Identification of Vascular Endothelial Growth Factor Determinants for Binding KDR and FLT-1 Receptors

GENERATION OF RECEPTOR-SELECTIVE VEGF VARIANTS BY SITE-DIRECTED MUTAGENESIS*

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Bruce A. Keyt†, Hung V. Nguyen, Lea T. Berleau, Carlos M. Duarte§, Jeanie Park, Helen Chen, and Napoleone Ferrara
From the Departments of Cardiovascular Research and Bio-organic Chemistry, Genentech, Inc.,
South San Francisco, California 94080

Vascular endothelial growth factor (VEGF) expression in various cell types is induced by hypoxia and other stimuli. VEGF mediates endothelial cell proliferation, angiogenesis, vascular growth, and vascular permeability via the endothelial cell receptors, kinase insert domain-containing receptor (KDR/Flk-1) and FLT-1. Alanine-scanning mutagenesis was used to identify a positively charged surface in KDR/Flk-1 and FLT-1. Alanine-scanning mutagenesis was used to identify a positively charged surface in KDR/Flk-1 and FLT-1. Alanine-scanning mutagenesis was used to identify a positively charged surface in KDR/Flk-1 and FLT-1. Alanine-scanning mutagenesis was used to identify a positively charged surface in KDR/Flk-1 and FLT-1. Alanine-scanning mutagenesis was used to identify a positively charged surface in KDR/Flk-1 and FLT-1. Alanine-scanning mutagenesis was used to identify a positively charged surface in KDR/Flk-1 and FLT-1.

VEGF is a specific mitogen for vascular endothelial cells in vitro and a potent angiogenic and vascular permeability enhancing factor in vivo (1–5). VEGF, also known as vascular permeability factor, is expressed in response to hypoxia and other stimuli by a variety of differentiated cells (1, 2, 6–14). VEGF expression has also been found in numerous human and rodent transformed cells (3, 4, 15–19). VEGF is encoded by a single gene, but it exists in four isoforms of 121, 165, 189, and 206 amino acids due to alternative mRNA splicing (20, 21). The two low molecular weight forms, VEGF121 and VEGF165, are secreted as soluble factors, while the other higher molecular weight forms, VEGF189 and VEGF206, are secreted but remain bound to extracellular matrix (22). Of the four isoforms, VEGF165 is the most abundantly expressed splice variant. VEGF165 is a heparin binding glycoprotein with a single glycosylation site (at Asn75) and is secreted as a homodimer of approximately 45 kDa (3, 17). VEGF165 can be cleaved by plasmin to yield VEGF105, which is equipotent to VEGF121 with respect to mitogenic activity on endothelial cells (22, 23). The amino acid sequence of VEGF110 exhibits limited but significant homology (17%) with PDGF. All eight cysteine residues, which are involved in intra- or interchain disulfides, are conserved among these growth factors. A cDNA sequence encoding a similar protein was identified by screening a human placental cDNA library (24). Placental growth factor (PLGF), with 47 and 18% sequence homology with VEGF and PDGF, respectively, is recognized as a member of the PDGF/VEGF family of growth factors.

Two tyrosine kinases, fms-like tyrosine kinase (FLT-1) and the kinase insert domain-containing receptor (KDR/Flk-1), have been identified as high affinity VEGF receptors (25–27). The murine homolog of KDR, fetal liver kinase 1 (Flk-1) has 85% homology with the human VEGF receptor (28). These receptors are localized on the cell surface of various endothelial cell types (29, 30). The FLT-1 and KDR/Flk-1 genes encode tyrosine kinase receptors, characterized by an extracellular domain containing seven immunoglobulin-like domains and a split tyrosine kinase intracellular domain (31). In many aspects, the VEGF receptors are structurally and functionally similar to the PDGF receptors, which have five immunoglobulin-like repeats in the extracellular domain (32, 33). In addition to VEGF, PLGF has also been demonstrated to bind FLT-1 with high affinity, but not to KDR/Flk-1 (34, 35). VEGF is a potent endothelial cell mitogen in vitro, while PLGF is 3 orders of magnitude less potent at inducing endothelial cell proliferation (4, 15–18). However, PLGF can significantly potentiate the in vitro and in vivo activity of low concentrations of VEGF (34).

