Vascular endothelial growth factor (VEGF) is a dimeric hormone that controls much of vascular development through binding and activation of its kinase domain receptor (KDR). We produced analogs of VEGF that show it has two receptor-binding sites which are located near the poles of the dimer and straddle the interface between subunits. Deletion experiments in KDR indicate that of the seven IgG-like domains in the extracellular domain, only domains 2–3 are needed for tight binding of VEGF. Monomeric forms of the extracellular domain of KDR bind ~100 times weaker than dimeric forms showing a strong avidity component for binding of VEGF to predimerized forms of the receptor. Based upon these structure-function studies and a mechanism in which receptor dimerization is critical for signaling, we constructed a receptor antagonist in the form of a heterodimer of VEGF that contained one functional and one non-functional site. These studies establish a functional foundation for the design of VEGF analogs, mimics, and antagonists.

Formation of the vasculature is one of the most intriguing and physiologically important processes in human biology. Induction of new blood vessels is clinically relevant following stroke or heart attack, whereas inhibition of vascularization is likely to curtail the growth of tumors or retinopathy disease (1, 2). Vascular endothelial growth factor (VEGF) regulates vascularization by acting as an endothelial cell mitogen and a vascular permeability factor (3). VEGF can bind to two different single-transmembrane receptors, kinase domain receptor (KDR) and fms-like tyrosine kinase-1 (Flt-1). Gene disruption experiments in mice show that both receptors are necessary for vascularization by acting as an endothelial cell mitogen and a dimeric hormone that controls much of vascular development. In-
binding buffer containing 0.5% bovine serum albumin, 0.05% Tween 20, 0.15 NaCl, and 20 mM Tris-HCl (pH 7.5). The mixture was transferred to a 96-well plate coated with anti-Fc antibody for KDR-IgG assays or MAKDS for KDR monomer assays and allowed 15 min to capture the complex. The plate was then washed and counted in Topcount Microplate Scintillation counter (Packard, Downers Grove, IL). Biotinylated VEGF<sub>1–109</sub> was used as tracer for some assays and horseradish peroxidase-conjugated strepavidin was added at the end. The concentration of receptor and <sup>125</sup>I-VEGF<sub>1–109</sub> or biotinylated VEGF<sub>1–109</sub> were adjusted so that they were at least a factor of four below the estimated K<sub>d</sub>. For mAb binding assays, KDR-IgG was captured on the rabbit anti-human Fc antibody Fab (Jackson ImmunoResearch Laboratory Inc., West Grove, PA) coated 96-well plate. Serial dilutions of mAbs were put in the plate and allowed to bind for 2 h and the plates washed thoroughly with incubation buffer. Horseradish peroxidase-conjugated rabbit anti-mouse Fab antibody was added which had been preabsorbed with human Fc.

**Purification and Refolding of the VEGF Variants**—The purification and refolding of VEGF<sub>1–109</sub> and VEGF<sub>8–109</sub> was performed as described in Muller et al. (9). The refolding of the VEGF<sub>1–109</sub> heterodimer was performed essentially as described by Potgens and co-workers (14). VEGF with the C51S/I46A/I83A mutations and VEGF<sub>1–109</sub> with C60S/F17A/E64A mutations were purified separately from *E. coli* by native gel filtration and SDS-PAGE under nonreducing conditions (data not shown). The fact that the monomer only 2-fold weaker than VEGF<sub>8–109</sub> (Fig. 2B) and was largely intact; mutational studies show this epitope is discontinuous and therefore requires proper folding of VEGF for binding to occur. The monomer was unable to compete with wild type VEGF<sub>1–109</sub> (<k<sub>d</sub> = 5 nM) for binding to KDR at concentrations approaching 1 μM (Fig. 2B). However, at much higher concentrations, monomeric VEGF could displace wild-type VEGF with an approximate IC<sub>50</sub> of ~20 μM (Table II). Thus, although the monomer appears to be properly folded its binding affinity for KDR is reduced ~1000-fold.

