Receptor-selective Variants of Human Vascular Endothelial Growth Factor

GENERATION AND CHARACTERIZATION*

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Vascular endothelial growth factor (VEGF) is a pleiotropic factor that exerts a multitude of biological effects through its interaction with two receptor tyrosine kinases, fms-like tyrosine kinase (Flt-1) or VEGF receptor 1 and kinase insert domain-containing receptor (KDR) or VEGF receptor 2. Whereas it is commonly accepted that KDR is responsible for the proliferative activities of VEGF, considerable controversy and uncertainty exist about the role of the individual receptors in eliciting many of the other effects. Based on a comprehensive mutational analysis of the receptor-binding site of VEGF, an Flt-1-selective variant was created containing four substitutions from the wild-type protein. This variant bound with wild-type affinity to Flt-1, was at least 470-fold reduced in binding to KDR, and had no activity in cell-based assays measuring autophosphorylation of KDR or proliferation of primary human vascular endothelial cells. Using a competitive phage display strategy, two KDR-selective variants were discovered with three and four changes from wild-type, respectively. Both variants had approximately wild-type affinity for KDR, were about 2000-fold reduced in binding to Flt-1, and showed activity comparable with the wild-type protein in KDR autophosphorylation and endothelial cell proliferation assays. These variants will serve as useful reagents in elucidating the roles of Flt-1 and KDR.

Vascular endothelial growth factor (VEGF) is a pleiotropic growth factor with multiple biological effects such as endothelial cell survival, proliferation and migration, calcium influx, formation of tube structures of endothelial cells, and vessel permeability and vasodilation. These multiple effects are exerted through the interaction of VEGF with two receptor tyrosine kinases (Flt-1 or VEGF receptor 1 and KDR or VEGF receptor 2). Knockout studies in mice show that both receptors are essential for survival and angiogenesis, but which biological effect(s) are mediated by which receptor is still not fully understood. It is widely accepted that KDR is the receptor that mediates the proliferation of endothelial cells, but besides the essential role of Flt-1 in embryogenesis, the function of Flt-1 is poorly understood. Several naturally occurring receptor-selective VEGF homologues, such as placental growth factor (PIGF), VEGF-B, and VEGF_{orf} (derived from the genomes of Orf viruses; Refs. 11–13), have been used in attempts to define the role(s) of the receptors, but some of the results remain controversial. Recent work with VEGF_{orf}, which is KDR-selective, has shown that activation of KDR alone is sufficient to stimulate the growth and migration of endothelial cells, the permeability of blood vessels, and the influx of calcium; in addition, VEGF_{orf} is fully angiogenic in vivo (11, 12). In contrast, Flt-1-specific PIGF has displayed great variability in mitogenic activity for endothelial cells (14–17). The identity of the receptor responsible for vessel permeability and dilation remains controversial. However, the importance of Flt-1 for angiogenesis has been indicated by the up-regulation of Flt-1 in endothelial cells under hypoxic conditions that stimulate angiogenesis (18), the ability of Flt-1-specific VEGF-B to regulate plasminogen activator (19), and the anti-angiogenic activity of Flt-1-specific ribozyme (20).

Interpretation of the results obtained with natural VEGF variants may be complicated by the presence in some variants of additional domains of unknown function, low sequence similarity (30–45%) to the wild-type protein, and the wide range of receptor affinities. Furthermore, some of these variants are unlikely therapeutic candidates due to expected immunogenic responses. In attempts to circumvent these disadvantages, the wild-type protein has been used as the starting point for the
introduction of point mutations in the receptor-binding region of the protein (21–23). The resulting variants showed moderate selectivity but also often lost binding affinity for the targeted receptor. Recently, structure-function studies have elucidated the three-dimensional structure of VEGF and defined its receptor-binding site (24–27), and we decided to use this information to guide further mutagenesis and phage display approaches to generate improved variants. Herein, we describe three receptor-selective VEGF variants with a minimal number of sequence changes compared with the wild-type. One mutant, containing four amino acid changes, binds with native affinity to Flt-1 and about 470-fold more weakly to KDR compared with wild-type VEGF. The other two variants, with three and four changes from the wild-type protein, respectively, have wild-type affinity for KDR but about 2000-fold reduced affinity for Flt-1. These variants are useful reagents for the further elucidation of the biological roles of KDR and Flt-1 and may have potential as therapeutic agents.