To gain a better understanding of the biological activity of VEGF, we began an analysis of structure/ activity relationships using site-directed mutagenesis of VEGF. In the current study, we report on the mutational analysis of VEGF and identify the receptor binding determinants for KDR/Flk-1 and FLT-1. Furthermore, the epitope mapping of a neutralizing monoclonal antibody is also described. We used the strategy of charged-to-alanine scanning mutagenesis to evaluate the role of various sites on VEGF in receptor binding. To confirm the results of alanine scanning, we have also introduced novel glycosylation sites in VEGF to inhibit receptor binding with additional carbohydrate. We report that KDR and FLT-1 receptors bind to...
Materials—Muta-gene phagmid in vitro mutagenesis kit, horseradish peroxidase-conjugated goat IgG specific for murine IgG, pre-stained low range M, standards, and Trans-Blot transfer medium (pure nitrocellulose membrane) were purchased from Bio-Rad. QIagen plasmid tip 100 kit and Sequenase version 2.0 were from QIagen (Chatsworth, CA, and U. S. Biochemical Corp., respectively). SDS gel (4–20%) polyacrylamide gel) and blotting paper were from Integrated Separations Systems (Natick, MA). SDS sample buffer (5X concentrate) and restriction enzymes were from New England Biolabs (Beverly, MA). O-Phenylene-diamine, citrate phosphate buffers, sodium dodecyl sulfate, and H$_2$O$_2$ substrates were purchased from Sigma. Buffer EZE formula 1 (transfer buffer) and X-Omat AR x-ray film were from Eastman Kodak Co. Maxisorb and Immunlon-1 microtiter plates were purchased from Nunc (Kamstrup, Denmark) and Dynatech (Chantilly, VA), respectively. Cell culture plates (12-well) and culture media (with calf serum-free medium), and cell monolayers were incubated for 72 h respectively. Polyethylene-20-sorbitan monolaurate (Tween 20) was prepared from Wako Chemicals (Osaka, Japan), and Amersham Corp., respectively. Bovine serum albumin (BSA) and rabbit IgG anti-human IgG (Fc specific) were purchased from Cappel (Durham, NC) and Calbiochem (La Jolla, CA), respectively. Plasmid vector (pRK5), competent Escherichia coli cells (DH5a and C1R2), cell culture medium, purified CHO-derived VEGF165, monoclonal (mAbs A4.6.1, 2E3, 4D7, 5C3, and 5F8), and polyclonal antibodies to VEGF165 were prepared at Genentech, Inc. (South San Francisco, CA). Construction, expression, and purification of FTL-1, FIK1, and KDR receptor-IgG chimeras were as described by Park et al. (34).

Site-directed Mutagenesis and Expression of VEGF Variants—Muta-tants were prepared using the Muta-Gene Phagmid in vitro mutagenesis kit according to the method of Kunkel and co-workers (36, 37). A plasmid vector pRK5 containing DNA for VEGF165 isoform was used for mutagenesis and transient expression. The pRK5 vector is a modified pUC118 vector and contains a cytomegalovirus enhancer and promoter (38, 39). The mutagenized DNA was purified using the QIagen Plasmid Midi kit tip 100, and the sequence of the mutations was verified using Sequenase version 2.0 kit. The mutated DNA was analyzed by restriction enzyme digestion as described by Sambrook et al. (40). Transient transfection of human fetal kidney “293” cells was performed in 6-well plates using the modified calcium phosphate precipitation method as described previously (42–44). Briefly, approximately 10$^6$ cells were incubated in 6-well plates containing a mixture of 15 µg of precipitated DNA. Cell culture supernatant was replaced with serum-free medium, and cell monolayers were incubated for 72 h at 37°C. Conditioned media (3 ml) was harvested, centrifuged, aliquoted, and stored at −70°C.

Quantification of VEGF165 Variants by ELISA—A radioimmunometric assay, described previously (45), was adapted for the quantification of VEGF mutants. Individual wells of a 96-well microtiter plate were precoated with 100 µl of a solution containing 10 µg/ml of rabbit IgG anti-human IgG (Fc-specific) at 4°C for 1 h in assay buffer (0.5% BSA, 0.03% Tween 80, 0.01% Thimerosal in PBS). The plates were blocked (300 µl/well) for 1 h in assay buffer (0.5% BSA, 0.03% Tween 80, 0.01% Thimerosal in PBS). After blocking, serial dilutions of VEGF165 mutants at varying concentrations (100 µl) and 127I-radioiodinated VEGF165 (5 × 10$^3$ cpm in 50 µl), which was mixed with VEGF receptor-IgG chimeric protein, FTL-1 IgG, or KDR-IgG (3–15 ng/ml), final concentration, 50 µl) in micronic tubes. An irrelevant antibody (1 µg IgG) and 1 µg IgG anti-His86) was used as a control for nonspecific binding of radioiodinated VEGF. Aliquots of these solutions (100 µl) were added to precoated microtiter plates and incubated for 4 h at 25°C. The supernatant was discarded, the plates were washed, and individual wells were counted by γ scintigraphy (LKB model 1277). The competitive binding between unlabeled VEGF165 (or VEGF165 mutants) and 127I-radioiodinated VEGF165 to the FLT-1 or KDR receptors was plotted and analyzed by four parameter fitting (Kaleidogaph, Abelbeck Software). The apparent dissociation constant for each VEGF mutant was estimated from the concentration required for 50% inhibition (IC$_{50}$). Nonspecific binding to an unrelated antibody was approximately 2% of maximal binding under similar conditions as binding to VEGF receptor-IgG fusion proteins.

