least squares best fit of the data (19).

**Inhibitor IC50 and K Determinations—**Unactivated (nonphosphorylated) KDR (10 nM) was incubated with 25 μM/μCi of [γ-33P]ATP, 0.3 mg/ml pEY, and inhibitors diluted in MeSO (final MeSO concentration of 5%) in kinase buffer for 15 min at 22 °C. KDR enzyme, activated as described above, was diluted to a final concentration of 0.15 nM in kinase buffer containing 25 μM/10 μCi of [γ-33P]ATP and 0.3 mg/ml pEY and then incubated in the presence of increasing concentrations of inhibitor for 15 min at 22 °C and processed as described above.

**Molecular Modeling**—Homology models of the unactivated (nonphosphorylated) and the activated (phosphorylated) forms of the KDR kinase were based on the published coordinates of the FGFR1 (21) and insulin receptor (22) kinases, respectively. The sequences of the FGFR1, insulin receptor kinase (IRK) and KDR were aligned by hand based on conserved structural elements, and the three-dimensional models were generated using the program LOOK (23). Docking of ligands into the models was done manually, based on the crystallographically established binding of staurosporine in PKA (24) and SU4984 in FGFR1 (25). The enzyme-ligand complex was then minimized using the CHARMM force field as implemented in Quanta97 (26). The ligand and the side chains of any residue within 5.0 Å were allowed to move, and the remainder of the enzyme was fixed. The models were considered to be minimized when the rms force was <0.01 kcal/mol/A.

**RESULTS**

**Identification of the Sequence of Enzymatically Active KDR Kinase**—The amino acid sequences deduced from three independent clones of human KDR from a human umbilical vein endothelial cell cDNA library each contain a valine residue at position 848 rather than the glutamic acid residue listed in the published sequence (Ref. 6; GenBank™ accession number L04947). This residue is located in the N-terminal portion of...
the kinase domain just C-terminal to the glycine-rich loop. Molecular modeling of the KDR kinase sequence into the known crystal structure of the homologous FGFR1 tyrosine kinase (21) positions the side chain of amino acid 848 in the ATP binding site in proximity of the adenine ring of ATP (data not shown). Valine is the most prevalent amino acid found at this position in both tyrosine and serine/threonine kinases (27), and it could stabilize the binding of ATP through hydrophobic interactions with the adenine ring. Modeling of the glutamic acid residue in this position generates a structure in which the negatively charged carboxyl group could interfere with ATP binding and suggests that the kinase might be less active. Although not obvious from the modeled structure because this carboxyl group could become protonated, purified KDRcyt-Glu848 is unable to autophosphorylate in the presence of 1 mM ATP (Fig. 1). The mutant protein also cannot catalyze phosphorylation of an exogenous peptide substrate but can itself be phosphorylated by a truncated version of the wild-type kinase (data not shown). In contrast, KDRcyt-Val848 isolated in a >99% tyrosine-dephosphorylated form is a functional kinase (Fig. 1), as demonstrated by autophosphorylation in the presence of 1 mM ATP/10 mM MgCl₂ and the ability to phosphorylate pEY. All subsequent characterization was carried out using the active cytoplasmic region of the KDR kinase containing Val848, denoted KDRcyt.

**KDR Tyrosine Kinase Activation**—The ability of KDRcyt to autophosphorylate and the effect of phosphorylation on catalytic activity of the kinase enzymatic activity were analyzed. KDRcyt was incubated with 10 mM MgCl₂ in the presence of increasing concentrations of ATP. Fig. 2 shows the quantitative densitometric scan of an anti-phosphotyrosine Western blot (Fig. 2, inset) measuring the ATP-dependent increase in tyrosine phosphorylation of the recombinant KDRcyt enzyme. A plot of phosphorylation versus ATP concentration (Fig. 2) yields an apparent 

\[ K_{\text{app,ATP}} = \frac{0.29 \text{ mm}}{\text{ATP}} \]