Variants of VEGF containing a single intermolecular disulfide bond were produced using a strategy reported by Potgens and co-workers (14). A C51S mutation was introduced into one subunit, and a C60S variant was produced in the other. C51S and C60S mutants were purified separately from *E. coli* and refolded together (see “Experimental Procedures”) and a heterodimer of VEGF containing two functional receptor binding sites (hV-2) was generated. The hV-2 heterodimer bound to KDR with virtually the same affinity as the wild-type dimer (Fig. 2B) and was fully active in cell-based assays (data not shown). Thus, the heterodimer containing only one intersubunit disulfide bonds is functional and properly folded. The individual cysteine mutants taken through the same refolding procedure do not bind KDR (up to 10 μM in concentration (data not shown), presumably because they cannot form a proper homodimer.

Next we produced single binding site heterodimers (hV-1) in which one pole on VEGF<sub>1–109</sub> was mutated at residues shown by alanine scanning to be most important for binding KDR (Fig. 1). One subunit contained the C51S mutation plus I46A and I83A, and the other subunit had C60S along with F17A and E64A. The hV-1 heterodimer was generated as hV-2 and confirmed by mass spectrometry. The hV-1 bound to the KDR monomer only 2-fold weaker than VEGF<sub>1–109</sub> (Fig. 2B), indicating that it can still bind the receptor with one pole intact. These data combined with the fact that monomeric VEGF binds much weaker to KDR strongly support a model where each KDR binds across the subunit-subunit interface and not exclusively to one of the subunits (Fig. 2A).

---

*Fig. 1. Epitopes for binding of KDR to the VEGF dimer. A space-filling rendition of the two VEGF subunits are shown in white and light gray (left). All residues that were alanine-scanned are colored: light blue (<1.0 kcal/mol impact on binding free energy), blue (1.0–2.0 kcal/mol), yellow (2.0–3.0 kcal/mol), and red (>3.0 kcal/mol). An end-on view generated by rotating the molecule up by 90° (right). The figure is reproduced with permission (9).*
amounts of biotinylated VEGF (1 nM) to KDR (0.5 nM) and incubated for respectively. Dilutions of these VEGF variants were added with fixed disulfide bonds in VEGF are indicated.

The inter-subunit and intra-subunit models for binding two molecules of KDR receptor to dimeric VEGF. The inter-subunit disulfide bonds in VEGF are indicated. Panel B, displacement of biotinylated VEGF_{1-109} from binding to monomeric KDR 1–7 by a monomeric form of VEGF_{1-109} containing the double mutant C51R/C60R, a heterodimeric form of VEGF_{1-109} that possesses either both binding sites (hV-2), or a single site at one pole of the hormone (hV-1) and the wild-type VEGF_{1-109} with IC_{50} values ~500, 10, 8.9, and 4.6 nm, respectively. Dilutions of these VEGF variants were added with fixed amounts of biotinylated VEGF (1 nm) to KDR (0.5 nm) and incubated for 18 h. The complex was captured with a mAb to KDR (MAKD9) as described under "Experimental Procedures."

The IgG-like Domains 2–3 in KDR Are Sufficient for High Affinity Binding—To determine the minimal IgG-like domain requirements for binding of KDR to VEGF_{1-109}, a series of deletions were produced in which each of the seven IgG-like domains were deleted from the carboxyl terminus of the extracellular domain. The deletion variants were expressed initially as dimeric proteins by fusion to the CH2-CH3 domain of an antibody (KDR-IgG). This was done to facilitate purification on a Protein A affinity column (12) and to compare their affinities to monomeric forms of KDR.

The choice of deletion junction was based on homology to other members of the IgG superfamily (16, 17). Systematic carboxyl-terminal domain deletions had virtually no effect on affinity for VEGF until IgG-like domain 3 was deleted (Table I); KDR 1–2 had an affinity that was >1000-fold reduced relative to KDR 1–3 but did show specific binding at concentrations above 2 μM (data not shown). A variant of KDR missing the first NH₂-terminal domain, KDR 2–3, bound nearly as well as the full-length KDR (Table I). These data suggest that domains 2–3 are most important for high affinity binding.

