Single-chain Vascular Endothelial Growth Factor Variant with Antagonist Activity*

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Vascular endothelial growth factor is a specific endothelial cell mitogen that is essential for the formation of the vascular system but in the adult individual is involved in several pathologic conditions, including cancer. It is a homodimeric protein that activates its receptor by binding two receptor molecules and inducing dimerization. By mixing two vascular endothelial growth factor monomers, each with different substitutions, heterodimers with only one active receptor binding site have previously been prepared. These heterodimers bind the receptor molecule but are unable to induce dimerization and activation. However, preparation of heterodimers is cumbersome, involving separate expression of different monomers, refolding the mixture, and separating heterodimers from homodimers. Here we show that a fully functional ligand can efficiently be expressed as a single protein chain containing two monomers. Single-chain vascular endothelial growth factor is functionally equivalent to the wild-type protein. By monomer-specific mutagenesis, one receptor binding site was altered. This variant competitively and specifically antagonizes the mitogenic effect of the wild-type protein on endothelial cells. The results obtained with the single-chain antagonist show the feasibility of the single-chain approach in directing alterations to single specific regions in natural homodimeric proteins that would be impossible to target in other ways.

Angiogenesis, the sprouting of new blood vessels from existing ones, is essential for the development of an organism. This is clearly demonstrated by the early embryonic death of transgenic animals in which angiogenesis is not regulated correctly (1–5). However, beneficial angiogenesis is a rare event in the adult individual, occurring only under special circumstances such as corpus luteum formation and wound healing (6, 7). In contrast, several pathologies are caused or promoted by excessive angiogenesis, including tumor growth, rheumatoid arthritis, diabetic retinopathy, and age-related macular degeneration (7). Therefore, angiogenesis has become a popular target for drug development, and several antiangiogenic compounds based on very different mechanisms of action are being developed (8).

Angiogenesis is regulated by a large number of both pro- and antiangiogenic cytokines and growth factors. One of these, vascular endothelial growth factor (VEGF), is highly specific for endothelial cells, and it has been shown to be one of the key inducers of angiogenesis (9). Many types of tumors produce VEGF to create the vascular network necessary to support tumor growth, and suppression of VEGF activity has been found to suppress tumor growth in animal models (7).

VEGF is a dimeric protein that exists in several isoforms. The core domain of VEGF that is involved in receptor binding and activation is ~110 amino acid residues/monomer (10). Two other domains of 24 and 44 amino acid residues are involved in binding of VEGF to the extracellular matrix and to cell surface heparin (11, 12). Differential RNA splicing leads to the formation of the isoforms VEGF121 (consisting of only the core domain) and VEGF165 (consisting of the core domain and the C-terminal heparin binding domain) (12). Less common splice variants are VEGF145 and VEGF189 that contain the 24-amino acid residue extracellular matrix binding domain located C-terminal to the core domain but N-terminal to the heparin binding domain (11, 12). Plasmin cleavage of VEGF leads to formation of VEGF114 which contains only the core domain (10). VEGF165 has been found to be a more potent inducer of angiogenesis than VEGF121 possibly because binding of the molecule to cell surface heparin presents the molecule to the receptor in a favorable way (10).

VEGF binds to and activates two tyrosine kinase receptors, kinase insert domain receptor (KDR) and fms-like tyrosine kinase receptor 1 (13). Most of the functions of VEGF are found to be mediated by KDR (14–16), and fms-like tyrosine kinase receptor 1 functions mainly as a decoy receptor, suppressing VEGF activity by capturing VEGF and thereby making it unavailable to KDR (15, 17–19). Activation of the VEGF receptors requires dimerization through binding of one receptor molecule to each of the two receptor binding sites on the VEGF dimer, located at the opposite ends of the VEGF dimer at the interface between the monomers (13).