Assay for Vascular Endothelial Cell Growth—The mitogenic activity of VEGF165 variants was determined using bovine adrenal cortical endothelial cells as target cells as described previously (1). Briefly, cells were plated sparsely (7000 cells/well) in 12-well plates and incubated overnight in Dulbecco’s modified Eagle’s medium with 10% calf serum, 2 µg/ml insulin, and antibiotics. The medium was exchanged the next day, and VEGF or VEGF mutants diluted in culture media from 100 ng/ml to 10ng/ml were layered in duplicate on the seeded cells. After 5 days at 37°C, the cells were dissociated with trypsin and quantified using a Coulter counter.

Molecular Modeling—A model of VEGF was generated with the Insight II and Discover software from Biosym Technologies (San Diego, CA). The model of VEGF was based on the crystal structure of PDGFβ solved by Oefner et al. (50). The sequences of VEGF and PDGF were aligned and the side chains of residues were to be replaced or deleted. The following amino acids were to be deleted: Asp13, Asp19, Glu38, Arg62, Lys84, and His93. At present, we are utilizing the modified monoclonal antibody (mAb A4.6.1) to test the microtiter plates. Immunoblotting of VEGF165 Mutants—Aliquots of conditioned cell media (16 µl) containing VEGF or VEGF mutant (~10 ng) were added to 1 ml of sample buffer (4 µl) and heated at 90°C for 3 min prior to loading on SDS gradient (4–20%) gels. Gels were electrophoresed and transferred to nitrocellulose paper in a Bio-Rad tank blotting buffer. The model of VEGF was based on the crystal structure of PDGFβ solved by Oefner et al. (50). The sequences of VEGF and PDGF were aligned and the side chains of residues were to be replaced or deleted (see Fig. 1). The side chains of six amino acids were substituted with those residues found in VEGF (i.e. Asp80, Glu64, Glu67, Arg62, Lys84, and His93).
Comparison of VEGF, PLGF, and PDGF Sequences—We have previously described studies that localize the receptor binding determinants for FLT-1 and KDR/Fk-1 in the amino-terminal (1–110) dimer of VEGF (23). Plasmin catalyzes the deavage of the carboxy-terminal, heparin-binding region (1–110) dimer of VEGF (23). Two gaps, inserted in the VEGF and PLGF sequences, are located in external loops respect to the eight cysteines shared by these proteins. Six cysteines form intrachain disulfides, and two cysteines participate in interchain covalent bonds (48, 49). Two gaps, inserted in the VEGF and PLGF sequences, are located in external loops based on the crystal structure of PDGFb dimer (50). VEGF110 shares 47, 15, and 19% sequence identity and 63, 24, and 28% similarity with PLGF, PDGFα, and PDGFβ, respectively (51). Inspection of sequence similarity and divergence among these sequences offers little insight as to the receptor binding determinants. We undertook the functional mapping of VEGF by site-directed mutagenesis.

Charged-to-Alanine Scan Mutagenesis—30 mutants of VEGF165, were constructed by site-directed mutagenesis where groups of between one and four neighboring charged amino acids (Arg, Lys, His, Asp, and Glu) were replaced with alanine. Table I lists the specific amino acid substitutions for each mutant and indicates the mean residue number for the position of the mutation(s). For a given mutant, this number is the average of the altered positions. Plasmid DNA encoding these mutants was transiently transfected in human 293 kidney cells, and the amount of VEGF in the conditioned cell media was determined using two VEGF-specific immunochemical assays. In Fig. 2, the results of a polyclonal/monoclonal assay, indicating that the epitope recognized by the neutralizing monoclonal antibody, A4.6.1, includes this determinant in VEGF. The amino acid substitution, R82A yields a mutant of VEGF exhibiting almost complete loss of immunochmical activity with mAb A4.6.1 (Fig. 2). The monoclonal immunoactivity was abolished by the triple alanine mutation, R82A/K84A/H86A and by an extraglycosylation mutation, R82N/K83L/K84S, as indicated in Fig. 2 by the mutants with mean residue numbers 84 and 83, respectively. Partial loss of mAb A4.6.1 immunoactivity was also observed with the combined mutant H90A/E93A VEGF (mean residue number 91.5).

