

Structure-Function Studies of Two Synthetic Anti-vascular Endothelial Growth Factor Fabs and Comparison with the AvastinTM Fab^{*[5]}

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In the quest to discover new research tools and to develop better agents in the fight against cancer, two antibodies, G6 and B20–4, were isolated from synthetic antibody phage libraries. Unlike the AVASTINTM antibody, a recently approved agent for the treatment of patients with colorectal cancer, B20–4 and G6 bind and block both human and murine vascular endothelial growth factor (VEGF). Here we have analyzed and compared the binding epitopes on VEGF for these three antibodies using alanine-scanning mutagenesis and structural analyses. The epitopes recognized by both synthetic antibodies are conserved between human and mouse VEGF, and they match closely to the receptor epitopes both structurally and functionally. In contrast, the Avastin epitope overlaps minimally with the receptor binding surface and centers around a residue that is not conserved in mouse. Our structural and functional analyses elucidate the cross-species reactivity of all three antibodies and emphasize the potential advantages of antibody generation using phage display as the resulting antibodies do not depend on sequence differences across species and preferentially target natural protein-protein interaction surfaces.

Angiogenesis, the process of new blood vessel formation, is not only essential for many physiologically important events but is also critical in a number of diseases such as tumor progression, diabetic retinopathy, and rheumatoid arthritis (1), (2). Starvation of tumors by inhibition of angiogenesis has been discussed for a number of years as a strategy in the fight against cancer, and dozens of anti-angiogenesis drugs are currently being tested in clinical trials (3). Vascular endothelial growth factor (VEGF)³ is one of the key players in angiogenesis (4). Blocking the activity of VEGF using specific antibodies or other protein entities to prevent VEGF binding and signaling through its receptors has been one of many approaches to reduce tumor growth (5–8). Recently, the AVASTINTM antibody (Bevacizumab), an antibody that binds human VEGF with high affinity, was approved for treating colorectal cancer

patients (9). This study for the first time validated an approach by which tumor starvation is induced through inhibition of VEGF.

VEGF (also called VEGF-A) and the members of the VEGF family such as VEGF-B (10), VEGF-C (11), VEGF-D (12), and PlGF (13) mediate their biological function by binding with different specificities to the three receptor tyrosine kinases VEGFR1, (also called flt-1) (14), VEGFR2 (also called KDR) (15), and VEGFR3 (16). Structural and functional studies have identified the receptor binding domain of VEGF (residues 8–109 fragment) as an anti-parallel homodimer (17, 18) that binds to VEGFR1 and VEGFR2 with two binding sites that are located on the opposite poles of the homodimer and are separated by >40 Å (19). The extracellular domain of the VEGF receptors consists of 7 immunoglobulin (Ig)-like domains with domains 2 and 3 being responsible for high affinity binding of VEGF. Alanine-scanning analyses and the crystal structures of VEGF in complex with the Fab fragment of the Avastin antibody (18) and with the second domain of VEGFR1 (19) showed that the binding epitopes on VEGF for the receptor and for the Avastin Fab are distinct and only partially overlapping. The most likely mechanism by which active Avastin antibodies inhibit receptor binding is steric hindrance (18).

Highly selective, neutralizing antibodies play an essential role in the quest to decipher the specific role of VEGF among its family members with overlapping activities. Therefore, besides its use as a drug in fighting cancer, Avastin could be a very important reagent to investigate angiogenic processes and the exact function of VEGF and its receptors. However, Avastin is specific to human VEGF and does not bind, for example, murine VEGF; thus its use for studies in mouse models is rather limited. The Avastin antibody is the humanized version of the monoclonal antibody A.4.6.1, which was derived using hybridoma technology by immunizing mice with human VEGF (5). This traditional approach excludes the possibility of producing antibodies that bind to murine VEGF, as the immune response in the mouse prohibits the generation of self-reactive antibodies. Thus, with this approach antibodies can be developed that only target binding areas with sequence differences in the proteins between host and target.

One solution to this limitation of the traditional method of raising monoclonal antibodies is a new technique that utilizes phage display of synthetic antibody libraries to find antibodies with high affinities against various targets of interest (20). The synthetic antibody library used in the approach described in Lee *et al.* (20) was built on the framework of a humanized antibody against human epidermal growth factor receptor 2 (erbB2), 4D5 (h4D5) (21). In this process a number of antibodies that bind murine VEGF with high affinity were identified. Two of these antibodies, termed G6 and B20–4, with unrelated sequences in their complement-determining regions, bind human and mouse VEGF with

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

The atomic coordinates and structure factors (code 2FJF, 2FJG, and 2FJH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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³ The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; h-VEGF, human VEGF; m-VEGF, mouse VEGF; CDR, complement-determining region.

equally high affinity.⁴ This property makes both antibodies useful research tools as they do not share the shortcomings of the Avastin antibody, which is unable to block the function of murine VEGF.