A number of reports describe investigation of VEGF by mutational analysis (10, 14, 20–24), and a few describe heterodimeric VEGF variants in which one but not the other receptor binding site has been altered (21, 22). The three-dimensional structure of VEGF has been determined by both NMR spectroscopy and x-ray crystallography (23, 25–29). Hence, extensive knowledge of VEGF structure-function relationships is available, and it has been demonstrated that a VEGF variant with only one functional receptor binding site is able to antagonize the function of wild-type VEGF by binding to the receptor without activating it (21, 22). However, due to the homodimeric nature of VEGF, obtaining VEGF variants that

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; KDR, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; KDR, kinase insert domain receptor; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
have specifically been altered in one but not the other receptor binding site is cumbersome, involving the expression and purification of two separate protein chains and refolding the two chains together, followed by a difficult isolation of correctly folded heterodimer (21, 22). This is not a procedure it is possible to use in combination with medium- or high-throughput screening. Moreover, it is not well suited for larger-scale manufacture of heterodimeric VEGF variants. Expressing two VEGF monomers as a single protein chain could be a solution to the problems associated with creation of heterodimeric VEGF and heteromers of other multimeric proteins, thereby allowing preparation of antagonists of a number of receptor ligands. The present work describes the construction and characterization of a single-chain VEGF molecule that acts as a VEGF antagonist. Even though this protein is not ideal, it represents an important step forward, and it shows the validity of the single-chain approach.

EXPERIMENTAL PROCEDURES

Materials—Primary human umbilical vein endothelial cells (HUVECs) from PromoCell were maintained in Endothelial Cell Growth Medium from PromoCell for two to five passages before being used in proliferation assays. WST-1 cell proliferation reagent for HUVEC proliferation assay and P40 polymerase for PCR were from Roche Molecular Biochemicals. Other cell culture reagents were from Invitrogen. Human VEGF121, VEGF165, bFGF, and antibodies against these proteins for use in Western blotting and KDR/Fc chimera were from R&D Systems. The Pichia pastoris protein expression system, including vectors, P. pastoris strains, and media components, was from Invitrogen, as were precast gels for SDS-PAGE. Toyopearl SP500C cation-exchange resin was from Tosoh Biotech. Resource S and NAP5 columns were from Amersham Biosciences. The Jupiter C18 column was from Phenomenex. DNA oligonucleotides used for gene synthesis, cloning, and mutagenesis were from TAG Copenhagen or DNA Technology. Modified trypsin was from Promega.

Gene Synthesis, Cloning, and Mutagenesis—Genes were synthesized by mixing equimolar amounts of alternating sense and antisense 70-base oligonucleotides covering the DNA sequence to be synthesized with 20-bp overlaps and running 35 cycles of PCR using P40 polymerase and varying concentrations of the oligonucleotide mixture. Different volumes of the primary PCR were used as template in a secondary, 35-cycle PCR using end primers. The resulting PCR reactions were run on agarose gels, and the reaction with the strongest band of the right size and the least background was selected. The correctly sized band was excised from the gel and cloned into the pPICZaA vector using the BamHI/XbaI restriction sites, and several clones were sequenced to find one without errors introduced during synthesis. Subsequent mutagenesis was done by PCR using DNA oligonucleotides containing the mutations of interest.

Protein Expression in P. pastoris—Plasmid encoding the protein of interest was transformed into the P. pastoris X-33 strain. Expression was induced by growing the cells in buffered, rich medium with methanol as the sole carbon source, as described in the Invitrogen protocols. Supernatants were harvested by centrifugation, and protein expression was checked by Western blot.

Protein Purification—P. pastoris culture supernatants (up to 500 ml) containing the protein of interest were adjusted to pH 4.4 using acetic acid and diluted to a conductivity of less than 15 millisiemens/cm in 100 mM sodium acetate, pH 4.4. The sample was applied to a 10 ml Toyopearl SP550C cation-exchange column, and bound protein was eluted in fractions with a linear gradient from 0 to 2 M NaCl in 100 mM sodium acetate, pH 4.4, using either an Akta Basic or Akta FPLC chromatography system from Amersham Biosciences. Fractons containing VEGF or VEGF variants were identified by Western blotting and pooled. For initial screening, the pooled fractions were concentrated, and buffer was changed to phosphate-buffered saline using VivaSpin concentration columns. Protein preparations were stored in aliquots at −20 °C.