Nearly all VEGF mutants were expressed with variable yield (from 293 cells) containing approximately 10–20 ng of VEGF or VEGF mutant were analyzed by nonreducing SDS-PAGE (Fig. 3). The gels were transferred and blotted as described under “Experimental Procedures,” using a mixture of five monoclonal anti-human VEGF165 antibodies. Autoradiography of the immunoblots indicated a major band at 45 kDa for wild type and mutant forms of VEGF. This immunopositive protein band co-migrated with purified, dimeric VEGF165 derived from CHO cells. For some mutants of VEGF, and for 293 cell-derived wild-type VEGF (but not VEGF derived from CHO cells), there appeared an additional minor band at approximately 70 kDa. Apparent molecular weights for all charged-to-alanine replacement mutants, as indicated by SDS-PAGE, were equivalent to that observed for wild-type VEGF165 derived from 293 or CHO cells. There was no indication of degraded forms of VEGF that would yield lower molecular weight species as has been observed for plasmid deavage of VEGF (22, 23).

VEGF Binding to KDR Receptor Is Primarily Mediated by Arg56, Lys84, and His86. The binding of VEGF mutants to soluble KDR-IgG was evaluated by competitive displacement of 125I-labeled VEGF165 in the absence or presence of heparin. The list of mutations is given in Table I. The results for 27 charged-to-alanine scan mutants of VEGF in studies of binding to KDR-IgG are shown in Fig. 4, plotted with respect to the position of the mutation(s). Wild-type VEGF165 expressed in 293 cells and CHO cells were equivalent with respect to displacement of 125I-labeled VEGF165 in KDR binding. The concentrations required to achieve half-maximal inhibition (IC50) were 31 and 29 pm for 293- or CHO-derived VEGF165, respectively (n = 8 replicates each). The IC50 values for wild-type VEGF were not significantly different in the absence versus the presence of 10 μg/ml heparin.

Many of the mutant proteins exhibited binding comparable with wild-type VEGF. In fact, the binding to KDR for 19 out of 25 alanine scan mutants was similar to that of wild-type VEGF; the average IC50 for mutants with wild-type phenotype was 29 ± 18 pm (n = 19). Three mutants (E42A, E44A, and
D63A) exhibited an apparent 4-6-fold increased binding to KDR receptor compared with wild-type VEGF. Since this effect (for two of these mutants) was observed for FLT-1 binding as well, the potential exists for under quantitation of selected mutants for the polyclonal/monoclonal ELISA. However, the most significant effect on binding was observed with the R82A, K84A, H86A mutant of VEGF, which exhibited 1000-fold decreased affinity for the KDR receptor in the absence of heparin, relative to that of wild-type VEGF (Fig. 4A). Interestingly, in the presence of heparin, the binding of this triple mutant was only 10-fold decreased compared with that of wild-type VEGF. These results are consistent with VEGF binding to KDR as a function of two sites of interaction, a heparin-independent site in the 1-110 dimer and a heparin-dependent binding site in the 111-165 domain. When the binding of VEGF to KDR is mediated entirely by the 1-110 region (in the absence of heparin) the mutations at 82, 84 and 86 severely compromise the binding of VEGF to KDR.

To evaluate the relative contribution of individual residues, single amino acid substitution mutants of VEGF were constructed. The single mutations, R82A, K84A, and H86A were found to display more modest decreases with respect to KDR binding (Table I). R82A VEGF exhibited wild-type KDR binding, while K84A VEGF and H86A VEGF were approximately 5.5- and 1.8-fold decreased in binding compared with that of wild-type VEGF, respectively. While the 84 position of VEGF was most dominant in the triple alanine mutant, the combination of mutations at 82, 84 and 86 clearly exhibited a synergistic effect on the interaction with the KDR receptor. In addition to the major KDR binding determinants, a minor site was observed in the 63-67 region. The triple mutant, D63A/E64A/E67A VEGF, was 2.4- and 3.0-fold reduced in binding to KDR in both the presence and absence of heparin. The single amino acid mutant E64A VEGF exhibited 7-fold decreased affinity for KDR, which implicated this site more than neighboring mutations in this region, i.e. D63A and E67A. Although modest in comparison to the majority of the effects observed with R82A/K84A/H86 VEGF, the most potent effects with single alanine replacement of charged amino acids were observed for E64A VEGF and K84A VEGF.