To determine the effect of autophosphorylation on the activation of the kinase catalytic activity, KDRcyt was first preincubated with increasing concentrations of ATP in the presence of MgCl₂, the reaction mixture was diluted, and ATP was adjusted to a final concentration of 10 μM. Phosphate incorporation into the exogenous polypeptide substrate pEY was measured. Without prior incubation with ATP, the level of KDRcyt autophosphorylation at 10 μM ATP was very low (Fig. 2), and any additional activation during the second reaction was minimal. The concentration of ATP required for half-maximal activation of the catalytic activity of the kinase was 0.52 mM, as shown in Fig. 3.

To further study the mechanism of activation of the KDR kinase, one or both of the tyrosines 1054 and 1059, known autophosphorylation sites in the activation loop (17), were changed by site-directed mutagenesis to phenylalanine residues. Activation of the purified mutant and wild-type enzymes was carried out with 1 mM ATP, and the resulting activities were measured using the exogenous pEY substrate. As shown in Fig. 4, the basal activities of these wild-type and mutant kinases were essentially equivalent. In contrast, prephosphorylated wild-type enzyme exhibited an 18-fold increase in tyrosine kinase activity, whereas the KDRcytY1054F and KDRcyt-Y1059F single mutants were each activated only 6-fold. The double mutant KDRcytY1054F/Y1059F showed less than a 2-fold increase in activity demonstrating the importance of both of these tyrosine residues for the activation of KDR tyrosine kinase activity. The increased activities resulting from preincubation in 1 mM ATP were not the result of instability of the enzyme in the absence of ATP at 37 °C because the activity of KDRcyt without the incubation step was equivalent to the activity of the enzyme incubated at 37 °C without ATP (data not shown). Despite substitution of the two activation loop tyrosines by phenylalanine residues, the double mutant still autophosphorylated (Fig. 5), consistent with the presence of additional previously identified KDR phosphotyrosine residues (17).

**Kinetic Mechanism**—The reaction mechanism of the activated kinase was investigated by a two substrate kinetic analysis varying the concentrations of both ATP and pEY. Double reciprocal plots of 1/v versus 1/[ATP] and 1/[pEY], displayed in Fig. 6, show an intersecting line pattern indicative of a sequential mechanism in which a ternary complex composed of the enzyme and both substrates forms before any products are released (20). These data rule out a ping-pong mechanism,
indicated by a parallel line pattern in this graphical analysis, in which one of the substrates binds, is converted to a product by modification of the enzyme or a co-enzyme, and is released prior to binding the second substrate.

![Graph showing [3P] Incorporation](Image)

**Fig. 4. Activation of wild-type and tyrosine to phenylalanine mutants of KDR<sub>cyt</sub>.** Wild-type and mutant enzymes were incubated either with (open bar) or without (filled bar) 1 mM ATP for 10 min at 37 °C. Each enzyme was then diluted 100-fold, the ATP concentration was adjusted to 10 μM containing 10 μCi of [γ-33P]ATP, and pEY was added to a final concentration of 0.30 mg/ml. The reaction was maintained at 37 °C for 5 min, and 33P incorporation into pEY was measured.

**Fig. 5. Autophosphorylation of the wild-type and Y1054F/Y1059F double mutant of KDR<sub>cyt</sub>.** Wild-type and mutant enzymes were incubated either with (+) or without (−) 1 mM ATP for 10 min at 37 °C, and reactions were stopped by the addition of sample buffer and boiled. The reaction products were separated by SDS-PAGE, analyzed on a Western blot probed with an anti-phosphotyrosine antibody, and visualized by autoradiography.