To understand how G6 and B20-4 are able to bind both mouse and human VEGF although the Avastin antibody is not, we carried out extensive alanine-scanning mutagenesis of VEGF to map the functional binding sites of both antibodies and to compare them with the Avastin binding epitope. In addition, we obtained the crystal structures of VEGF in complex with the B20-4-Fab and the G6-Fab to identify structural determinants for high affinity binding of both antibodies. Our results show that G6 and B20-4 recognize VEGF at a similar "hot spot" that is utilized by VEGFR1_{D2}, emphasizing the importance of this surface area for future drug discovery efforts. This epitope only partially overlaps with the binding area of Avastin, and, unlike the Avastin binding epitope, it is conserved in the protein sequences of human and mouse VEGF. We have further shown that G6 adopts a canonical conformation in its unliganded form but adopts a highly unusual conformation in the VEGF-bound form.

MATERIALS AND METHODS

Alanine Scanning of VEGF—The relative binding affinities of the G6-Fab or B20-4-Fab toward individual alanine-substituted VEGF mutants *versus* wild type VEGF were measured using VEGF phage enzyme-linked immunosorbent assay as described previously (18). Briefly, human VEGF (residues 1–109) coded in phagemid vector pB2105 was mutated using the Kunkel mutagenesis method, and the various mutants were displayed on phage. Serial dilutions of phage displaying VEGF mutants were bound to 96-well plates that were coated with G6, B20-4, Y0317, A.4.6.1 (the murine IgG precursor of the Avastin antibody), or VEGFR1_{D1-3} (Ig domain 1–3 of VEGFR1). The bound phage were detected using horseradish peroxidase conjugated to an anti-M13 antibody (Amersham Biosciences). Based on this titration, a subsaturating concentration of phage was chosen to incubate with serial dilutions of G6 or B20-4 Fab in binding buffer (phosphate-buffered saline with 0.5% bovine serum albumin and 0.05% Tween 20) at room temperature for 1 h. Then the mixtures were transferred to Fab-coated 96-well plates and incubated for 10 min to capture unbound VEGF phage. The phage bound to the plate were detected using the anti-M13 antibody horseradish peroxidase conjugate as above. Relative affinities (EC_{50}) were calculated as the concentration of Fab resulting in half-maximal phagemid binding. The ratio of the EC_{50} of mutant *versus* wild type VEGF represents the relative difference of the various binding affinities. To test for structural integrity of VEGF mutants with severely reduced binding affinities toward a subset of the tested antibodies, we captured the unbound mutants with a binding partner capable of binding as determined in the initial titration. Some VEGF mutants have exchanges that abrogate binding toward G6 or B20-4 completely (e.g. F17A and Y21A, etc.); we verified the structural integrity of these mutants by retesting their ability to bind A.4.6.1. or VEGFRs.

Expression, Purification, Crystallization, and Data Collection—The receptor binding fragment of human VEGF comprising residues 8–109 (VEGF-(8–109)) were expressed, refolded, and purified as described previously (23). G6 and B20 Fabs were expressed in *Escherichia coli* and purified as described previously (24). Complexes between Fab and VEGF-(8–109) were obtained by mixing the purified components in a 1:1 molar ratio and further purified as described previously (24). Crystals of the G6-Fab-VEGF-(8–109) and the B20-4-Fab-VEGF-(8–109)

complexes were obtained at 19 °C using the vapor diffusion method in sitting drops. For crystallization of G6-Fab-VEGF-(8–109), crystallization buffer containing 2.0 M ammonium sulfate and 5% isopropanol was mixed in equal volume with protein solution (8 mg/ml). Small crystals appeared after 3 days and belonged to space group P3₁21 with cell dimensions of $a = 117.9 \text{ \AA}$ and $c = 212.6 \text{ \AA}$. These crystal forms contained one complex comprising a VEGF dimer and two Fab molecules in the asymmetric unit. Before data collection, single crystals were briefly dipped into artificial mother liquor containing 25% glycerol and flash frozen in liquid nitrogen. Data were collected at beamline 9–1 of the Stanford Synchrotron Radiation Laboratory. For crystallization of the B20-4-Fab-VEGF-(8–109) complex, crystallization buffer with 0.1 M sodium acetate, pH 4.6, and 1.6 M ammonium sulfate was mixed in 1:1 ratio with protein solution (20 mg/ml). Crystals appeared in 1–2 days and grew to $500 \times 400 \times 400 \text{ \mu m}$. The crystals were soaked in 0.1 M sodium acetate, pH 4.6, and 2 M sodium malonate overnight and flash frozen in liquid nitrogen. Crystals diffracted to $3.1 \text{ \AA}$ at the ALS Synchrotron Source on beamline 8.3.1.

