Mapping of the Sites Involved in Ligand Association and Dissociation at the Extracellular Domain of the Kinase Insert Domain-containing Receptor for Vascular Endothelial Growth Factor*

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The kinase insert domain-containing receptor (KDR) for vascular endothelial growth factor (VEGF) has been shown to be involved in vasculogenesis and angiogenesis. This receptor is characterized by seven immunoglobulin (Ig)-like domains within its extracellular region. To identify the domains involved in VEGF binding, we constructed various deletion mutants of the extracellular region fused with the crystallizable fragment portion of an IgG and then examined the binding affinity with VEGF by means of the BIAcore biosensor assay. Deletion of the COOH-terminal two or three Ig-like domains out of a total of seven affected ligand dissociation rather than association. Further deletion of the fourth domain caused a drastic decrease in the association rate. Binding ability was abolished completely with removal of the third domain. The mutant KDR proteins lacking the NH₂-terminal Ig-like domain exhibited a slightly higher association rate compared with those of the mutants having this domain. Deletion of the first two NH₂-terminal Ig-like domains caused a drastic reduction in the association rate, but affinity to VEGF was retained. These results suggest that the third Ig-like domain is critical for ligand binding, the second and fourth domains are important for ligand association, and the fifth and sixth domains are required for retention of the ligand bound to the receptor molecule. The first Ig-like domain may regulate the ligand binding.

Vascular endothelial growth factor (VEGF),¹ known as a specific mitogen for vascular endothelial cells in vitro and a potent angiogenic and vascular permeability-enhancing factor in vivo (1–5), plays roles in a variety of processes involving vasculogenesis and angiogenesis (6, 7). Two tyrosine kinases, Flt-1 (8, 9) and KDR (murine Flk-1) (10–13), have been identified as high affinity VEGF receptors. Experiments with knockout mice deficient in either receptor revealed that KDR/Flk-1 was essential for the development of endothelial cells, whereas Flt-1 was necessary for organization of the embryonic vasculature (14, 15). Both receptors are characterized by seven immunoglobulin (Ig)-like domains in the extracellular region and a split kinase intracellular domain (8–13). It has been indicated that the receptor-binding determinants of VEGF are localized in the amino-terminal portion (amino acids 1–110), and KDR and Flt-1 bind to different sites on VEGF (16). The $K_d$ for KDR has been reported to be 75–770 pM (10, 12, 13, 17), which is about 10–90 times higher than that for Flt-1, which is about 10 pM (9, 17). Flt-1 and KDR have been shown to have different signal transduction properties (17, 18). KDR undergoes strong ligand-dependent tyrosine phosphorylation in intact cells, whereas Flt-1 exhibits a weak response (9, 10, 12, 17, 18).

Elucidation of how VEGF activates the receptors is essential for understanding the sequence of events that result in vasculogenesis and angiogenesis. The functional roles of the Ig-like domains within the extracellular region of Flt-1 have been examined by means of domain deletion studies. The ligand binding function was found to reside within the first three Ig-like domains (19–23), the region further downstream being required for dimer formation (20, 22). According to the model proposed by Wiesmann et al. (23), the second and third Ig-like domains of Flt-1 are involved in the direct contact with VEGF. Recently, Fuh et al. (24) reported that two monomeric forms of KDR dimerized across the subunit interfaces of the VEGF and initiated signaling. Furthermore, they showed that the second and third Ig-like domains of KDR were sufficient for tight binding with VEGF. However, the functional roles of the other Ig-like domains have not yet been made clear. In this study, we constructed various deletion mutants of the extracellular Ig-like domains of KDR and examined their binding affinity with VEGF using a BIAcore biosensor. Based on the results of this study, we discuss the functions of the Ig-like domains of KDR in ligand binding.