VEGF Binding to FLT-1 Receptor Involves Interaction with Asp63, Glu64, and Glu67—As was observed for KDR binding, most of the alanine scan mutants of VEGF bound FLT-1 with similar affinity as wild-type VEGF (Fig. 4B). The IC50 values for wild-type VEGF were 22 ± 8 and 15 ± 2 pm in the absence and presence of heparin, respectively (n = 13). Analysis of alanine scan VEGF mutants indicated two sites of interaction with FLT-1 that co-localized with the KDR binding determi-
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The binding of N75A VEGF was indistinguishable from that of wild-type VEGF as indicated by the 30-fold reduction in affinity with D63A/E64A/E67A VEGF in the absence of heparin. This is in contrast to the results with KDR, which indicated that mutations in the 63–67 region of VEGF exhibited only modest effects on KDR binding. The major site of KDR interaction (82–86 region) yielded only minor effects with respect to FLT-1 binding. Additional mutational sites at the carboxyl terminus were associated with minor effects on FLT-1 binding. The relative roles of major and minor receptor binding sites are reversed for FLT-1 in comparison with that for KDR.

The Effect of Glycosylation on Receptor Binding—An unglycosylated form of VEGF was constructed, expressed in 293 cells, and visualized by SDS-PAGE and immunoblotting (Fig. 3). This mutant, N75A VEGF, appeared to have a lower molecular weight consistent with the lack of glycosylation at position 75. The binding of N75A VEGF was indistinguishable from that of wild-type VEGF for both KDR and FLT-1. For the wild-type protein, N-linked carbohydrate at Asn75 does not appear to play a role in mediating VEGF receptor binding.

We inserted potential neoglycosylation sites to observe the effects of carbohydrate addition at or near putative receptor binding sites. Surface accessible sites were considered optimal in extracellular loops or turns as predicted on the basis of the crystal structure of PDGFb dimer (50). One such site (42–44 region) was selected as a control since no receptor binding determinants were identified in this region by charged-to-alanine scanning mutagenesis. The neocarbohydrate site in E42N/E44S VEGF was apparently glycosylated, as indicated by the increased molecular weight observed on SDS-PAGE immunoblots (Fig. 3). The N-linked carbohydrate at position 42 did not interfere with binding to KDR or FLT-1 receptors as indicated by IC50 values of 15 and 13 pM, respectively.

Potential Glycosylation Site at Position 82 Results in Severely Decreased KDR Binding—The extent of extraglycosylation was not apparent on the immunoblot for R82N/I83L/K84S VEGF (Fig. 3). Although the R82N/I83L/K84S mutation had minimal effect on apparent molecular weight, the effect on KDR binding was quite significant. R82N/I83L/K84S VEGF exhibited only partial displacement of the labeled VEGF in KDR binding (Fig. 5A). The half-maximal inhibitory concentration for R82N/I83L/K84S VEGF was estimated to be 100,000 pM. However, in the presence 10 μg/ml heparin, the relative affinity of R82N/I83L/K84S VEGF for KDR was 50-fold decreased compared with that of wild-type VEGF. Interestingly, this putative extraglycosylation mutant exhibited normal affinity for FLT-1 (Fig. 5B). Mutations in the 82–86 region (R82A, K84A, H86A, and R82N/I83L/K84S) confer significantly decreased interaction with KDR and normal binding to FLT-1.

Extraglycosylation at Position 64 Decreases FLT-1, but Not KDR Binding—E64N/L66S VEGF was observed as a faint band with apparent increased molecular weight on SDS-PAGE (Fig. 3). E64N/L66S VEGF exhibited approximately 45-fold decreased binding to FLT-1 in the presence or absence of heparin, respectively (Fig. 5B, and Table I). This mutant having FLT-1-specific effects exhibited modestly decreased binding with KDR receptor. The relative binding of D63A/E64A/E67A VEGF and E64N/L66S VEGF to soluble KDR was approximately 3- and 5-fold decreased, respectively. These changes are small in comparison with their effects on FLT-1 binding, where this region is the major binding determinant.