Amino Acid Sequence Analysis—N-terminal amino acid sequence analyses of protein samples were carried out following SDS-PAGE and electroblotting onto polyvinylidene difluoride membranes using an Applied Biosystems 494 Protein Sequencer equipped with a blot cartridge and operated according to the manufacturer’s instructions. N-terminal amino acid peptide sequence analyses were carried out on glass fiber filters pretreated with Polybrene in the same protein sequencing using a normal cartridge. All cysteine residues indicated were positively identified as S-carboxamido-cysteines.

MALDI-TOF Mass Spectrometry—MALDI-TOF mass spectrometry was done using an Applied Biosystems Voyager DE-PRO mass spectrometer operated in linear mode for protein and peptide identification. Sample preparation for MALDI-TOF mass spectrometry consisted of mixing 1 μl of sample with 1 μl of matrix solution. The matrix solution was a saturated solution of c-cyano-4-hydroxy cinnamic acid in 49.5% H2O/49.5% acetonitrile:0.1% trifluoroacetic acid. Mass spectra were calibrated externally with proteins and peptides of known masses.

Amino Acid Analysis—Lyophilized protein samples were hydrolyzed for 16 h at 110 °C in 6 M HCl containing 1% phenol in N2-blanketed glass vials before quantification of the liberated amino acids by amino acid analysis using the AccQTag system from Waters.

Enzymatic Degradation and Peptide Purification—Before degradation with modified trypsin, 460 μg of single-chain VEGF variant 1E64R/2:I46R was denatured, reduced, alkylated, and desalted. A lyophilized sample was denatured in 400 μl of 6 M guanidinium-HCl, 0.3 M Tris-HCl, pH 8.3, and incubated overnight at 37 °C before the addition of 50 μl of 0.2 M dithiothreitol in the same buffer. After a 2-h incubation at ambient temperature to reduce the disulfide bonds, 50 μl of 1.0 M iodoacetamide in denaturation buffer was added, and the free thiol groups were alkylated for 30 min at ambient temperature. Finally, the sample was buffer-changed into freshly made 50 mM NH4HCO3, 0.4 M urea, pH 8.3, on a NAP5 column. Enzymatic degredations of reduced and alkylated single-chain VEGF variant 1E64R/2:I46R with modified trypsin were carried out for 16 h at 37 °C in a thermomixer using 2.3 μg of enzyme. The peptides generated by the enzymatic degradation were separated using a Jupiter C18 column (2 × 50 mm) and eluted with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. Collected fractions were analyzed using MALDI-TOF mass spectrometry and amino acid sequence analysis.

Endothelial Cell Proliferation Assay—HUVECs were seeded at a density of 4,000–5,000 cells/well in 96-well plates in 100 μl of Dulbecco’s modified Eagle’s medium/F-12 containing 0.5% fetal bovine serum and growth factors (VEGF 121, VEGF 165, or bFGF) were included on each plate. WST-1 cell proliferation reagent was added to each well, the plates were incubated at 37 °C for 1.5 h, and then A500 was read in an enzyme-linked immunosorbent assay reader. Relevant standard curves of wild-type growth factors (VEGF121, VEGF165, or bFGF) were included on each well, and background absorbance of 0 and 1 μl of either VEGF121, VEGF165, or bFGF was included. All curves displayed were representative of at least three independent experiments.

BiaCore Receptor Binding Assay—The interaction of single-chain VEGF proteins and a VEGF-Fc receptor dimer (R&D Systems) was analyzed using a BIAcore 3000 analyzer (BIAcore). VEGF-Fc was coupled to a BIAcore CM5 chip, and equivalent molar amounts (133 nm) of a VEGF single-chain wild-type and an antagonist mutant were injected over the receptor. Sensorsgrams of association (in the presence of ligand) and dissociation (subsequently, in the absence of ligand) were recorded.