EXPERIMENTAL PROCEDURES

Mutagenesis—Site-directed mutagenesis was performed using the method of Kunkel et al. (29) in the background of the receptor-binding domain (residues 1–109) of VEGF. All mutations were verified by DNA sequencing. The VEGF residues that were individually mutated were Ala-5, Lys-5, Lys-10, Lys-15, Met-15, Met-18, Tyr-21, Gin-22, Tyr-25, Ile-43, Ile-46, Phe-47, Lys-48, Asp-63, Glu-64, Glu-65, Leu-66, Gin-79, Met-81, Ile-83, His-86, Gin-89, Ile-91, Lys-101, Gin-103, Arg-105, and Pro-106. A combination mutant with four residues changed to Ala (Ile-43, Ile-46, Gin-79, and Ile-83) was made using site-directed mutagenesis (29).

Selection of KDR-specific VEGF Variants—VEGF alanine-scanning data were used to select residues for randomization in phage-displayed libraries. Libraries were constructed following the method described by Sidhu et al. (30), using a phagemid (pB2105; Ref. 24) that allows for the phage display of VEGF as a fusion to the carboxyl-terminal domain of the gene-3 minor coat protein. Briefly, for each library, a “stop template” phagemid was constructed in which codons chosen for randomization were replaced with stop codons using site-directed mutagenesis (29). The mutations were verified by DNA sequencing. The resulting phagemid was then used as the template for a second round of mutagenesis with oligonucleotides designed to replace all stop codons with appropriately designed degenerate codons. Successful mutagenesis at all sites produced library members with randomized codons at all selected positions, and unsuccessful mutagenesis at one or more sites resulted in the generation of stop codons at those positions that did not display VEGF. Approximately 70% of the clones contained mutations at all the designed sites. Two types of degenerate codons were used. VEGF positions that were not important for KDR binding (see “Results”) were “hard randomized” using degenerate codons (NNS, where N = A/C/G/T and S = G/C) that encodes all twenty natural amino acids. Positions that were important for KDR binding (see “Results”) were “soft randomized” using oligonucleotides designed to maintain a bias toward the wild-type sequence while allowing a 50% mutation rate at each position. This mutation rate was attained by synthesizing the oligonucleotides such that each base in the doped region is coupled with a mixture containing 70% of the base corresponding to the wild-type sequence and 10% of each of the other three bases. Four libraries were constructed, with four or five varied residues per library (see Table 1). Each library contained 1.5 * 10^10 unique members and was thus sufficiently large to represent all possible random sequence combinations. Standard phage display methods (30) were used to cycle phage from each library through rounds of binding selection with domains 1–3 of KDR (KDR (1–3)) coated on 96-well Maxisorb immunoplates as the capture target. Each library was sorted separately in the presence of 100 nM domains 1–3 of Flt-1 (Flt (1–3)) as competing ligand, sufficient to prevent about 95% of the phagemids from binding to KDR. After washing to remove unbound phage, bound phage were eluted with 0.2 M glycine, pH 2.0. Neutralized phage were propagated in Escherichia coli XL1-Blue with M13-VCS helper phage. After 5–6 rounds of sorting, individual phage clones were isolated and analyzed with a phage ELISA. Phage clones that bound specifically to immobilized KDR (1–3) in the presence of Flt (1–3) in solution were subjected to DNA sequence analysis.