**EXPERIMENTAL PROCEDURES**

Construction of Plasmids Carrying Various Deletion Mutants of the KDR Extracellular Domain

cDNA encoding a fusion protein consisting of a deletion mutant of the extracellular domain of KDR and amino acids 234–460 of the human IgG heavy chain (25) (denoted Fe) was cloned under the polyhedrin promoter (XhoI-NotI site) in the baculovirus transfer vector pVL1393 (PharMingen, San Diego, CA). The EcoRI 2.8-kb fragment of BCMGNeo-KDR (26) covering the extracellular domain of KDR was inserted into the EcoRI site of pUC18. Then the XhoI site of the plasmid, located at 12 base pairs upstream of the open reading frame of the KDR, was converted to an XbaI site by digestion with XhoI followed by treatment with Klenow fragment and...
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Expression of the Mutant KDR by Insect Cells

To construct the recombinant baculovirus containing the mutant KDR cDNA, the transfer vector was cotransfected with BaculoGold viral DNA (Pharmingen) into Sf9 cells as described (28). The recombinant virus was prepared from the supernatant and purified by plaque purification as described (28). Confluent monolayers of Sf21 cells grown in serum-free medium Ex-Cell 400 (JRH Biosciences, Lenexa, KS) were infected with the recombinant virus (multiplicity of infection = 10) and then incubated at 27 °C for 72 h.

Purification of the Mutant KDR

The culture medium of the recombinant baculovirus-infected Sf21 cells was applied to a protein G-Sepharose (Amersham Pharmacia Biotech) column, and the positive fractions were pooled and then dialyzed against PBS. The purity of the VEGF thus obtained was above 90%.

Preparation of VEGF

VEGF<sub>165</sub> was expressed by baculovirus-infected insect cells as described previously (26). The culture supernatant was applied to a heparin-Sepharose CL-6B (Amersham Pharmacia Biotech) column. The bound proteins were eluted with a gradient of 0–1 M NaCl in 20 mM sodium phosphate, pH 7.3. Each fraction was analyzed by SDS-PAGE, and the positive fractions were pooled and then dialyzed against 20 mM sodium phosphate, pH 7.3, containing 1 M (NH₄)₂SO₄. A sample was applied to a butyl-Sepharose (Amersham Pharmacia Biotech) column, and the bound proteins were eluted with a gradient of 1–0 M (NH₄)₂SO₄. The positive fractions were pooled and then dialyzed against PBS. The purity of the VEGF thus obtained was above 90%.
Ligand Binding Assays with 125I-VEGF

The mutant KDR proteins were diluted to 0.2–10 μg/ml with PBS. Aliquots (50 μl) were applied to the surface of methanol-treated 96-well plates (MultiScreen-IP, Millipore, Bedford, MA) for 12 h at 4 °C. The plates were then washed with PBS, and nonspecific sites were blocked with PBS containing 1% bovine serum albumin for 30 min at 25 °C. An aliquot (50 μl) of 4 ng/ml 125I-labeled VEGF (1,200–1,800 Ci/mmol; Amersham Pharmacia Biotech) was added to each well followed by incubation for 1.5 h at 25 °C. The wells were washed with PBS containing 0.05% Tween 20 and then with PBS and then dried at 50 °C. An aliquot (10 μl) of Microscint-O (Packard, Meriden, CT) was added to each well, and then the bound 125I-labeled proteins were quantified with TopCount (Packard). Competition was examined by incubating 50 μl of 4 ng/ml 125I-labeled VEGF and 50 μl of 0.02–10 μg/ml mutant KDR proteins on plates coated with 0.2 μg of 7N-KDR protein.