**Fig. 6. Kinetic mechanism of KDR<sub>cyt</sub> and KDR<sub>cyt</sub>Y1054F/Y1059F.** A two substrate analysis of the mechanism of KDR<sub>cyt</sub> is shown. Enzyme reactions were carried out with wild-type (A and B) or mutant (C and D) enzymes for 5 min at 37 °C either varying ATP concentrations at a series of fixed pEY concentrations (0.063 (○), 0.125 (●), 0.25 (■), and 0.50 (▲) mg/ml) (A) or varying pEY concentrations at several fixed ATP concentrations (0.05 (○), 0.10 (●), 0.20 (■), and 0.30 (▲) mM) (B). In the case of the mutant enzyme, reactions were carried out either varying the concentrations of ATP at fixed pEY concentrations (0.19 (○), 0.38 (●), 0.75 (■), and 1.5 (▲) mg/ml) (C) or varying the concentrations of pEY at fixed ATP concentrations (0.12 (○), 0.25 (●), 0.50 (■), and 1.0 (▲) mM) (D). Incorporation of 33P was measured, and the values were fit to the equation for ternary complex formation (Equation 1) and plotted as V<sub>o</sub> versus 1/[S].

The kinetic constants, determined by nonlinear curve fitting (19), are displayed in Table I. The Michaelis constants K<sub>ATP</sub> and K<sub>pEY</sub> are similar to the dissociation constants K<sub>ATP</sub>(K<sub>H</sub>) and K<sub>pEY</sub>(K<sub>M</sub>) calculated from Equation 1, indicating that each substrate binds independently. This is consistent with a rapid equilibrium model in which association and dissociation of each substrate is rapid compared with catalysis and binding is random. Patterns of product inhibition can be used to distinguish reaction mechanisms (20). The results of inhibitor studies using the product ADP and the dead-end substrate AMP-PCP are plotted in Fig. 7. Both inhibitors show a competitive pattern versus ATP and are noncompetitive with the peptide substrate, further supporting a random substrate addition mechanism. A product inhibitor of the peptide substrate, which is not available, would be required to rule out an ordered mechanism in which ATP binds first (20). The reaction mechanism of the wild-type unactivated form of the kinase could not be determined because autophosphorylation and consequently enzyme activation would occur during the reaction at high ATP concentrations, as shown in Fig. 2.

A two substrate analysis was done for the KDR<sub>cyt</sub>Y1054F/Y1059F double mutant in an effort to model the unactivated kinase (Fig. 6). The K<sub>ATP</sub> and K<sub>pEY</sub> values and K<sub>ATP</sub> and K<sub>pEY</sub> were marginally different (Table I), consistent with some synergy between substrates (28). Similar to the wild-type enzyme, ADP and AMP-PCP are both competitive with respect to ATP and are noncompetitive with the peptide substrate (data not shown). As shown in Table I, the K<sub>s</sub> values for ATP and pEY in the double mutant are increased by 6.9- and 2.7-fold, respectively, compared with the wild-type enzyme, with essentially no change in the turnover numbers.

**Inhibition of Unactivated Versus Activated Enzyme—**The ability of kinase antagonists to inhibit selectively either the activated or unactivated form of the enzyme was tested. Two commercially available inhibitors, staurosporine and the indolinone 3-(4-dimethylamino-benzylidene)-1,3-dihydro-indol-2-one (Fig. 8), were found to bind competitively with ATP and noncompetitively with the polypeptide substrate (data not shown). KDR<sub>cyt</sub> either was used without a prior activation step or was activated with 1 mM ATP as described under “Experimental Procedures.” The reaction measuring the incorporation of phosphate into the pEY substrate was done at a low ATP concentration (25 μM), at which little, if any, autophosphorylation (Fig. 2) and activation (Fig. 3) occurred in the time course...
of the assay. Under these conditions, staurosporine exhibits a modest 6-fold selectivity for the activated \( K_i = 2.5 \text{ nM} \) compared with the unactivated \( K_i = 16 \text{ nM} \) form of the enzyme (Table II). In contrast, the indolinone is an approximately 100-fold more potent inhibitor of the unactivated compared with the activated enzyme under these same assay conditions, consistent with a higher affinity for the unactivated form \( K_i = 0.04 \mu M \) compared with the activated form of the enzyme \( K_i = 4 \mu M \).