To determine if these deletions had caused misfolding of the molecules, we analyzed their binding to three different anti-KDR monoclonal antibodies (Table I), one of which (MAKD6) blocks binding of VEGF. The antibodies bound to nearly all the deletion variants with affinities virtually identical to the full-length KDR 1–7 (EC_{50} ~1 nM). Deletion of domain 1 caused complete disruption for binding of the non-neutralizing antibodies (MAKD1 and MAKD5) but not the neutralizing antibody (MAKD6). Thus, the deletions do not grossly disrupt the structure of the molecules and locate the epitopes for MAKD1 and MAKD5 to domain 1 and for MAKD6 to domain 2. The fact that the antibody MAKD6, which blocks binding of VEGF, binds to domain 2 further supports the importance of domain 2 for binding VEGF.

To facilitate preparation of monomeric forms of KDR, a Genenase 1 protease cleavage site (18) was engineered at the junction of the last KDR IgG domain and the CH2 domain (19). The cleaved KDR was shown to be monomeric based on its mobility in nonreducing SDS-PAGE and gel filtration. Both the KDR 1–7 and KDR 1–3 monomers bound all three mAbs and equally well to VEGF (Table I). These results show the first three IgG-like domains are sufficient for binding of VEGF whether in monomeric or dimeric forms.

One VEGF Dimer Binds Two Molecules of the Extracellular Domain of KDR—To determine the stoichiometry of binding of VEGF_{1-109} to the extracellular domain of KDR, we systematically varied the ratio of VEGF to KDR and determined the apparent size of the complexes by gel filtration. The glycosylated monomeric KDR 1–7 migrated as a single peak by gel filtration chromatography with an apparent molecular mass of ~250 kDa (Fig. 3A). By comparison, the dimeric KDR 1–7-IgG migrated as a 600-kDa peak (data not shown). Upon addition of one equivalent of VEGF (dimer) per two equivalents of KDR (monomer), a single complex peak was formed of apparent molecular mass ~400 kDa. A minor shoulder was seen that might represent the slight excess KDR monomer in the mixture. The fact that the 2:1 complex is smaller by gel filtration than expected from the sum of the component molecular masses (520 kDa) may be that VEGF aligns the receptor subunits in a more compact fashion.

Further additions of 2 and 3 equivalents of VEGF did not change the position of the high molecular weight peak, and excess VEGF accumulated as the free dimeric hormone (Fig. 3A). The height of the free VEGF peak was small because VEGF contains no tryptophan residues and therefore has a small molar absorbance at 280 nm. When the ratio of KDR to VEGF exceeded 2:1, free KDR 1–7 accumulated as a shoulder. The hV-1 heterodimeric variant of VEGF forms a 1:1 complex with monomeric KDR. This complex migrated at a position that was intermediate between the free KDR 1–7 and the 2:1

![FIG. 2](image)

Panel A, inter-subunit and intra-subunit models for binding two molecules of KDR receptor to dimeric VEGF. The inter-subunit disulfide bonds in VEGF are indicated. Panel B, displacement of biotinylated VEGF_{1-109} from binding to monomeric KDR 1–7 by a monomeric form of VEGF_{1-109} containing the double mutant C51R/C60R, a heterodimeric form of VEGF_{1-109} that possesses either both binding sites (hV-2), or a single site at one pole of the hormone (hV-1) and the wild-type VEGF_{1-109} with IC_{50} values ~500, 10, 8.9, and 4.6 nm, respectively. Dilutions of these VEGF variants were added with fixed amounts of biotinylated VEGF (1 nm) to KDR (0.5 nm) and incubated for 18 h. The complex was captured with a mAb to KDR (MAKD9) as described under "Experimental Procedures."
KDR-VEGF complex. When the ratio of the hV-1 to KDR 1–7 exceeded unity, the free heterodimer accumulated in the chromatogram.