Phage ELISA—Phage ELISA protocols were adapted from a previous work (24). Cultures of E. coli XL1-Blue harboring phagemids were grown for 8 h at 37 °C in 1 ml of 2x yeast tryptone broth, 50 μg/ml carbenicillin, 10 μg/ml tetracycline, and 30 μl of the same medium, supplemented with M13-VCS helper phage (10^10 phage/ml), and grown overnight at 37 °C. Phage were harvested from the culture supernatant by precipitation twice with polyethylene glycol/NaCl and resuspended in 1.0 ml of phosphate-buffered saline, 0.5% BSA, 0.1% Tween 20 (binding buffer). Maxisorb 96-well immunoplates were coated with capture target protein (KDR (1–3) or Flt (1–3)) overnight at 4 °C (100 μl at 5 μg/ml in 50 mM carbonate buffer, pH 9.6). The plates were then blocked for 1 h with 0.5% BSA in PBS and washed eight times with PBS, 0.05% Tween 20. Serial dilutions of KDR (1–3) or Flt (1–3) with a subsaturating concentration of phage particles displaying VEGF or VEGF variants were added to the wells in 100 μl of binding buffer. After 1 h, the plates were washed eight times with PBS, 0.05% Tween 20, incubated with 100 μl of dilute horseradish peroxidase/anti-M13 antibody conjugate in PBS blocking buffer for 30 min, and then washed eight times with PBS, 0.05% Tween 20 and two times with PBS. Plates were developed using an o-phenylenediamine dihydrochloride/H_2O_2 solution (100 μl), stopped with 2.5 M H_2SO_4 (50 μl), and read spectrophotometrically at 492 nm. Affinities were estimated as IC_{50} values: the concentration of KDR (1–3) or Flt (1–3) that blocked 50% of phage binding.

Protein ELISA—Microtiter plates were coated with purified VEGF (at 5 μg/ml) in 50 mM sodium carbonate (pH 9.6) at 4 °C overnight. Plates were blocked with 0.5% BSA, and serial dilutions of competing VEGF alanine mutants and a subsaturating concentration (100 pM) of biotin-labeled receptor (KDR (1–3) or Flt1(1–3)) were added to wells in 100 μl of binding buffer (0.5% Tween 20, 0.5% BSA in PBS). After 1 h, the plates were washed, and the bound protein was stained with streptavidin horseradish peroxidase conjugate (Amersham Pharmacia Biotech) and assayed. Affinities were estimated as IC_{50} values: the concentration of KDR (1–3) or Flt (1–3) that blocked 50% of protein binding.

VEGF Refolding and Purification—VEGF or VEGF mutants were produced in E. coli 27C7 shake flask cultures and isolated as refractile bodies. Each protein was refolded as described previously (31). Briefly, the protein was solubilized and unfolded in 6.0 M guanidine HCl, 1.0 M oxidized glutathione, pH 6.0. The solution was dialyzed for 10 h against 10 volumes of 2.0 M urea, 2.0 M reduced glutathione, 0.5 M oxidized glutathione, 20 mM Tris-HCl, pH 8.0. Urea was removed by overnight dialysis at 4 °C against 20 volumes of 20 mM Tris-HCl, pH 8.0. The refolded dimer was further purified using anion exchange chromatography (Pharmacia HiTrap Q, 1.0 ml) to remove traces of misfolded monomer. The purity and identity of the refolded dimer were assessed by SDS-polyacrylamide gel electrophoresis and mass spectrometry.

Radioimmunoassay—A previously described RIA (24) was used to measure the binding affinities of VEGF mutants for KDR and Flt-1 receptors in native VEGF-(1–121) IgG Fc fusion of the extracellular domain of KDR or Flt were incubated with 5 pM 125I-labeled VEGF and varying concentrations of VEGF mutant (18 h at room temperature in 20 mM Tris-HCl, 150 mM NaCl, 0.5% BSA, 0.05% Tween 20). The solution was then transferred to a 96-well plate coated with anti-Fc antibody and incubated for 30 min at room temperature. The plates were washed five times with PBS containing 0.05% Tween and counted with a Topcount microplate scintillation counter (Packard Instrument Co.).