BIACore Biosensor Assays

All experiments were performed at 25 °C using HBS buffer comprising 10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20. VEGF was coupled to a CM-5 biosensor chip by amine coupling using an amine coupling kit (Pharmacia Biosensor Ltd., Uppsala, Sweden) with the following modifications. VEGF was diluted to 2 and 4 μg/ml with 10 mM sodium acetate, pH 6.0; after immobilization, the surfaces were conditioned with 0.1 M glycine-HCl, pH 1.5, for 4 min. This gave 770 and 1,390 response units, respectively. The KDR proteins were diluted to 0.1, 0.2, 0.3, and 0.4 μM with HBS buffer. For the 3N-, 2N-, 1N-, 5N–2N, and 4N–2N-KDR proteins, 3 and 4 μM solutions were also prepared. Each sample was injected over the VEGF surface at a flow rate of 20 μl/min. Sensorgrams were recorded and normalized as a base line of 0 response units. An equivalent volume of each protein dilution was also injected over an activated and ethanolamine-quenched surface for determination of the bulk refractive index background. At the end of each cycle, bound KDR was removed by injecting 5 μl of 50 mM HCl at a flow rate of 20 μl/min to regenerate the chip. To determine the association and dissociation rate constants (k_on and k_off, respectively), the sensorgrams were analyzed by nonlinear least squares curve fitting using BIAevaluation 2.1 software (Pharmacia Biosensor). The equation \( R = R_0 \exp(-k_{off}(t-t_0)) \) was used for the dissociation phase, where \( t_0 \) is the start time for dissociation and \( R_0 \) is the response at the start of the dissociation. \( k_{on} \) was calculated using the model A + B = AB and by fitting the data to the equation \( R = R_\infty \frac{1}{1+\frac{C}{k_{on}}} + k_{off}(t-t_0) \), where C is the molar concentration of the analyte and \( R_\infty \) is the steady-state response level. The final \( k_{on} \) and \( k_{off} \) values were calculated from the means of these values for each doublet injection of an injection series. The apparent Kd value was determined from the ratio of these final kinetic constants (k_off/k_on).

Other Methods

Protein concentrations were determined by the method of Bradford (29) using bovine serum albumin as a standard. SDS-PAGE was performed with a ready made 5–20% (w/w) polyacrylamide gradient gel (ATTO Corp., Tokyo, Japan) according to the method of Laemmli (30), with PBS containing 1% bovine serum albumin for 30 min at 25 °C. An aliquot (50 μl) of Microscint-O (Packard, Meriden, CT) was added to each well followed by incubation for 1.5 h at 25 °C. The wells were washed with PBS containing 0.05% Tween 20 and then with PBS and then dried at 50 °C. An aliquot (10 μl) of Microscint-O (Packard, Meriden, CT) was added to each well, and then the bound 125I-labeled proteins were quantified with TopCount (Packard). Competition was examined by incubating 50 μl of 4 ng/ml 125I-labeled VEGF and 50 μl of 0.02–10 μg/ml mutant KDR proteins on plates coated with 0.2 μg of 7N-KDR protein.

RESULTS

Preparation of Mutant KDR Proteins—To examine the binding sites and characteristics of the Ig-like domains in the extracellular region of KDR, a set of Ig-like domain deletions was constructed as fusions with the Fc portion of a human IgG (Fig. 1) and expressed in baculovirus-infected insect cells. We chose the deletion juncture of KDR based on the putative Ig-like domains of the murine counterpart Flk-1 (11). All of the recombinant KDR proteins could be highly purified from the culture medium using a protein G-Sepharose column (Fig. 2). We also analyzed the proteins by nonreducing gel electrophoresis and confirmed that all constructs migrated as dimeric proteins (data not shown). The apparent molecular masses of the proteins shown in Fig. 1 were estimated from the results of SDS-PAGE analysis.

Binding of the Mutant KDR Proteins to 125I-VEGF—We examined the region of the receptor responsible for VEGF binding by means of a solid phase binding assay. The KDR proteins were fixed on plastic plates, and then the bound 125I-VEGF was measured (Fig. 3). The 7N- and 6N-KDR proteins bound to the ligand in almost the same manner. However, the 5N- and 4N-KDR proteins bound with lower affinity than the 7N form, and the 3N-, 2N-, and 1N-KDR proteins showed almost undetectable binding with this method. The first Ig-like domain of 7N-, 5N-, and 4N-KDR was dispensable for binding to the ligand. High affinity binding ability rather tended to be obtained on removal of this domain. Deletion of the first and second Ig-like domains from 4N-KDR led to undetectable binding to the ligand. This effect was almost the same when the mutant KDR had the fifth Ig-like domain.