Parallel experiments were carried out with the monomeric form of KDR 1–3 (Fig. 3B). When no VEGF was present, KDR 1–3 migrated as a single peak of apparent molecular mass of ~70 kDa. Addition of 1 equivalent of VEGF dimer to 2 equivalent of KDR 1–3 resulted in forming a peak with apparent molecular mass of ~160 kDa. Addition of 2 and 3 equivalents of VEGF did not change the position of the complex peak, but free VEGF accumulated. Increasing additions of KDR 1–3 in excess of the 2:1 ratio to VEGF dimer showed increasing appearance of free KDR 1–3. A similar set of experiments with the hV-1 showed it maximally formed a 1:1 complex (Fig. 3B); when the ratio of either the variant or KDR 1–3 was skewed from unity, the free excess component accumulated. These experiments explicitly show that two molecules of KDR bind to one VEGF dimer, and that form of KDR lacking IgG-like domains 4–7 are capable of producing the 2:1 complex in solution. When VEGF is engineered to have only one functional binding site (hV-1) it cannot dimerize the receptor in vitro.

VEGF Binds Avidly to Dimeric versus Monomeric Forms of KDR—Given the fact that the VEGF dimer binds two molecules of receptor we wished to determine to what extent predimerization of the receptor influenced affinity. This can be readily seen by comparing the binding constants for the monomeric and dimeric forms of KDR (Table I). KDR-IgG fusions containing domains 1–7 or 1–3 bound 50–100-fold stronger

---

**Fig. 3.** Panel A, gel filtration chromatography of various ratios of KDR 1–7 monomer and VEGF dimer (upper six chromatograms) or VEGF heterodimer (hV-1) containing a single functional binding site (lower two chromatograms). The concentration of KDR 1–7 monomer was 1 μM except at ratios of 3:1 and 4:1, where the concentration of KDR 1–7 was 1.5 and 2 μM, respectively. The quantitation of protein was determined by amino acids hydrolysis and absorbance at 280 nm. Panel B, gel filtration chromatography of various ratios of KDR 1–3 monomer and VEGF dimer (six chromatograms on the left) or hV-1 (five chromatograms on the right). The concentration of KDR 1–3 monomer was held constant at 1 μM except at ratios of 3:1 and 4:1, where KDR 1–3 monomer was 1.5 and 2 μM, respectively.
than their monomeric counterparts. The affinity of the single-site heterodimeric VEGF for binding to the dimeric KDR IgG fusion was about 200-fold weaker than wild-type VEGF (Fig. 4). In contrast, binding to monomeric KDR for the heterodimer was only 2-fold weaker than native VEGF (Fig. 2B). These data, summarized in Table II, show that binding of dimeric VEGF to predimerized KDR is ~100-fold stronger than when either the hormone or receptor contains a single binding site.

VEGF Binds Virtually the Same Way to Monomeric and Dimeric KDR—Given the strong avidity component to binding of VEGF to its receptor, we wished to determine if VEGF binds the same way to monomeric and dimeric forms of KDR 1–7. We have previously reported the alanine scan of VEGF for binding to KDR-IgG (9). Here we analyze the binding of these same alanine mutants to monomeric KDR (Table III). The data show that the same set of alanine mutants that are most disruptive for binding to KDR-IgG are also strongly disruptive to binding monomeric KDR.

There are some subtle and systematic differences in the way the alanine mutants bind the monomeric versus dimeric KDR. For example M18A, I43A, I46A, E64A, and I83A were more disruptive to affinity (by factors ranging from 2–8-fold) when tested against the monomeric versus dimeric KDR. Only F17A was more disruptive to the dimer than the monomer (by ~2-fold). The biased suppression of the disruptive effects of the alanine mutations when binding to the dimer is likely caused from avidity in binding. We conclude there are no gross differences in the way monomeric and dimeric forms of KDR bind to VEGF.