VEGF Mutants with Decreased KDR Receptor Binding Are Weak Endothelial Cell Mitogens—Mitogenic activities of VEGF and mutants of VEGF were determined using bovine adrenal cortical capillary endothelial cells. Wild-type VEGF, derived from 293 cells or CHO cells, induced half-maximal proliferation at 28 ± 10 pM (n = 6) and 16 ± 8 pM (n = 9), respectively. Conditioned cell media from mock-transfected 293 cells did not
induce endothelial cell proliferation. The half-maximally effective concentrations (EC50) for most VEGF mutants were similar to wild-type VEGF (Table I). Significant effects on endothelial cell proliferation were observed with mutations in the 82–86 region. The mitogenic potency of R82A/K84A/H86A VEGF was decreased 20-fold compared with wild-type VEGF. Proliferation by R82N/I83L/K84S VEGF was reduced to such an extent that 50% of maximal growth was not achieved at the highest concentration tested (Fig. 6). To quantitate the potency of R82N/I83L/K84S VEGF, we compared the concentration of the mutant required to achieve 20% of maximal VEGF-induced stimulation. The difference in EC20 values for wild-type VEGF and R82N/I83L/K84S VEGF (4 pM versus 230 pM, respectively) indicated 60-fold reduced potency for the mutant with a neoglycosylation site in the region specific for KDR binding. The effect of these mutations on endothelial cell growth is consistent with the KDR binding data. The affinity of R82A/K84A/H86A VEGF and R82N/I83L/K84S VEGF with soluble KDR (in the presence of heparin) was reduced 10- and 50-fold, respectively, compared with that observed with wild-type VEGF. Since endothelial cells in vitro express surface and matrix associated heparan sulfate-containing proteoglycans (53), it is appropriate to compare the mitogenic response of endothelial cells to VEGF or VEGF mutants with the binding data for soluble VEGF receptors in the presence of heparin. Taken together, the mutational analysis of VEGF by alanine scanning and extra-glycosylation provide strong evidence that binding to KDR receptors on endothelial cells is a triggering event for the induction of proliferation observed with VEGF.

VEGF Mutants with Decreased FLT-1 Receptor Binding Are Fully Active Endothelial Cell Mitogens—Alanine scan substitutions in the 63–67 region of VEGF were shown to have normal binding to KDR and decreased binding to FLT-1 (Figs. 4 and 5). Triple and single alanine mutants (D63A/E64A/E67A VEGF, D63A/E64A/E67A VEGF, and E67A VEGF) were evaluated for induction of endothelial cell growth. All of these mutants exhibited mitogenic potency similar to that of wild-type VEGF (Fig. 6 and Table I). The mutant with a putative extraglycosylation site in the 63–67 region; E64N/L66S VEGF also exhibited normal activity on endothelial cells. These data reinforce the observation that FLT-1-deficient mutants of VEGF induce endothelial cell proliferation similar to wild-type VEGF. Furthermore, these data suggest that VEGF binding to FLT-1 receptors on endothelial cells is unrelated to mitogenesis and proliferation. This mutational analysis has identified VEGF variants that are relatively selective for KDR or FLT-1 receptors. The data in this report suggest an electrostatic component of VEGF-receptor interaction, such that the determinants for KDR and FLT-1 include predominantly positive or negatively charged regions of VEGF, respectively.

**Fig. 4.** Receptor binding of alanine scan mutants of VEGF<sub>165</sub>. The VEGF mutants were expressed in 293 cell culture, and the conditioned cell medium was used to displace wild-type VEGF from binding receptor-IgG chimeras. The mean residue number is the average of the amino acid positions that were altered by mutation. The values are expressed as the concentration required to half-maximally inhibit (IC<sub>50</sub>) the binding of radiolabeled CHO-derived VEGF to KDR-IgG (panel A) or FLT-1 IgG (panel B). The binding assays were done in the presence (filled circles) or the absence (open boxes) of heparin at 10 μg/ml. These experiments were performed in triplicate; error bars indicate standard deviation.

**Fig. 5.** Competitive displacement of <sup>125</sup>I-labeled VEGF<sub>165</sub> from KDR or FLT-1 with triple alanine scan or glycosylation mutants. Displacement curves with KDR-IgG (panel A) or FLT-1 IgG (panel B) binding labeled VEGF in competition with wild-type VEGF (filled circles), D63A/E64A/E67A VEGF (open circles), D64N/L66S VEGF (open triangles), R82A/K84A/H86A VEGF (open triangles), R82N/I83L/K84S VEGF (filled triangles). These experiments were performed in duplicate in the absence of heparin.
DISCUSSION

KDR and FLT-1 receptor binding determinants on VEGF, identified by site-directed mutagenesis, were localized on a model of VEGF based on the crystal structure of a homologous protein, PDGFb. VEGF and PDGFb display 28% amino acid sequence homology with conserved alignment of eight cysteines within the receptor binding domain (i.e. 1–110 of VEGF). The crystal structure of PDGFb dimer has been elucidated at a 3.0-Å resolution (50). A model of the three-dimensional structure of PDGFb dimer is shown (see Fig. 7), with the appropriate amino acids of VEGF highlighted that are involved in receptor binding as identified by mutational analysis.