To confirm that the binding characteristics of the mutant KDR proteins were not caused by the efficiency of their immobilization on the plastic plate, each KDR protein was added as a competitor in the binding assay system with the 7N form fixed on the plastic plate and 125I-VEGF (Fig. 4). The 7N-, 6N-, 5N-, and 4N-KDR proteins blocked the ligand binding, the IC50 values being 45, 79, 210, and 790 ng/ml (0.18, 0.34, 1.17, and 5.34 μM, respectively). Deletion of the first Ig-like domain from the amino termini of the 7N-, 5N-, and 4N-KDR proteins caused slightly stronger inhibition (IC50 = 0.10, 0.70, and 2.28 μM, respectively). The 3N-, 2N-, 1N-, 5N–2N, and 4N–2N KDR proteins at concentrations up to 5 μg/ml did not block the ligand binding significantly. Consequently, in this assay system the binding characteristics of the KDR proteins were essentially the same as those observed with the plate-fixed method described above.

Analysis of the Interaction of the Mutant KDR Proteins with VEGF by BIACore—VEGF was immobilized directly to a carboxylated dextran matrix chip through the primary amine group on VEGF, and the mutant KDR proteins were injected over the VEGF surface. Representative sensorgrams are shown in Fig. 5. The effects of deletion of the Ig-like domains of KDR on VEGF binding observed in this experiment (Fig. 5, A–D) were essentially consistent with those with the competitive binding assay described above. However, the binding of 3N-, 4N–2N, and 5N–2N-KDR, which was not detected with the plate-fixed method or the competitive binding assay described above, was observed in this system with high concentrations of the analytes (Fig. 5, E and F). The binding of the 2N- and 1N-KDR proteins was not detected with concentrations up to 4 μM (Fig. 5E). These results suggest that the third Ig-like domain is critical for VEGF binding.

The kinetic parameters analyzed in the sensorgrams are
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summarized in Table I. With extension of the COOH-terminal deletion of the extracellular region, the $K_\text{a}$ value increased gradually. In the cases of the 6N-, 5N-, and 4N-KDR proteins, the decreases in $k_{\text{on}}$ were of the same order of magnitude; however, the increase in $k_{\text{off}}$ was affected greatly, especially when the deletion extended to the sixth or fifth Ig-like domain. Thus, the increase in $K_\text{a}$ on deletion of the COOH-terminal two or three Ig-like domains was mainly the result of an increase in $k_{\text{off}}$. As for 3N-KDR, the $K_\text{a}$ value of which was about 15-fold higher than that of 4N-KDR, the reduction in the ligand affinity was attributed mainly to the 10-fold reduction in $k_{\text{on}}$. The kinetic parameters were also affected by deletion of the NH$_2$-terminal Ig-like domains. On removal of the first Ig-like domain from the 7N-, 5N-, and 4N-KDR proteins, $k_{\text{on}}$ increased slightly. However, deletion of the NH$_2$-terminal two domains caused a 10-fold decrease in $k_{\text{on}}$. $k_{\text{off}}$ was also influenced by the NH$_2$-terminal deletion, but the effect differed among the mutant KDR proteins. On deletion of the first Ig-like domain, $k_{\text{off}}$ of the 7N- and 5N-KDR proteins increased, but it decreased in the case of 4N-KDR. The increase in $k_{\text{on}}$ on deletion of the NH$_2$-terminal two Ig-like domains from 5N-KDR was more than that in the case of 4N-KDR. Taking all of the facts together we conclude that the second and fourth domains contribute by maintaining the high association rate of the receptor-ligand interaction, and the NH$_2$-terminal domain has a slightly negative effect on the association. The downstream region, especially the fifth and sixth Ig-like domains, is important for retention of VEGF bound to the receptor molecule.