KDR Domains 1–3 Are Sufficient for Signaling in Cells—NIH 3T3 fibroblast cells that contain the extracellular domain of colony-stimulating factor receptor fused to the transmembrane and intracellular domain of the Flt-4 receptor incorporate [3H]thymidine and proliferate when treated with the colony-stimulating factor receptor fused to the transmembrane and intracellular kinase domain of Flt-4. At low concentrations of VEGF this cell line incorporated [3H]thymidine with an EC50 of ~100 pm (Fig. 5A); high concentrations of VEGF (>1 μM) showed inhibition. Such a bell-shaped dose-response curve is anticipated for a two-site hormone dimerizing two identical receptors (21). The hV-1 was inactive (Fig. 5A).

A similar construct was produced in which only domains 1–3 of KDR were linked to Flt-4. These cells also incorporated [3H]thymidine in response to VEGF (Fig. 5B) but did so with a higher EC50 (~10 nm) and lower maximal response. We did not go to high enough concentrations to see inhibition by VEGF. The hV-1 was virtually inactive. Primary human umbilical vein endothelial cells (HuVEC) showed a bell-shaped dose-response curve (Fig. 5C). We resist making quantitative comparisons between HuVEC and KDR expressed NIH 3T3 cells given the fact that the HuVEC contain both KDR and Flt-1 receptors (22).

The difference in EC50 values and maximal response for the KDR 1–7 and KDR 1–3 cell lines likely resulted from the fact that the number of functional receptors on the KDR 1–3 cell line was at least 10-fold lower based upon binding of 125I-VEGF (data not shown). To explore the effect of receptor number on signaling directly we isolated three different clones of cells that varied over a range of 12-fold in the amount of the KDR 1–7 that specifically bound 125I-VEGF (Fig. 6A). The maximal levels of [3H]thymidine incorporation correlated with the number of receptors expressed on these cells and the EC50 values correlated inversely with the number of receptors (Fig. 6B). It is interesting that the basal levels of [3H]thymidine incorporation correlated with the receptor number as well, suggesting that receptors can preassociate and signal weakly in the absence of exogenous VEGF. All of the transfecants containing the KDR 1–3 construct expressed much lower levels of receptors which may suggest that domains 4–7 are important for high level expression and display of the receptor.

Antagonism of VEGF Receptors by the Single-site Heterodimer of VEGF—Given the ability of hV-1 to bind but not dimerize and activate KDR (Fig. 5), we studied its ability to antagonize signaling of KDR. Indeed the heterodimer antagonizes [3H]thymidine incorporation in the 3T3 cells transfected with the chimeric KDR (1–7)-Flt-4 receptor and HuVEC with an IC50 of ~300 and ~20 nm, respectively (Fig. 7). The fact that the heterodimer is less effective on the 3T3-transfected cells versus HuVEC likely reflects the fact that the former expresses much higher levels of receptors (Fig. 6A).
DISCUSSION

KDR Binds Across the VEGF Dimer Interface—The data here combined with previous mutational analysis (9) suggest that binding occurs across the VEGF dimer interface (Fig. 2A). It may be a general feature of the cystine-knot hormones that the receptor binds at the interface between hormone subunits. A structure of domain 2 of the Flt-1 receptor bound to VEGF shows it binds across the dimer interface (23). A heterodimer containing one molecule of VEGF linked to its homolog, PLGF, is only 20–50-fold reduced as a mitogen on HuVEC cells (EC50; 50 nM) whereas the PLGF homodimers are inactive (24). The fact the VEGF/PLGF heterodimer shows any activity can be rationalized from our mutational analysis (Table III). Some of the critical binding determinants (Phe-17, Glu-64, Gln-79, and Ile-83) are conserved in PLGF and others are reasonably conservative substitutions (M18Q, I43V, Y45H and I46M). These later substitutions would likely have a much more dramatic effect when present in both subunits, thus accounting for the absence of significant mitogenic activity for the PLGF homodimer up to the concentrations that were tested (<1 μM). Similar observations have been made for homodimers and heterodimers of PDGF (isoforms AA, AB, and BB) for binding the PDGF-α and -β receptors (25). Mutational analysis of nerve growth factor, another member of the cystine-knot family of dimeric hormones, shows a broad patch of residues involved in binding receptor that spans the interface between subunits (26).