RESULTS

Creating a Single-chain Form of VEGF121—A synthetic single-chain gene consisting of coding sequences for two VEGF121 monomers connected by a 14-residue (42-bp; amino acid sequence, GSGSGKSKSSGKKGG) linker was prepared as described under “Experimental Procedures” (Fig. 1B). The codon usage was optimized for expression in yeast systems (such as Saccharomyces cerevisiae or P. pastoris) while maximizing the difference in codon usage between the two monomers to reduce the risk of recombination events between the two monomers in vivo and facilitate monomer-specific PCR on the plasmid constructs. The synthesized gene was cloned into the pPICZaA P. pastoris expression vector after the KEX2 signal cleavage site,
endothelial cells in vitro was found to be indistinguishable from that of wild-type VEGF121 in both potency and efficacy, as shown in Fig. 3. This indicates that connecting the two monomers with a peptide linker does not affect the interaction between VEGF and the KDR receptor; in other words, single-chain VEGF functions as effectively as a wild-type VEGF homodimer.

Creating a Single-chain VEGF Antagonist—Initially, nine single-chain VEGF variants were prepared by site-directed mutagenesis aimed at creating an antagonist by altering one of the receptor binding sites of the single-chain VEGF to make that site unable to bind the receptor (Fig. 4A). Positions are given relative to wild-type VEGF121 for each monomer, so that 1:E64 is glutamic acid 64 in the first (N-terminal) monomer, and 2:I46 is isoleucine 46 in the second (C-terminal) monomer. The 1: and 2: tell whether the alteration is present in the N-terminal (1) or C-terminal (2) monomer. 2:MR(81–83)del means that the Met-Arg-Ile sequence at position 81–83 in the C-terminal monomer was deleted. 2:180I means that a methionine was inserted after isoleucine 80 in the C-terminal monomer.

These variants were expressed in P. pastoris as described for the wild-type single-chain VEGF and partially purified by a single cation-exchange step. This step removes most of the media components and some proteins and allows analysis by mass spectrometry and N-terminal amino acid sequencing. However, the purity is not sufficient to allow quantification by amino acid analysis or A280 absorption measurement. Thus, whereas it is possible to evaluate the efficacy in HUVEC proliferation assay, it is not possible to make an estimate of agonist or antagonist potency. Most of these variants were able to stimulate HUVEC proliferation in a way that was indistinguishable from wild-type VEGF121 (Fig. 4A). However, single variant, 1:E64R/2:I46R, demonstrated only a very modest induction of HUVEC proliferation and was able to fully antagonize VEGF121-induced proliferation (Figs. 4B and 5).

Protein Characterization—SDS-PAGE and MALDI-TOF mass spectrometry of the variants of single-chain VEGF always show the presence of two components like those seen for wild-type single-chain VEGF. N-terminal amino acid sequence analysis of the two components after SDS-PAGE and electroblotting gave the same N-terminal sequence (EGGGQNHHEVKFMD) corresponding to amino acid residues 5–19 of wild-type VEGF121.

In MALDI-TOF mass spectrometry, both components are seen to be heterogeneous with respect to their masses. The shapes of the two peaks clearly indicate that glycosylation of the components is the basis for the mass heterogeneity. The smaller component has an average mass of 30.8 kDa, with masses ranging from 30 to 31.5 kDa, whereas the larger component has an average mass of 32.8 kDa, with masses ranging from 32 to 34 kDa. The theoretical mass of the polypeptide part of single-chain VEGF starting at Glu5 is 28915 Da. Because single-chain VEGF contains two potential N-glycosylation sites (one in each monomer), incomplete glycosylation of either of these sites is the likely explanation for the two components. In some mass spectra, the nonglycosylated version of single-chain VEGF is sometimes observed in minute amounts.

The ability of single-chain VEGF to induce proliferation of
are potential N-glycosylation sites. After degradation of the antagonistic single-chain VEGF with trypsin, the peptides containing these two amino acid residues (amino acid residues 1:G65-1:R82 and 2:C57-2:R82) were purified and characterized. Both 1N:75 and 2:N75 were found to be partially glycosylated because both a glycosylated and a nonglycosylated version of each peptide were purified. Based upon the relative amounts of the nonglycosylated and glycosylated versions of each peptide in the high pressure liquid chromatography purification, it was clear that 1:N75 was almost fully glycosylated, whereas 2:N75 was glycosylated to a lesser extent.