DISCUSSION

We constructed various truncated mutants of the extracellular Ig-like domains of VEGF receptor KDR to gain an insight into the structure/function relationship of VEGF binding. The KDR variants were fused with the Fc portion of a human IgG. The receptor-ligand interactions were analyzed by means of three assay systems: a solid phase binding assay, a competitive solid phase binding assay, and analysis of the binding using a BIAcore biosensor. Essentially identical results were obtained with these systems. With the BIAcore biosensor assay, we can analyze the rates of association and dissociation of two molecules; therefore, it is useful for characterizing a receptor-ligand interaction. This work is the first to involve the BIAcore system for identifying the ligand binding sites of a receptor. The apparent $K_\text{a}$ value determined from the ratio of $k_{\text{on}}$ and $k_{\text{off}}$ ($k_{\text{off}}/k_{\text{on}}$) of 7N-KDR, having the full-length extracellular domain, was 0.19 nM (Table I), which was within the range reported for membrane-bound full-length KDR (10, 12, 13, 17, 26). Therefore, the properties of the truncated KDR variants on the ligand binding observed in the assay system must reflect those of the intact KDR. Based on the results of our study, the deduced function of the Ig-like domains of KDR in VEGF binding is summarized in Fig. 6.

![Fig. 3. Solid phase binding assaying of $^{125}$I-VEGF binding to the mutant KDR proteins.](image)

![Fig. 4. Inhibition of specific $^{125}$I-VEGF binding to 7N-KDR by the mutant KDR proteins.](image)
We showed that 3N-KDR, composed of the first three NH2-terminal Ig-like domains, and 4D1–2N-KDR, which has only the third and fourth domains, could bind to VEGF with 1,000-fold lower affinity than 7N-KDR. However, removal of the third domain abolished completely the receptor’s ability to bind to the ligand. Therefore, the third domain is critical for minimal ligand binding under those experimental conditions (Fig. 6).

Fuh et al. (24) reported that the second and third domains of KDR were sufficient for high affinity binding, and the mutant KDR, which has only the NH2-terminal two domains, could bind to the ligand with more than 1,000-fold decrease in affinity. The difference between our results and theirs might be because of the different definitions of the domains. The second and third domains of their mutant KDR contained an additional 9 and 22 amino acids, respectively, on the COOH terminus of each domain in our definition. In addition, it might be attributed to the difference of the post-translational modifications of the KDR proteins because they used the mutant KDR proteins expressed by the human kidney 293 cell lines, whereas we used insect cell-produced KDR proteins.

From the results of kinetic analysis of the receptor-ligand interaction, we found that the second and fourth domains play major roles by maintaining the high association rate of the interaction (Fig. 6). Davis-Smyth et al. (19) suggested that specific ligand-binding determinants of KDR are located in the second Ig-like domain. In addition to the domain, the fourth domain might also be required for primary recognition of the ligand, and both domains might determine the ligand specificity. Because removal of the first Ig-like domain resulted in an increase in the $k_{on}$ value, it is possible that this domain partially shields the ligand recognition domain and thus regulates the association rate of ligand binding (Fig. 6). Deletion of the downstream Ig-like domains caused decreases in the affinity with VEGF, which was mainly the result of an increase in the dissociation rate. Thus, these Ig-like domains, especially the fifth and sixth ones, play major roles in retention of VEGF bound to the receptor molecule (Fig. 6). It should be noted that removal of the first Ig-like domain also influenced the dissociation rate, but the effect differed according to the COOH-terminal structure of the mutant KDR; that is, the rate of increase in the $k_{off}$ value decreased gradually with extension of the COOH-terminal deletion (Table I). The first domain might

![Fig. 5. Binding of the mutant KDR proteins to VEGF immobilized on the surface of a plasmon resonance sensor chip. VEGF was immobilized (1,390 response units, RU) on a carboxylated dextran matrix chip, and then the mutant KDR proteins were each injected over the VEGF surface at the flow rate of 20 μl/min. Sensorgrams were recorded and normalized as to the base line of 0 response units, and then the bulk refractive index background was subtracted as described under “Experimental Procedures.” The profiles of 0.4 μM 7N-, 6N-, 5N-, 4N-, and 3N-KDR (panel A), 0.4 μM 7N- and 7D1N-KDR (panel B), 0.4 μM 5N-, 5D1N-, and 5D1–2N-KDR (panel C), 0.4 μM 4N-, 4D1N-, and 4D1–2N-KDR (panel D), 4 μM 3N-, 2N-, and 1N-KDR (panel E), and 4 μM 5D1–2N- and 4D1–2N-KDR (panel F) are shown.](image)