The binding modes of staurosporine and the indolinone docked into the ATP binding site of the KDR model are shown in Fig. 9. These models suggest that the bidentate hydrogen bonds formed by N1 and N6 of ATP are mimicked by the lactam moiety of the inhibitors. In addition to these hydrogen bonds, there are several hydrophobic contacts between Leu\(^{840}\), Val\(^{848}\), Lys\(^{868}\), Val\(^{899}\), Val\(^{916}\), Val\(^{918}\), Cys\(^{919}\), Gly\(^{922}\), Leu\(^{1035}\), Cys\(^{1045}\), and the aromatic regions of the inhibitors (not shown). Comparisons of the KDR ATP binding sites from the FGFR1- and IRK-derived models revealed that although the hydrogen bonds and the hydrophobic pocket formed by Val\(^{848}\), Lys\(^{868}\), Val\(^{899}\), and Val\(^{916}\) are preserved in the unactivated FGFR1-like enzyme, the position of Leu\(^{840}\) is much closer to the binding site in the activated IRK-like conformation. Because staurosporine is flat in the region of the binding site formed by Leu\(^{840}\), the change in the position of this residue on activation does not generate unfavorable steric interactions. In contrast, the dimethylamino-phenyl ring from the inhibitor 3-(4-dimethylamino-benzylidene)-1,3-dihydro-indol-2-one occupies and extends beyond the same region of the binding site. In the more open conformation of the unphosphorylated kinase, the phenyl moiety of the indolinone can also be accommodated. However, in the active phosphorylated form of the enzyme, this ring appears to be partially occluded by the Leu\(^{840}\) side chain. Presumably, the energy required for the enzyme conformational change that facilitates the binding of this inhibitor lowers its affinity for this form of the kinase. Consistent with this hypothesis, the crystal structure of staurosporine in complex with PKA (24) shows inhibitor induction of a more open kinase binding site conformation.

**DISCUSSION**

KDR is critical for VEGF-induced mitogenic and chemotactic signaling in vascular endothelial cells. To study the regulation and enzymatic activities of the tyrosine kinase portion of this receptor, we cloned the cytoplasmic domain and expressed it as a GST fusion protein. The GST domain not only provided a simple and rapid affinity purification of the kinase but also dimerized the protein (data not shown) in a manner that is presumably similar to that which is promoted by binding of the dimeric VEGF ligand, thereby facilitating transphosphorylation. Kinase domains from other transmembrane tyrosine kinase receptors have been studied and found to be good models for investigating the catalytic activity and activation of full-length receptor kinases (29, 30).

Comparing the predicted amino acid sequence of the KDR cDNA clone that we isolated to the published sequence (6) revealed a difference at amino acid position 848. In our cDNA clone, the glutamic acid was replaced by a valine residue. The equivalent valine residue is very conserved in both the tyrosine and serine/threonine kinases families of kinases and is reported to be a valine in the amino acid sequences deduced from both of the published mouse KDR (flk-1) sequences (31, 32) (GenBank\(^\text{TM}\) accession numbers X59397 and X70842) and the closely related fli-1 (GenBank\(^\text{TM}\) accession number X51692) and fli-4 (GenBank\(^\text{TM}\) accession number X68203) receptor cDNA sequences (5, 33). Molecular modeling of the KDR kinase domain based on the FGFR1 coordinates (21) suggests that a negatively charged glutamic acid side chain at this position could diminish ATP binding. The purified GST-KDR\(_{\text{cyt}}\)Glu\(^{848}\) fusion protein was soluble and could be phosphorylated by a truncated KDR “core” kinase but was unable either to auto-phosphorylate or to catalyze phosphate incorporation into an exogenous polypeptide substrate. The lack of activity of GST-KDR\(_{\text{cyt}}\)Glu\(^{848}\) might be attributable either to inability of the properly folded enzyme to bind substrate or to improper folding of the kinase domains. The cytoplasmic domains of KDR contain 19 tyrosine resi-
doses, 4 of which are known to be autophosphorylation sites (17). Two of these sites (951 and 996) are located in a poorly conserved sequence known as the type III receptor tyrosine kinase insert loop (18). Phosphorylation of homologous tyrosine residues in PDGF β-receptor provide docking sites for other downstream signal transduction molecules to form a signaling complex (18). These tyrosine residues might have a similar function in KDR. The remaining two tyrosine phosphorylation sites (1054 and 1059) are located in the activation loop of the kinase. In other receptor tyrosine kinases, phosphorylation of tyrosine residues in the activation loop has been associated with an activation of kinase activity (34–36).