Cell Binding Assays—KDR- or Flt-1-transfected NIH3T3 cells were made as described previously (27). Cells were maintained in F12 medium with 10% fetal bovine serum with 400 μg/ml G418 (Life Technologies, Inc.). For binding assays, cells were plated in 12-well plates at 1 * 10^6 cells per well to reach confluency the next day. Cells were washed and blocked in Hanks’ buffered saline with 1% BSA for 1 h before adding the mixture of 125I-VEGF109 with increasing concentration of unlabeled VEGF variants. The plates were incubated at 4 °C for 3 h and then washed twice with Hanks’ buffered saline with 0.5% BSA. The bound labeled VEGF was collected by solubilizing the washed plate with 1 N NaOH and counted in a gamma counter (Isodata, ICM). 125I-VEGF109 was made by iodination of chloramine-T. Each 50 μl labeled VEGF was used for KDR and Flt-1 cell binding, respectively.

Endothelial Cell Proliferation Assay—The mitogenic activity of VEGF variants was determined using human umbilical vein endothelial cells (HUVECs) (Clonetics). An early passage (less than six) of HUVEC was seeded in 96-well plates (about 3000 cells per well) in F12 with 1% fetal bovine serum and starved for 18 h. The cells were fasted

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in CS-C medium (Cell Systems, Kirkland, WA) without growth factors and supplemented with 2% dialyzed fetal bovine serum for 24 h at 37 °C with 5% CO₂ before replacing the medium with fresh fasting medium. VEGF variants at several concentrations in the same fasting medium were added to the wells to bring the volume up to 150 μl per well. The cells were incubated for 18 h, pulsed with [³H]thyminidine (0.5 μCi per well) for 24 h, harvested, and counted with a Topcount microplate scintillation counter (Packard Instrument Co.).

### Kinase Receptor Activity Assay—Activity of the VEGF variants was determined by measuring their ability to induce KDR phosphorylation in a kinase receptor activity assay. Serially diluted VEGF-(1–109) variants (0.01–10 μM) were added to Chinese hamster ovary cells that express KDR with a peptide epitope flag (gDflag) fused to the amino terminus. Cells were lysed using 0.5% Triton X-100, 150 mM NaCl, 50 mM HEPES, pH 7, and phosphorylated gDflag-KDR in the lysate was captured with anti-gDflag monoclonal antibody coated on an ELISA plate. The wells were incubated with biotinylated monoclonal antiphosphotyrosine antibody for 2 h, followed with peroxidase-labeled streptavidin. Phosphorylation of gDflag-KDR was detected by measuring absorbance at 450 nm on a plate reader.

### Smooth Muscle Cell MMP-9 Assay—Human aorta smooth muscle cells (Clonetics) were maintained in SM2 medium (Clonetics) at 37 °C in 5% CO₂ and 95% ambient air in the presence of 10% fetal bovine serum in 6-well polystyrene plates (Becton Dickinson). When cells attained 90% confluence, they were growth-arrested for 24 h in serum-free medium containing 0.2% BSA. VEGF-(1–109), PIGF or VEGF-(1–109) variants were added at a final concentration of 40 ng/ml, and the cells were cultured for an additional 24 h in the serum-free medium containing 0.2% BSA. Gelatinase in the conditioned medium was analyzed by zymography. Media were collected and concentrated, and 25 μl- aliquots were mixed with 2 × sample buffer without reducing agent or heating. Samples were loaded on a 10% polyacrylamide gel containing 0.1% gelatin (Novex, San Diego, CA) for electrophoresis. In addition to using regular molecular weight markers, the MMP-2 and MMP-9 zymographic standards (Chemicon, Temecula, CA) were used as standards for gelatinases. After electrophoresis, proteins were renatured by incubation of the gels for 30 min at room temperature in renaturing buffer (Novex) overnight at 37 °C. The gels were stained with 0.25% Coomassie Brilliant Blue (Sigma). Gelatinase activity was identified as lightly stained or clear bands following destaining.