![Fig. 6. Schematic representation of a model for the deduced function of the Ig-like domains of KDR in VEGF binding. Numbers 1–7 indicate the respective Ig-like domains, and the intradomain disulfide bonds are shown as S S.](image)

**Table I** Kinetic constants for the interaction of the mutant KDR proteins with VEGF

| KDR variant | 7N  | 6N  | 5N  | 4N  | 3N  | 2N  | 1N  | 7D1N | 5D1N | 4D1N | 5D1–2N | 4D1–2N |
|------------|-----|-----|-----|-----|-----|-----|-----|------|------|------|--------|--------|
| $k_{on} \times 10^{8}$ (M$^{-1}$·s$^{-1}$) | 6.64 | 5.95 | 5.17 | 3.15 | 0.30 | ND  | ND  | 8.43 | 6.46 | 5.75 | 0.44   | 0.63   |
| $k_{off} \times 10^{5}$ (s$^{-1}$) | 1.27 | 2.80 | 12.7 | 42.2 | 63.2 | ND  | ND  | 2.70 | 13.4 | 35.5 | 59.2   | 64.1   |
| $K_d$ (nM) | 0.19 | 0.47 | 2.46 | 13.4 | 211  | ND  | ND  | 0.32 | 2.07 | 6.17 | 135    | 102    |

We showed that 3N-KDR, composed of the first three NH$_2$-terminal Ig-like domains, and 4D1–2N-KDR, which has only the third and fourth domains, could bind to VEGF with 1,000-fold lower affinity than 7N-KDR. However, removal of the third domain abolished completely the receptor’s ability to bind to the ligand. Therefore, the third domain is critical for minimal ligand binding, and the mutant KDR, which has only the NH$_2$-terminal two domains, could bind to the ligand with more than 1,000-fold decrease in affinity. The difference between our results and theirs might be because of the different definitions of the domains. The second and third domains of their mutant KDR contained an additional 9 and 22 amino acids, respectively, on the COOH terminus of each domain in our definition. In addition, it might be attributed to the difference of the post-translational modifications of the KDR proteins because they used the mutant KDR proteins expressed by the human kidney 293 cell lines, whereas we used insect cell-produced KDR proteins.

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also be involved in the regulation of the ligand dissociation together with the fifth and sixth domains. It might be possible that the conformation of the first domain differs among the mutant KDR proteins.

Domain deletion studies on another VEGF receptor, Flt-1, indicated previously that the fourth domain and a region further downstream contribute to receptor dimer formation (20, 22). Fuh et al. (24) reported that dimerized forms of KDR bound to VEGF about 100-fold more tightly than the corresponding monomeric forms. As 5N-KDR binds about 100-fold more tightly than 3N-KDR (Table I), the fourth to fifth Ig-like domains of KDR must also be involved in receptor dimerization. The mutant KDR proteins used in this study form dimers through disulfide bonds in the Fc portion. However, the mutant KDR lacking the downstream Ig-like domains could not form tightly associated dimeric molecules at the KDR portion; therefore, VEGF bound to the receptor might easily be released from the molecule. To identify more precisely the region that contributes to receptor dimerization of KDR we need to analyze the structures of a series of deletion mutants not fused with an IgG.

In this study, we characterized the Ig-like domains within the extracellular region of KDR on VEGF binding and could roughly classify them based on the results of ligand binding analysis. Further dissection of the extracellular domain by means of internal deletions should make it possible to define more precisely the receptor-ligand interaction. Alternatively, crystallization of the recombinant KDR proteins and the KDR-VEGF complexes may provide molecular insights into the binding of VEGF to KDR.

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