In an effort to investigate the activation mechanism of KDR, we changed the two tyrosines in the activation loop, individually and together, to phenylalanine residues and compared their ability to activate to that of the wild-type protein. The GST-fused KDR<sub>cyt</sub>V<sup>1054F</sup>/V<sup>1059F</sup> wild-type kinase isolated in the dephosphorylated state could undergo autophosphorylation with concomitant 18-fold activation at physiologic magnesium/ATP concentrations. Each of the kinases containing a single tyrosine phosphorylated state could undergo autophosphorylation with the glycine-rich flap including the Leu<sup>840</sup> side chain highlighted in red. The shifted position of the glycine-rich flap including the Leu side chain, modeled from the phosphorylated IRK coordinates, is shown in yellow. The two models were aligned by superposition of the energy-minimized position of staurosporine (white). It was not possible to minimize the indolinone (green) in the IRK based model because of unfavorable steric interactions.

The kinetics of the KDR reaction were studied in a series of experiments with the preactivated wild-type enzyme and the double mutant. Results from a two substrate analysis are typical for a reaction mechanism that proceeds through formation of a ternary complex, indicating a sequential mechanism (20) in which both substrates bind prior to release of products. Inhibitor profiles from experiments with either the product ADP or the dead-end substrate AMP-PCP are consistent with either a steady state ordered mechanism with ATP bound first or a rapid equilibrium Bi Bi (reaction with two substrates producing two products) random mechanism (20). A suitable peptide substrate product inhibitor is not available that can be used to distinguish between the obligatory initial binding of ATP and random addition of substrates. The catalytic mechanism of the full-length ligand-stimulated activated PDGF β-receptor kinase is also found to be a sequential rather than ping-pong mechanism (37), and the epidermal growth factor receptor (38) and insulin receptor kinase (39) reactions are reported to occur by a sequential Bi Bi rapid equilibrium random mechanism. In addition, sequential mechanisms are reported for other tyrosine kinases, such as csk (40) and pp60c-src (41), and for the serine/threonine kinases, cAMP-dependent protein kinase (42), and p38 kinase (43), the latter utilizing an ordered sequential mechanism in which the peptide substrate binds first (43).

Measurement of kinetic rate constants for the nonactivated KDR kinase is difficult because activation can occur at high ATP concentrations during the course of the experiment. The double mutant provides a model of the unactivated kinase that cannot be substantially activated by autophosphorylation and appears to retain an unactivated ATP binding site that resembles the wild-type enzyme, as reflected by its similar intrinsic catalytic activity and binding of inhibitors. Therefore, the mechanism of catalytic activation can be studied by comparing kinetic constants of the wild-type and double tyrosine mutant enzymes. Activation of wild-type KDR by autophosphorylation is accompanied by 7- and 3-fold decreases in $K_{m,\text{ATP}}$ and $K_{m,\text{Mg}^{2+}}$, respectively.
respectively, with no alteration in $k_{\text{cat}}$. These data suggest that activation of KDR is a result of an increased affinity for both substrates and not a change in the intrinsic catalytic activity of the enzyme, as is reported for the full-length ligand-stimulated epidermal growth factor receptor kinase (38). In addition, ligand activation of PDGF-β-receptor is reported to result in 4- and 2.3-fold decreases in the $K_m$ values for ATP and peptide substrate, respectively (37). However, Kovalenko et al. (44) found no change in the $K_m$ for the peptide substrate but did observe a 3-fold decrease in the $K_{\text{mATP}}$ on activation.