A very interesting observation was that both the nonglycosylated and the glycosylated version of the peptide containing 2:N75 were purified in two forms distinguished by a mass difference of 17 Da. Because the N-terminal amino acid residue in the peptide (amino acid residue 2:57) is an S-carboxamido-methyl-Cys residue, we propose that the mass difference is caused by cyclization of the S-carboxamidomethyl-Cys residue with concomitant loss of NH3 (mass, 17 Da) in a reaction analogous to the cyclization of N-terminal glutamine residues.

N-terminal amino acid sequence determination of the peptide with the highest mass gave the expected amino acid sequence (CGGCCNDEGLECVPTEES[N]ITMQIMR; [N] is found either glycosylated or nonglycosylated) with the 4 Cys residues identified as S-carboxamidomethyl-Cys residues. A peptide with cyclized N-terminal S-carboxamidomethyl-Cys residue should be inaccessible to N-terminal amino acid sequencing. However, upon N-terminal amino acid sequencing of the peptides with the supposedly cyclized S-carboxamidomethyl-Cys residue at the N terminus, it was possible to obtain sequence information for the peptide, but with residue 1 identified as an S-carboxymethyl-Cys residue. An N-terminal S-carboxymethyl-Cys resi-
FIG. 5. VEGF antagonism by single-chain VEGF 1:E64R/2:I46R. A shows the ability of single-chain VEGF 1:E64R/2:I46R to antagonize the mitogenic effect of VEGF121 (●) and bFGF (○) on endothelial cells. The curves have been normalized to wild-type VEGF121 and bFGF standard curves, respectively. This panel demonstrates that the apparent antagonist effect of the variant is VEGF-specific and is not due to the presence of unspecific, cytotoxic impurities in the protein preparation. Furthermore, it demonstrates that the variant is a full antagonist because the level of endothelial proliferation is reduced to the level observed without stimulation. B shows the antagonist activity of the single-chain VEGF variant against low (1 nM, ●) or high (200 nM, ○) concentrations of wild-type VEGF121. The reduced potency of the antagonist when the concentration of agonist is increased (as seen from the right shift of the curve) demonstrates that the single-chain VEGF variant has competitive antagonist behavior. Furthermore, it shows that the apparent antagonist behavior is not due to a bell-shaped agonist behavior of the wild-type VEGF protein or another type of high-dose inhibition, in which case an increase in the concentration of agonist would not have led to a right shift of the antagonist curve.

due can only be explained by in-sequence hydrolysis of an cyclized N-terminal S-carboxamidomethyl-Cys residue to an S-carboxymethyl-Cys residue. This specific observation clearly supports the interpretation that the N-terminal S-carboxamidomethyl-Cys residue can undergo cyclization just like N-terminal Gln residues. It should be stressed that all other Cys residues covered by amino acid sequencing were clearly identified as S-carboxamidomethyl-Cys residues without any presence of S-carboxymethyl-Cys residues, ruling out the possibility for contamination of the iodoacetamide used with iodoacetic acid. Such a contamination also would not explain the observation of the loss of 17 Da in the peptide mass.

MALDI-TOF mass spectrometry of the glycosylated peptides showed that the glycan structures attached to 1:N75 and 2:N75 were almost identical and heterogeneous in accordance with the data obtained from intact single-chain VEGF. The masses of the glycan structures are in accordance with glycan structures composed of 2 N-acetylgalactosamine residues and 6–14 hexose residues. Taking into account that the single-chain VEGF is produced in P. pastoris, it is highly likely that the hexose residues are mannose residues.

Further Characterization of the 1:E64R/2:I46R Variant—To verify that the antagonist activity of the 1:E64R/2:I46R variant is specific and not caused by cytotoxic impurities in the protein preparations, antagonist assays were run against variable doses of VEGF121 and against bFGF. As shown in Fig. 5A, the single-chain VEGF variant is able to fully antagonize the mitogenic effects of VEGF121 in a dose-dependent manner. In contrast, the mitogenic effect of bFGF is not affected, demonstrating that the apparent antagonism is VEGF-specific and not due to cytotoxic impurities in the protein preparation. As shown in Fig. 5B, the antagonist potency of the single-chain VEGF variant is reduced (the IC50 is increased) when the concentration of VEGF121 is increased. Together, these assays demonstrate that the single-chain VEGF variant is a specific, competitive VEGF antagonist, in accordance with the proposed model for how a VEGF molecule with only one active receptor binding site would affect the interaction between VEGF and its receptor.