### RESULTS

#### Alanine Scan of the Receptor-binding Site—An alanine scan (32) was used to define the relative importance of individual VEGF residues for KDR versus Flt-1 binding. The residues selected for mutagenesis included the 22 contact residues observed in the crystal structure of the complex between the receptor-binding domain of VEGF and domain 2 of Flt-1 (26) as well as Phe-47 and Glu-64, which had previously been identified as KDR-binding determinants (24). These 22 residues were individually mutated to Ala in the background of the receptor-binding domain of VEGF (residues 1–109) (24, 33). Each mutant protein was produced and purified to homogeneity, and an ELISA was used to determine binding affinities for domains 1–3 of KDR and Flt-1 (these three domains contain the entire VEGF-binding site (26, 27)). The results of this analysis are shown in Table I. The mutated region consists of five segments of polypeptide chain, which we define based on the VEGF structure as the “20s helix,” the “40s loop,” the “60s loop,” the “80s loop,” and the “100s loop” (Table I). Overall, the residues found to be most important for high affinity binding to KDR are in good agreement with those previously identified in a study using the entire extracellular portion (domains 1–7) of KDR (24). The first four segments defining the receptor-binding site, with the largest reductions in binding (greater than 10-fold) observed for Ala substitution of Phe-17, Tyr-21, Glu-64, and Leu-66 (Table I).

### Table I

| Residue  | KDR(1–3)  | Flt(1–3)  |
|----------|-----------|-----------|
| VEGF(1–109) | 1 (10 nM) | 1 (6 nM) |

- 20s helix:  
  - Lys-16: 1  
  - Phe-17*: 45  
  - Met-18**: 5  
  - Tyr-21**: 19  
  - Glu-22**: 6  
  - Tyr-25**: 6  

- 80s loop:  
  - Ile-42*: 21  
  - Ile-46*: 96  
  - Phe-47**: 5  
  - Lys-48**: 1  

- 100s loop:  
  - Gln-79*: 55  
  - Met-81**: 9  
  - Ile-83*: 89  
  - His-86**: 2  
  - Gln-89: 1  
  - Ile-91: 1

Correlating these results reveals that some residues within the receptor-binding site are important for high affinity binding to both receptors, and a subset consisting of Ile-43, Ile-46, Gln-79, and Ile-83 is critical for KDR but not Flt-1 binding. In contrast, mutation of the critical Flt-1-binding determinants also tended to significantly reduce affinity for KDR (Table I).

### Generation of an Flt-1-specific Variant—The alanine scan data suggested that a VEGF variant with high selectivity for the Flt-1 receptor could be readily generated by combining the four mutations that greatly affected KDR but not Flt-1 binding. Therefore, a variant (hence denoted “Flt-1-sel”) was constructed with alanine substitutions at positions 43, 46, 79, and 83. Quantitative binding measurements were carried out using a soluble RIA using receptor constructs consisting of the first three domains of the extracellular portion. In these assays, wild-type VEGF-(1–109) has affinities for KDR and Flt-1 of 0.5 nM and 0.4 nM, respectively (Fig. 1, A and B). Flt-1-sel was found to have at least 470-fold reduced KDR binding affinity in this assay (Fig. 1A). Somewhat surprisingly, because small reductions in receptor binding had been observed for the individual point mutants in the ELISA, the affinity of the variant for Flt-1 was essentially identical to that of the wild-type protein (Fig. 1B). The activity of this variant was tested in cell binding assays. Consistent with the RIA data, Flt-1-sel showed no detectable binding to KDR-transfected 3T3 cells and slightly improved binding to Flt-1-transfected cells (Fig. 1, C and D).
Table I suggests that a combination of alanine mutations to generate a KDR-selective mutant is unlikely to succeed because of the overlap in critical binding determinants for KDR and Flt-1. Therefore, to identify variants that bind tightly to KDR and not to Flt-1, a strategy was chosen based on competitive phage display. Four random libraries were generated of phagemids displaying VEGF-(1–109), varying 4 to 5 residues from one of the segments of the receptor-binding site in each library. These libraries are here denoted the 20s helix library and the 40s, 60s, and 80s loop libraries (see Table I). In each library, the individual residues chosen to be randomized were those that were most important for VEGF binding to Flt-1. Of these, residues that are only marginally important for KDR binding (Met-18, Tyr-21, Gln-22, Tyr-25, Phe-47, Lys-48, Asp-63, Gly-65, Leu-66, Met-81, and His-86) were completely randomized. For residues that are critical KDR-binding determinants (Phe-17, Ile-43, Ile-46, Glu-64, Glu-79, Ile-83), a “soft randomization” scheme was used that introduced a 50% mutation rate while maintaining a bias toward the wild-type residue at each position.