Requirements and Consequences for Receptor Dimerization—Hormone-induced receptor dimerization is a general mechanism for activation of tyrosine kinase receptors (27). All receptors that bind cystine-knot hormone dimers are presumed to be activated by receptor dimerization (6). Here, gel filtration experiments provide in vitro evidence that VEGF binds two molecules of the extracellular domain of KDR. The dimerized complex appears to be very stable since excess VEGF is unable to dissociate the complex to 1:1 complex. This dimerization reaction is critical for signaling because the VEGF heterodimer, hV-1, with only one functional site is inactive in cell-based assays and antagonizes the action of wild-type VEGF. Receptor dimerization is also supported by the observation that cell-based assays show a bell-shaped dose-response curve with respect to VEGF. PDGF isoforms have been shown to induce...
dimerization of the extracellular domains of the PDGF-α and -β receptors in vitro (28). Binding of the dimeric hormone, SCF, to the extracellular domain of the Kit receptor, a tyrosine kinase receptor of the IgG class, causes dimerization in vitro (29–31), and induces a bell-shaped dose-response curve in vivo (30).

Predimerized forms of KDR bind VEGF 100-fold more tightly than monomeric forms of KDR showing a strong avidity component in binding. Dimeric receptor fusion proteins, such as IgG fusions, or receptors bound to monoclonal antibodies are often used as convenient assay reagents for hormones and their variants. The data presented here show that there is a significant avidity component to binding in these fusions that affects the affinity constants. The avidity effect observed here is not the result of an alternate way that VEGF binds the dimeric KDR because alanine mutations in VEGF that are most disruptive to binding monomeric KDR are also the ones that most affect binding to dimeric KDR (Table III).

We observed that wild-type VEGF1–109 binds about 100-fold more tightly than the single-site heterodimer to cells expressing KDR 1–7 (not shown). This suggests that receptors on cells may be loosely associated. Moreover, NIH 3T3 cells expressing larger numbers of VEGF receptors showed a higher basal level of [3H]thymidine incorporation in the absence of VEGF (Fig. 6B), suggesting that receptors on cells have an intrinsic ability to dimerize in the absence of ligand. Similar observations have been made for cells overexpressing various tyrosine kinase receptors, such as variants of the EGF receptor (27). Overexpression of the PDGF receptors can induce receptor autophosphorylation in the absence of ligand, and it is even possible to cross-link small amounts of the extracellular domains in the absence of PDGF (28). The fact we did not see evidence for dimerization by gel filtration of the ecodomains of KDR 1–7 or 1–3 in the absence of VEGF may only reflect the sensitivity of the method and that the receptors have a much higher effective concentration on cells than in our solution experiments (∼1 μM).

Deletion experiments showed that domains 2–3 of KDR are sufficient and necessary for high affinity binding of VEGF (Table I). Cells can signal when transfected with KDR domain 1–3 linked to Flt-4, even with low receptor expression, suggesting that domains 4–7 are not essential for signaling. We cannot rule out other roles for these domains; they may stabilize the signal transduction complex and/or provide for better display and expression of the receptor. Systematic deletion experiments have been conducted on at least four other tyrosine kinase receptors of the IgG class, and generally show that binding is dominated by IgG-like domains 2–3. Deletion experiments showed the first three of the five IgG-like domains in the Kit receptor are required for binding of SCF, but there is uncertainty regarding the role of domain 4 in signaling (30, 31). An antibody directed toward domain 4 blocked signaling in cells transfected with Kit, and deletion of domain 4 blocked signaling but not stem cell factor binding (30). In contrast, biophysical experiments (31) showed that Kit 1–3 can dimerize in solution with stem cell factor and both the enthalpy and free energy of binding were indistinguishable from Kit 1–5. In either case, both groups agree that the ligand-binding site for stem cell factor is contained in the first three IgG-like domains.