To further characterize the interaction between VEGF and the KDR receptor, BiaCore analyses were run in which single-chain VEGF and the 1:E64R/2:I46R variant were floated over immobilized KDR/Fc chimera, which is dimeric. As shown in Fig. 6, wild-type single-chain VEGF displays a biphasic dissociation with half-lives of ~55 s and 43 min, respectively, corresponding to VEGF dimer bound with one or two sites. The 1:E64R/2:I46R variant also displays biphasic dissociation. However, whereas the half-life of the fast dissociation is indistinguishable from that of wild-type single-chain VEGF, the half-life of the slow dissociation is 23 min, i.e. only half of that of the wild-type single-chain VEGF. This supports the proposed model for how the antagonist should work: the 1:E64R/2:I46R variant has one intact receptor binding site, and hence variant ligand bound with only one (intact) site dissociates with the same kinetics as the wild-type ligand. However, the variant ligand bound with two sites dissociates much faster than the wild-type ligand because one of the sites has reduced affinity for the receptor.

FIG. 6. Interaction between the KDR receptor and single-chain VEGF. The interaction was analyzed using a BiaCore system as described under “Experimental Procedures.” The two curves represent the dissociation of single-chain VEGF and the 1:E64R/2:I46R variant from predimerized receptor (KDR/Fc chimera). Both molecules show biphasic dissociation corresponding to a mix of ligands bound with either one or two receptor binding sites. The two molecules show an indistinguishable fast dissociation (half-life, ~55 s), in accordance with the expectation that they both have at least one intact high-affinity receptor binding site. However, when both sites are used for binding, the dissociation of the 1:E64R/2:I46R variant is much faster (half-life, 23 min) than that of the wild-type single-chain (half-life, 43 min), demonstrating that the arginine substitutions reduce the affinity for the receptor.
substitutions. To investigate this, a single-chain VEGF variant with only the 1:E64R substitution was created. This variant was expressed and purified by the two cation-exchange steps described above. The ability of this variant to stimulate endothelial cell proliferation was compared with that of the 1:E64R/2:I46R variant (Fig. 4B). It is clearly seen that the mitogenic effect of the 1:E64R variant is much higher than that of the 1:E64R/2:I46R variant.

**DISCUSSION**

**Single-chain VEGF**—Application of the single-chain antagonist generation approach to VEGF requires that single-chain VEGF be expressed as a molecule that is functionally equivalent to wild-type VEGF. Therefore, the initial experiments were aimed at creating a single-chain form of wild-type VEGF with functional properties identical to those of normal dimeric VEGF.

Several parameters can be varied in the design of a single-chain VEGF, including the selection of which monomer to use (e.g. VEGF_{110}, VEGF_{121}, or VEGF_{165}) and the choice of peptide (if any) to link the two monomers. Based on the crystal structure of the core domain of VEGF (i.e. most of VEGF_{121}), in which the C terminus of one monomer is located in close proximity to the N terminus of the other monomer, it was anticipated that two VEGF_{121} monomers could be expressed as a single-chain dimer connected by a short linker peptide. In contrast, it is difficult to contemplate the feasibility of preparing single-chain dimers of VEGF_{165} because the structures of the two domains of this molecule have not been determined together. Of course, because the difference between the VEGF_{121} and VEGF_{165} monomers is a domain located at the C terminus of the monomer, a single-chain dimer in which the N-terminal monomer is VEGF_{121} and the C-terminal monomer is VEGF_{165} would have the same requirements for the connection between the monomers as would a single-chain dimer of VEGF_{121} monomers.