To select KDR-specific variants, phagemids were isolated from the randomized libraries that were able to bind to immobilized KDR (domains 1–3) in the presence of 100 nM competing Flt-1 (domains 1–3) in solution. This amount of Flt-1 was sufficient to block about 95% of the nonresistant phagemids, as monitored by comparison to the amount of phagemid bound in the absence of competing receptor. After 5–6 rounds of sorting, only the 20s helix and the 60s loop libraries exhibited substantial resistance to the added Flt-1, consistent with the observation from the mutagenesis data that these two libraries include the most important Flt-1-binding determinants.

The relative receptor binding affinities of the phagemids displaying the individual variants were assessed using a competition phage ELISA on nine clones each from the 20s and 60s libraries (data not shown). From each library the clone that displayed the greatest reduction in binding to Flt-1 and unaffected binding to KDR was selected for further analysis, and the sequences of the randomized residues were determined. The clone from the 20s library contained four mutations from wild-type VEGF, namely Met-18 to Glu, Tyr-21 to Leu, Gln-22 to Arg, and Tyr-25 to Ser (variant “KDR-sel1”). The clone from the 60s library had three changes: Asp-63 to Ser, Gly-65 to Met, and Leu-66 to Arg (“KDR-sel2”). For both clones, protein was made and purified, and receptor binding affinities were measured...
ured using an RIA (Fig. 1). Both variants had approximately wild-type binding affinity for KDR (Fig. 1A) but bound 1700 and 1900-fold more weakly, respectively, to Flt-1 (Fig. 1B). KDR-sel2 was further examined in receptor-transfected cell binding assays and showed wild-type binding to cells displaying KDR (Fig. 1C) yet 200-fold reduced binding to cells transfected with Flt-1 (Fig. 1D). The difference in Flt-1 binding compared with the RIA measurement is probably because of the reduced dynamic range of the cell binding assay caused by avidity effects of the high density cell surface receptor.

Activity of the Receptor-selective Variants—To assess the biological potency of the receptor-selective VEGF variants, we tested their ability to stimulate autophosphorylation of KDR in KDR-transfected Chinese hamster ovary cells (Fig. 2) and proliferation of HUVEC (Fig. 3). Both KDR-selective variants show activity equivalent to or modestly higher than wild-type VEGF-(1–109), as expected from their measured high affinities for KDR. (Some enhancement in activity may be expected if the KDR-selective mutants suffer less from depletion resulting from interactions with Flt-1 receptors present on HUVEC.) Consistent with the results of the binding assays, the Flt-1-selective variant was inactive in these cell-based assays (Figs. 2 and 3). To assess the activity of the Flt-1-selective variant, we used an assay that measures the secretion of matrix metalloprotease 9 following activation of Flt-1 expressed on human smooth aorta muscle cells (35). In contrast to KDR-sel2, Flt-1-sel was fully active in this assay when compared with the activity of the wild-type VEGF-(1–109) or PlGF (Fig. 4).

**DISCUSSION**

Previous structure-function studies have shown that VEGF has two symmetrical receptor-binding sites straddling the VEGF homodimer interface at the poles of the molecule (24). The crystal structure of VEGF bound to the second Ig-like domain of Flt-1 (26) revealed that the binding surface for this domain overlapped almost completely with the functional epitope for KDR as deduced through a mutagenesis analysis (24). Thus, VEGF appears to use a shared receptor-binding site for binding to both KDR and Flt-1. This situation is analogous to what has been observed for human growth hormone, which can interact through a common binding site with either the growth hormone receptor or the prolactin receptor (28, 36, 37). In such cases, creation of receptor-selective variants with minimal changes from the wild-type protein is a difficult undertaking, and earlier efforts involving mutation of charged VEGF residues to alanine (21, 22) or grafting from PlGF (23) have only achieved limited success.