Surprisingly, the largely unactivable double mutant could undergo autophosphorylation at high ATP concentrations. The unactivated kinase retains a basal level of activity and can still phosphorylate the exogenous substrate, albeit at a much reduced rate compared with the activated kinase at low ATP concentrations. However, the $k_{\text{cat}}$ values of the wild-type and double mutant enzymes are essentially the same, indicating that at saturating concentrations of substrates, the same $V_{\text{max}}$ is achievable. Phosphorylation of the activation loop tyrosines in KDR leads to a lowering of the $K_m$ for both substrates, which might ensure that the kinase is operating either at or near saturating substrate concentrations. The resulting increased level of phosphorylation could be required to outpace dephosphorylation by phosphatases, thereby sustaining a phosphorylation signal long enough to form a competent signal transduction complex. Autophosphorylation of the intact PDGF-β-receptor stimulated by PDGF containing a similar activation loop tyrosine was reported to be important for the activation of kinase activity but not for autophosphorylation. Tyrosines in the juxtamembrane region that are known to be phosphorylation sites are critical for autophosphorylation, leading to the suggestion that multiple events are required for full activation of PDGF-β-receptor. By analogy, phosphorylation of other tyrosines residues in KDR may be required for additional activation events in vivo. Phosphorylation events mediated by other kinases in vivo might also play a role in KDR activation.

Finally, we find that inhibitors that are competitive with ATP can interact differently with the activated and unactivated forms of the kinase. Staurosporine, a nonselective kinase inhibitor, has an approximately 6-fold higher affinity for the activated than for the unactivated wild-type kinase and double mutant. ADP, as expected, binds with higher affinity to the activated form of the enzyme, mimicking ATP binding. However, a more selective kinase inhibitor of the indolinone structural class (46) binds with an approximately 40- and 100-fold higher affinity to the double mutant and unactivated wild-type enzymes, respectively, compared with the activated wild-type kinase. These results indicate that conformational changes in the ATP binding site probably occur upon activation consistent with molecular modeling in which staurosporine appears to be better accommodated by a slightly more open binding site in the active kinase, whereas the indolinone would require a conformational change in the position of the glycine-rich flap. The partial occlusion of the inhibitor binding site by Leu-840 and the generation of unfavorable steric interactions is likely to decrease the affinity of the indolinone for the activated kinase. The double mutant and unactivated wild-type enzymes bind inhibitors, including ADP, with similar affinities, suggesting that the double mutant is a good surrogate for the unactivated wild-type enzyme.

Kovalenko et al. (44) found that a tyrphostin (AG1295) displays a different mode of inhibition of activated and nonactivated PDGF-β-receptor, indicating conformational changes in the ATP binding site that alter inhibitor binding. Furthermore, they observed less than a 2-fold decrease in the $K_i$ of this inhibitor on receptor kinase activation with ligand. The KDR inhibitor 3-(4-dimethylamino-benzylidene)-1,3-dihydro-indol-2-one, however, shows no change in the mode of inhibition but does display a dramatic decrease in $K_i$ for the activated kinase. Inhibition of the unactivated form of the kinase might be therapeutically sufficient. Such an inhibitor has the advantage of competing with a form of the enzyme that has decreased affinity for ATP and could prevent catalytic activation of the enzyme. These data demonstrate that not only can inhibitors be found that are kinase-selective, but also conformational selectivity within the same molecule can be achieved.

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