A described above, the first single-chain VEGF that was tested consisted of two VEGF_{121} monomers connected by a 14-residue linker peptide. This protein was successfully expressed in *P. pastoris* and found to stimulate endothelial cell proliferation in a manner indistinguishable from that of wild-type VEGF_{121}, indicating that this construct would provide a suitable scaffold for the preparation of single-chain VEGF_{121} heterodimers. The lack of N-terminal residues of wild-type VEGF_{121} that were removed from the protein during posttranslational processing was not found to influence the activity of the molecule, in good accordance with previous studies on VEGF (30). Mass spectrometry of the expressed protein revealed two main forms, a monoglycosylated form and a diglycosylated form. The nonglycosylated form was only observed in minute amounts. Analysis of the glycosylation at the glycopeptide level identified the glycosylation as being well-known *P. pastoris* N-glycan structures. No observation of large mann nan structures that are sometimes observed on proteins expressed in *P. pastoris* was made. Similar patterns of glycosylation were observed for all of the variants prepared. However, because previous studies have shown that glycosylation does not affect VEGF activity (31–33), no attempts were made to obtain homogenously glycosylated protein preparations. Later, peptide map studies on the 1:E64R/2:I46R variant revealed a difference in the frequency of glycosylation of the two monomers. The most N-terminal glycosylation site had a higher frequency of glycosylation than the most C-terminal site, a property that is likely to apply to wild-type single-chain VEGF too. Taking the closeness of 2:N75 to the C terminus of the protein into consideration, this is hardly surprising.

**Preparing a Single-chain Antagonist**—As mentioned above, extensive structure-function information regarding the interaction between VEGF and its receptors is available in the literature. Based on this information, two strategies were used to make one of the receptor binding sites nonfunctional. One strategy was to substitute arginine residues into one of the receptor binding sites on positions known from previously published studies to be important for the VEGF-receptor interaction (mainly from alanine scanning studies). A different strategy was to insert or delete one or more residues in the receptor binding site to try to disrupt the structure of the entire region.

One of the nine variants that were initially prepared, 1:E64R/2:I46R, showed almost no agonist activity and was able to fully antagonize the activity of wild-type VEGF_{121}. Antagonist assays against bFGF and elevated concentrations of VEGF_{121} demonstrated that the observed effect is due to a VEGF-specific, competitive antagonist. Subsequently, BioCore analysis of dissociation of wild-type single-chain VEGF and the 1:E64R/2:I46R variant from the KDR receptor supported the notion that the antagonist variant contains one intact receptor binding site and one site with reduced receptor binding affinity. Together, these data clearly show that the 1:E64R/2:I46R single-chain VEGF variant is effectively a VEGF variant with only a single receptor binding site.

Interestingly, all eight other variants had full agonist activity. Whereas effects such as receptor saturation may hide a difference between some of the variants and wild-type VEGF, there is obviously a huge difference between 1:E64R/2:I46R and the other variants. This is in interesting contrast to earlier alanine scanning studies on VEGF, in which Glu^{46} and Ile^{46} do not appear to be much more important than the other residues that were substituted in this study (Tyr^{45}, Gln^{79}, and Ile^{31}). The subsequent preparation of a single-chain VEGF with 1:E64R as the only substitution revealed that both 1:E64 and 2:I46 must be substituted to arginines at the same time to eliminate agonist activity.

The effect of arginine substitutions on receptor binding obviously cannot be predicted from alanine scanning mutagenesis results, emphasizing that alanine scanning alone presents a biased picture of the relative importance of the residues in a protein and that arginine scanning, glutamate scanning, or tryptophan scanning mutagenesis would provide complementary data for structure-function studies. A different consequence of this observation is that to improve the antagonist identified in this study by reducing its low agonist activity further, a more complete arginine scanning is necessary. This is the case because the results of previous alanine scanning studies cannot be used to predict the positions in which arginine substitution will have the greatest effects.

The single-chain VEGF antagonist variant prepared in this study is not an optimal solution to the problem of preparing a VEGF variant with antagonist properties. For example, the variant shows limited agonist activity within a narrow concentration range, and the antagonist potency is low. However, the construction of this variant demonstrates the feasibility of using the single-chain approach for creating VEGF antagonists, and the variant may serve as a scaffold for further improvement of antagonistic properties.

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