We based our approach on an alanine scan of all the residues
in the common binding site as observed in the structure of the complex with Flt-1 domain 2 (26). This analysis indicated that the importance of each individual residue for high affinity binding sometimes varied for KDR and Flt-1. Indeed, mutation to Ala of Ile-43, Ile-46, Gln-79, or Ile-83 showed that the side chains of these residues are critical for tight binding to KDR but unimportant for Flt-1 binding. Based on these results, we constructed an Flt-1-selective variant with four changes from the wild-type protein by introducing alanine residues at these positions. The mutational analysis also revealed VEGF residues whose mutation affected Flt-1 binding significantly more than KDR binding. This finding enabled us to target these residues in a randomization and competitive selection strategy using phage display methods, resulting in two KDR-selective variants with only three and four changes from wild-type VEGF, respectively. In neither variant do the changes correspond to the amino acids observed in the naturally occurring KDR-selective VEGF-like proteins, VEGForf (Fig. 5), suggesting that considerable variability can be tolerated at these positions while maintaining tight binding to KDR. With the substitutions Asp-63 to Ser and Leu-66 to Arg, variant KDR-Sel2 has decreased negative charge in the 60s loop compared with wild-type VEGF, supporting the notion that negative charge in this region is important for Flt-1 binding (21). In contrast, the changes in the 20s helix are charge-neutral, so no such generalizations can be made for KDR-Sel1. Interestingly, it is the combination of all these changes together rather than any individual substitution that is responsible for the increased selectivity. When the changed residues (Fig. 5) were individually introduced in the wild-type protein in an attempt to identify the most important selectivity determinants, we found that all single point mutants had binding affinities to KDR and Flt-1 similar to those of the wild-type protein (data not shown). This result underscores the power of phage display methods to engineer modified specificity profiles by introducing concerted changes that could not easily have been predicted based on structural or sequence information. In an attempt to obtain further enhanced selectivity, we combined the mutations from both KDR-selective variants. However, the resulting variant with seven mutations was nearly inactive in cell-based assays and had considerably reduced (more than 200-fold) KDR binding affinity (data not shown). Because the crystal structure of wild-type VEGF shows that the 20s helix and 60s loop are in close proximity (24, 25), we believe the observed non-additivity of the two sets of mutations results from disruption of the local structure of the protein. Numerous biological effects of VEGF have been reported beyond the proliferative activities attributed to KDR. These activities include reduction in blood pressure upon VEGF administration, vessel permeabilization, migration of endothelial cells, and others (4). The receptor-selective variants described here serve as valuable reagents for determining the relative importance of KDR and Flt-1 for the many biological effects triggered by VEGF. Furthermore, given the limited number of changes from wild-type VEGF, these variants could be important tools for the design of therapeutic agents with fewer side effects than the native protein.