The polypeptide chain of each monomer is folded into two twisted anti-parallel pairs of β-strands (shown as green ribbons, Fig. 7). The amino termini of the PDGFb dimer were not resolved, and the structure begins at residue 17 in VEGF numbering. Three intramolecular disulfide bonds are arranged in a knotted configuration, shown in yellow for Cys26–Cys68, Cys57–Cys102, and Cys60–Cys104 (VEGF numbering, see Ref. 48 for PDGF disulfides). Two intermolecular disulfide bonds form the covalent linkage of the homodimeric structure via cysteines in the (1–110) domain of VEGF dimer (50). Shown as filled circles (VEGF numbering, see Ref. 48 for PDGF disulfides). Two intermolecular disulfide bonds form the covalent linkage of the homodimeric structure via cysteines in positions 51 and 60. Dimerization leads to an elongated structure with three surface loops that cluster at each end of the molecule. Defner and co-workers (50) suggest that these loops would probably form receptor recognition sites for PDGF. The surface loops, denoted as I, II, and III in PDGFb, include the corresponding regions of VEGF from 36 to 46, 61 to 68, and 84 to 87, respectively (VEGF numbering). Loop I is not resolved in the PDGFb structure and is denoted by a gap in the peptide strand from residues 37 to 45 (VEGF numbering). Loops II and III in VEGF are involved in receptor recognition as indicated by the binding studies with mutants D63A/E64A/E67A VEGF and R82A/K84A/H86A VEGF, respectively. Interestingly, the loop II residues Asp63, Glu64, and Glu67 that mediate VEGF binding predominantly to FLT-1, form a negatively charged surface at one end of each monomer (residues 63, 64, and 67 shown in red, see Fig. 7). In contrast, the loop III associated residues, Arg82, Lys84, and His86, which predominantly mediate binding to KDR, cluster to form a positively charged surface at the other end of the VEGF monomer (residues 82, 84, and 86 shown in blue). These oppositely charged surface loops are at distal ends of the monomer, but they are in close proximity in the dimeric form of VEGF. Although the receptor binding regions of loop II and III predominantly mediate binding of FLT-1 and KDR, respectively, there is indication that KDR interacts with residues in both loops. For example, the VEGF mutant with a potential extraglycosylation site at position 64 exhibits reduced affinity for KDR as well as for FLT-1 receptors (6- and 40-fold, respectively). Altogether, the functional analysis of VEGF by site-directed mutagenesis indicates that the VEGF dimer exhibits receptor binding regions at both ends of the protein.

Two sets of receptor binding sites on VEGF interact predominantly with FLT-1 or KDR. These sites, shown schematically as A and B in Fig. 8, display binding to KDR and FLT-1, respectively. Site A, composed of basic amino acids (including Arg82, Lys84, and His86) predominantly mediates the interaction of VEGF with KDR. It is interesting to note that in recent studies, a cationic series of amino acids in a pentapeptide sequence of PDGF (Val158-Arg159-Lys160-Lys161-Pro162, PDGF numbering) has been demonstrated to play a critical role in ligand binding to the PDGFα receptor (52). This sequence is perfectly conserved between PDGFα and PDGFb, but is not found in either VEGF or PLGF (see Fig. 1). The cationic sequence is located in the loop III region of the PDGF structure, which corresponds to amino acids 80–90 of VEGF. PDGF and VEGF appear to share a requirement for basic amino acids in the loop III region (site A) in binding to PDGFα receptor and KDR, respectively. These data do not exclude significant con-
Fig. 8. VEGF displays different receptor binding sites for KDR and FLT-1. This schematic diagram provides a functional description of the two receptor epitopes identified by mutational analysis. The A site, composed of basic residues (region 82–86), mediates the binding to KDR. The B site, composed of acidic residues (region 63–67), mediates the interaction with FLT-1 receptor. This diagram illustrates bivalent epitopes at opposite ends of VEGF, suggesting a possible mechanism for ligand-induced dimerization of receptors.

Contributions by noncharged amino acids, such as Gln\textsuperscript{79}, Ile\textsuperscript{80}, Met\textsuperscript{81}, Ile\textsuperscript{82}, and/or Pro\textsuperscript{85}.