Deletion analysis of PDGF-α receptor, which contains five IgG-like domains, has shown that domains 2–3 are sufficient for binding PDGF isoforms although the presence of domain 1 has a small differential effect on binding PDGF-AA versus PDGF-BB (32). Deletion analysis of the fibroblast growth factor receptor, which contains three IgG-like domains, showed that domains 2–3 are sufficient for high affinity binding of fibroblast growth factor (33). Deletion experiments in Flt-1, which like KDR contains seven IgG-like domains, have shown that the VEGF-binding site is located among the first three IgG-like domains (34–36) and domain 2 of Flt-1 alone can bind VEGF tightly (29). Thus, domain 2 may play a dominant role in all five of these tyrosine kinase receptors that have IgG-like domains and may be general to the other members of this class.

Mechanism-based Antagonists of VEGF—Antagonists to VEGF may be very useful in preventing tumor angiogenesis and retinopathy diseases. Here, we have elucidated the functional requirements for receptor binding and activation and designed an antagonist, hV-1, for the proliferation of HuVEC cells based on this knowledge. The fact the IC50 of hV-1 for inhibiting VEGF in HuVEC (∼20 nM) is ∼100-fold higher than the EC50 of VEGF stimulating growth (∼0.2 nM) likely reflects the avidity effect described above. We believe the hV-1 antagonize VEGF stimulation of HuVEC by blocking the dimerization of KDR since KDR is more important for signaling mitogenesis. However, the hV-1 does bind Flt-1 with near wild-type affinity and we are currently looking at its ability to activate Flt-1. Alkaline scanning of both receptor-binding sites on VEGF suggests that the binding sites for KDR and Flt-1 overlap and are not identical (9, 23, 37). Based on these results it should also be possible to design receptor specific antagonists and further elucidate the functions of the two receptors. Overall, these studies provide a basis from which we can produce new analogs of VEGF to both probe its biology and generate new and potent therapeutics.

Acknowledgments—We thank Toni Klassen and Jin Kim for mAbs to KDR, Hans Christinger for providing purified VEGF, Napoleone Ferrara for providing the plasmid pHEBO23-KDR-IgG and pHEBO23-Flt-1-IgG, Jennifer Singh, Richard DeMarco, Michael Clasen for technical support, the DNA Synthesis Group at Genentech for oligonucleotides, and David Wood for the graphics.
REFERENCES