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REFERENCES

1. Risau, W. (1997) Nature 386, 671–674
2. Ferrara, N. (1999) Kidney Int. 56, 794–814
3. Folkman, J. (1995) Nat. Med. 1, 27–31
4. Ferrara, N., and Davis-Smyth, T. (1997) Endo. Rev. 18, 4–25
5. Bauters, C. (1997) Clin. Cardiol. 20, 52–57
6. Ferrara, N., and Alitalo, K. (1999) Nat. Med. 5, 1359–1364
7. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. (1993) Nature 362, 841–844
8. Frank, N. (1997) Ophthalmo. Res. 29, 341–353
9. Fong, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) Nature 376, 66–70
10. Shiota, M., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995) Nature 376, 62–66
11. Meyer, M., Claus, M., Leppe-Wienhues, A., Wenken, J., Augustin, H. G., Ziche, M., Lanz, C., Buttin, M., Rizita, H. J., and Dehio, C. (1999) EMBO J. 18, 363–374
12. Ogawa, S., Oku, A., Sawano, A., Yamaguchi, S., Yazaki, Y., and Shibuya, M. (1998) J. Biol. Chem. 273, 31273–31282
13. Wise, L. M., Veikko, T., Mecer, A. A., Savory, L. J., Fleming, S. B., Caesar, C., Vitali, A., Makinen, T., Alitalo, K., and Stacker, S. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3071–3076
14. Keyt, B. A., Nguyen, H. V., Berleau, L. T., Duarte, C. M., Park, J., Chen, H., and Ferrara, N. (1999) J. Biol. Chem. 274, 25646–25654
15. Sawano, A., Takahashi, T., Yamaguchi, S., Asunuma, M., and Shibuya, M. (1996) Cell Growth Differ. 7, 213–221
16. Landgren, E., Schiller, P., Cao, Y., and Claesson-Welsh, L. (1998) Oncogene 16, 2569–2577
17. Gerber, H. P., Condorelli, F., Park, J., and Ferrara, N. (1997) J. Biol. Chem. 272, 23659–23667
18. Ogawa, S., Korpelainen, E., Pepper, M. S., Mandriota, S. J., Aase, K., Kumar, V., Gunji, Y., Jelsch, M. M., Shibuya, M., Alitalo, K., and Eriksson, U. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11709–11714
19. Parry, T. J., Cushman, C., Gallegos, A. M., Agrawal, A. B., Richardson, M., and Anderson, L. E., Monley, L. R., Mokler, V. R., Winec, F. E., and Pavco, P. A. (1999) Nucleic Acids Res. 27, 2569–2577
20. Keyt, B. A., Nguyen, H. V., Berleau, L. T., Duarte, C. M., Park, J., Chen, H., and Ferrara, N. (1996) J. Biol. Chem. 271, 5638–5646
21. Shen, B.-Q., Lee, D., Gerber, H. P., Keyt, B.A., Ferrara, N., and Zongcheck, T. F. (1998) J. Biol. Chem. 273, 29979–29985
22. Stacker, S. A., Vitali, A., Caesar, C., Domagala, T., Groesen, L. C., Nix, E., Achen, M. G., and Wilks, A. F. (1999) J. Biol. Chem. 274, 34884–34892
23. Muller, Y. A., Li, B., Christinger, H. W., Wells, A. J., and de Vos, A. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7192–7197
24. Muller, Y. A., Christinger, H. W., Keyt, B. A., and de Vos, A. M. (1997) Structure 5, 1325–1338
25. Wiesmann, C., Fuh, G., Christinger, H. W., Eigenbrot, C., Wells, A. J., and de Vos, A. M. (1997) Cell 89, 695–704
26. Fuh, G., Li, B., Crowley, C., Cunningham, B., and Wells, J. A. (1998) J. Biol. Chem. 273, 11197–11204
27. Somers, W., Ultsch, M. A., and de Vos, C. M. A. F. (1994) Nature 372, 678–681
28. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
29. Shibahara, S. B., Lowman, H. B., Cunningham, B. C., and Wells, J. A. (2000) Methods Enzymol. 328, 333–363
30. Cao, Y., Chen, H., Zhou, L., Chiang, M. K., Anand-Apte, B., Weatherbee, J. A., Wang, Y., Fang, P., Flanagan, J. G., and Tsang, M. L. (1996) J. Biol. Chem. 271, 3154–3162
31. Wells, J. A. (1991) Methods Enzymol. 202, 390–411
32. Keyt, B. A., Berleau, L. T., Nguyen, H. V., Chen, H., Heinesh, H., Vanden, R., and Ferrara, N. (1996) J. Biol. Chem. 271, 7788–7795
33. Gallop, M. A., Berrett, R. W., Dower, W. J., Fodor, S. P. A., and Gordon, E. M. (1994) J. Med. Chem. 37, 1233–1251
34. Wang, H., and Keiser, J. A. (1996) Circ. Res. 83, 832–840
35. de Vos, A. M., Ultsch, M. H., and Kossiaff, A. A. (1992) Science 255, 306–312
36. Kossiaff, A. A., Somers, W., Ultsch, M., Andow, K., Muller, Y. A., and de Vos, A. M. (1994) Proteins Sci. 3, 1697–1705