The B site of VEGF, composed of acidic residues (including Asp\textsuperscript{63}, Glu\textsuperscript{64}, and Glu\textsuperscript{67}) is shown in Fig. 8 as weakly interacting with the KDR receptor. This second site, however, appears to be directly involved with the binding of VEGF to FLT-1. LaRochelle et al. (54), utilizing site-directed mutagenesis of PDGF, have described similar findings with respect to mapping the α/β receptor binding determinants. Mutations of Asn\textsuperscript{136} and Arg\textsuperscript{137} in PDGF, which correspond to Asp\textsuperscript{63} and Glu\textsuperscript{64} of VEGF (see Fig. 1), have been shown to be essential for the α/β receptor specificity of PDGFα and PDGFβ. In the present study, the effect of mutations within the loop II of VEGF (site B) did not strongly affect the binding of VEGF\textsubscript{165} to FLT-1 as compared with the effect of loop I mutations (site A) on KDR binding. The alanine replacement of Asp\textsuperscript{63}, Glu\textsuperscript{64}, and Glu\textsuperscript{67} resulted in 30-fold decreased affinity with FLT-1, whereas the R82A/K84A/H86A mutant exhibited 1000-fold decreased binding to KDR (see Table 1). Noncharged amino acids such as Asn\textsuperscript{62} and Leu\textsuperscript{66} may also contribute to the interaction of VEGF with FLT-1. These data suggest additional site(s) of FLT-1 interaction, possibly in the carboxyl-terminal domain, residues 111–165 of VEGF. Amino acids in site A appear not to interact with the FLT-1 receptor, as indicated by the wild-type phenotype associated with R82A/K84A/H86A and R82A/I83A/K84A mutations.

The 2-fold symmetry of PDGFββ localizes the three loop regions on either end of the molecule (50). The evidence of receptor binding sites at both ends of the dimer is consistent with the growth factors of the PDGF/VEGF family exhibiting bivalency. Fretto et al. (55) have established that PDGF is bivalent with respect to PDGFR receptor binding (55). Bivalent binding of VEGF provides a mechanism for dimerization of the KDR and/or FLT-1 tyrosine kinase receptors on endothelial cells. Consistent with cross-linking of tyrosine kinase receptors followed by tyrosine phosphorylation, VEGF has been demonstrated to stimulate tyrosine phosphorylation of a 200-kDa membrane protein from endothelial cells (56, 57). It remains to be seen if KDR and FLT-1 can form heterodimeric receptors on endothelial cells, as has been postulated by Watanabeberger et al. (57).

The concept of heterodimerization of receptor tyrosine kinases is well documented, particularly in the case of α/β heterodimers of PDGF receptors, which differentially bind PDGFα and PDGFβ (58). The recently reported finding of VEGF/PLGF heterodimers adds another layer of complexity with respect to various forms of VEGF and PLGF, which have differential specificity for FLT-1 and/or KDR (59).

In the present study, we describe VEGF mutants that are relatively selective for KDR or FLT-1 receptors. These mutants, with differential receptor specificity, will provide molecular tools to probe the relative functional significance of KDR and FLT-1 receptors. The role of KDR and FLT-1 in stimulating endothelial cell proliferation has been addressed using the VEGF mutants. R82A/K84A/H86A and R82N/I83L/K84S mutants of VEGF are significantly decreased in both KDR binding and endothelial cell growth assays (Figs. 5 and 6 and Table I). Furthermore, we have observed that the FLT-1 deficient mutants (D63A, E64A, E67A, and E64N/L66S forms of VEGF) display full activity with respect to endothelial cell proliferation. These results are consistent with the lack of endothelial growth in response to PLGF, which is specific for FLT-1 but does not bind KDR (34). These data, with the present study, correlate the mitogenic and proliferative effects of VEGF with KDR binding and subsequent receptor phosphorylation. In another study, it was shown that KDR-expressing cells respond to VEGF stimulation with marked changes in cell morphology, actin organization, chemotaxis, and mitogenesis, whereas FLT-1 expressing cells lacked these responses to VEGF (57). The significance of the regulation of endothelial function by receptor tyrosine kinases is unclear, especially with respect to the role of FLT-1 in angiogenesis. The pivotal role of both FLT-1 and KDR/Flk-1 in development has been clearly demonstrated by the recent reports on mice deficient in these endothelial-specific receptor kinases.

Homozygous mutations that inactivate the FLK gene result in failure of blood island formation and vasculogenesis, whereas homozygous mutations that inactivate the FLT-1 gene result in defective assembly of endothelial cells into tubes (41, 60). Both mutations result in intrauterine death at day 8.5. Clearly, both FLT-1 and KDR/Flk-1 are critical for growth and development of normal vascular endothelium. We anticipate further analysis of the biological significance of KDR and FLT-1-mediated responses to VEGF in vitro and in vivo through the use of receptor-selective VEGF mutants.

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