1. Aiello, L. P., Avery, R. L., Arrigg, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. R., Thieme, H., Iwamoto, M. A., and Park, J. E. (1994) *N. Engl. J. Med.* 331, 1489–1497
2. Folkman, J. (1995) *Nat. Med.* 1, 27–31
3. Ferrara, N., Houck, K., Jakeman, L., and Leung, D. W. (1992) *Endocr. Rev.* 13, 18–32
4. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M. L., and Schuh, A. G. (1995) *Nature* 376, 62–66
5. Fong, G.-H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) *Nature* 376, 66–74
6. Sun, P. D., and Davies, D. R. (1995) *Annu. Rev. Biophys. Biomol. Struct.* 24, 269–291
7. Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V., and Ferrara, N. (1989) *Science* 246, 1306–1309
8. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991) *Mol. Endocrinol.* 5, 1806–1814
9. Muller, Y. A., Li, B., Christinger, H. W., Wells, J. A., Cunningham, B. C., and De Vos, A. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 7192–7197
10. Park, J. E., Chen, H. H., Winer, J., Houck, K. A., and Ferrara, N. (1994) *J. Biol. Chem.* 269, 25646–25654
11. Kunkle, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 488–492
12. Chamow, S. M., and Ashkenazi, A. (1996) *Trends Biotechnol.* 14, 52–60
13. Lucas, B. K., Giere, L. M., DeMarco, R. A., Shen, A., Chisholm, V., and Crowley, C. W. (1996) *Nucleic Acids Res.* 24, 1774–1779
14. Potgens, A. J., Lubsen, N. H., van Altena, M. C., Vermeulen, R., Bakker, A., Schoenmakers, J. G. G., Ruiter, D. J., and de Waal, R. M. W. (1994) *J. Biol. Chem.* 269, 32879–32885
15. Graham, F. L., and Van der Eb, A. J. (1973) *Virology* 52, 456–467
16. Williams, A. F., and Barclay, A. N. (1988) *Annu. Rev. Immunol.* 6, 381–405
17. Finnerty, H., Kelleher, K., Morris, G. E., Bean, K., Merberg, D. M., Kri, R., Morris, J. C., Stooked, H., Turner, K. J., and Wood, C. R. (1993) *Oncogene* 8, 2293–2298
18. Carter, P., and Wells, J. A. (1987) *Science* 234, 394–399
19. Blechman, J. M., Lev, S., Younger, L., Van Der Eb, A. J., and De Vos, A. M. (1997) *Cell* 91, 695–704
20. Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L., and Alitalo, R. (1994) *Oncogene* 9, 3545–3555
21. Fuh, G. Cunningham, B. C., Fukumura, R., Nagata, S., Goeddel, D. V., and Wells, J. A. (1992) *Science* 256, 1677–1680
22. Soker, S., Fidder, H., Neufeld, G., and Klagesbrun, M. (1996) *J. Biol. Chem.* 271, 5761–5767
23. Weismann, C., Fuh, G., Christinger, H. W., Eikgenhort, C., Wells, J. A., and De Vos, A. M. (1997) *Cell* 91, 695–704
24. Cao, Y., Chen, H., Zhou, L., Chiang, M.-K., Anand-Apte, B. Weatherbee, J. A., Wang, Y., Fang, F., Flanagan, J. G., and Tsang, M. L.-S. (1996) *J. Biol. Chem.* 271, 3154–3162
25. Heldin, C. H. (1995) *Cell* 80, 213–223
26. Bradshaw, R. A., Murray-Rust, J., Ibanez, C. F., McDonald, N. Q., Lapatte, R., and Blundell, T. L. (1994) *Protein Sci.* 3, 961–913
27. Ullrich, A., and Schlessinger, J. (1990) *Cell* 61, 203–212
28. Herren, B., Rooney, B., Weyer, K. A., Berg, N., Schmidt, G., and Pech, M. (1993) *J. Biol. Chem.* 268, 15088–15095
29. Lev, S., Yarden, Y., and Givol, D. (1992) *J. Biol. Chem.* 267, 15970–15977
30. Blechman, J. M., Lev, S., Berg, J., Eisenstein, M., Vales, B., Vogel, Z., Givol, D., and Yarden, Y. (1995) *Cell* 80, 103–113
31. Lemmon, M. A., Pinchasi, D., Zhou, M., Lax, I., and Schlessinger, J. (1997) *J. Biol. Chem.* 272, 6311–6317
32. Mahadevan, D., Yu, J.-C., Saldanha, J. W., Thanki, N., McPhie, P., Uren, A., LaRochelle, W. J., and Heidaran, M. A. (1995) *J. Biol. Chem.* 270, 27595–27600
33. Wang, F., Kan, M., Xu, J., Yan, G., and McKeehan, W. L. (1995) *J. Biol. Chem.* 270, 27595–27600
34. Davis-Smyth, T., Chen, H., Park, J., Presta, L. G., and Ferrara, N. (1996) *EMBO J.* 15, 4919–4927
35. Cunningham, S. A., Stephan, C. C., Arrate, M. P., Ayer, K. G., and Brock, T. A. (1997) *Biochem. Biophys. Res. Commun.* 231, 596–600
36. Barleon, B., Totzke, F., Herzog, C., Blanke, S., Kremmer, E., Siemeister, G., Marne, D., and Martiny-Baron, G. (1997) *J. Biol. Chem.* 272, 10382–10388
37. 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