Crystals of the G6-Fab in its unbound form were obtained from protein that was further purified using a Superdex 200 column in 30 mM Tris, pH 7.5, and 0.4 M NaCl. The protein was concentrated and used in crystallization trials. Crystals used for the structure determination were grown at 19 °C in sitting drops by mixing equal volumes of protein solution (10 mg/ml) and crystallization buffer containing 25% polyethylene glycol 3350 and 0.1 M sodium acetate (pH 4.7). These crystals belong to space group C2 with cell parameters $a = 275 \text{ \AA}$, $b = 192 \text{ \AA}$, $c = 154 \text{ \AA}$, and $\beta = 117^\circ$. Before data collection crystals were dipped in artificial mother liquor containing 30% ethylene glycol and flash frozen in liquid nitrogen. Annealing the crystals by interrupting the cold stream for 30 s dramatically improved the diffraction quality of the crystals. Data to $2.65 \text{ \AA}$ were collected at the beam line 5.0.2 of the Advanced Light Source (Berkeley).

Data Processing, Structure Determination, and Refinement—All data were processed using Denzo and Scalepack (25). The structure of the G6-Fab-VEGF-(8–109) and the B20-Fab-VEGF-(8–109) complexes were solved using AMoRe (26), using the coordinates of VEGF from a previously described VEGF-Fab complex (1B11) and Fab fragments containing either the variable domains V_H/V_L or the constant domains C_{H1}/C_L of the VEGF-Fab complex 1TZI. The structure of G6-Fab in its unbound form was solved using fragments of the G6-Fab-VEGF-(8–109) structure as search models. Model building and refinement were done using programs Refmac (27) and O (28), respectively.

Crystal Packing Arrangement of G6—The structure of the G6-Fab in its unbound form is not unusual; however, its packing arrangement in the crystal is quite remarkable. The asymmetric unit of the crystal contains 12 Fab molecules (see supplemental Fig. S1). When superimposed separately, the variable and the constant domains of all 12 Fabs superimpose well, but the elbow angles of the 12 Fabs vary significantly and range from 142 to 175° (see supplemental Fig. S1) and cluster around 144° (3 copies), 155° (3 copies), and 174° (6 copies). In the crystal packing arrangement, the variable domains of the three Fabs assemble around a 3-fold axis. Located in the center of this trimer is the CDR-H2, but all other CDRs of both the light and the heavy chain contribute to this interface as well. The resulting Fab- V_H/V_L trimer has the shape of a rather flat sheet with the constant domains protruding away in different angles. Each of these flat sheets represents one face of a regular tetrahedron; the entire dodecameric complex has a diameter of $\sim 170 \text{ \AA}$, and the central cavity of the dodecamer is large enough to hold a sphere with a diameter of $\sim 25 \text{ \AA}$ (see supplemental Fig. S1).

⁴ Liang, W. C., Wu, X., Peale, F. V., Lee, C. V., Meng, G., Gutierrez, J., Fu, L., Malik, A. K., Gerber, H. P., Fuh, G., and Ferrara, N. (2006) *J. Biol. Chem.* **281**, 951–961.

VEGF residues	Relative affinity (EC ₅₀ _{ala mut} /EC ₅₀ _{wt})				
	G6	B20-4	VEGFR1	VEGFR2	A4.6.1
WT	1.0	1.0	1.0	1.0	1.0
E30	0.8	• 3.1			
I43	1.0	1.2		5.6	1.2
Y45	3.7	1.1		1.6	• 2.2
I46	2.2	1.3	• 4.0	NB	2.1
F47	3.5	1.8	3.0	3.6	1.1
K48	• 0.3	• 1.0	• 1.0	0.7	• 0.7
P53	1.7	0.8			
Q79	0.7	• 0.9	• 3.0	NB	• 0.7
M81	• 1.3	• 1.8	• 6.9	2.0	• NB
R82	1.3	1.3		0.6	• 3.3
I83	• 40	• 0.9	• 5.4	100	• 1.0
K84	0.8	1.5		2.2	• 1.8
P85				5.0	1.0
H86	• 7.1	0.8	• 1.0	1.3	• 1.9
Q87	• 2.3	1.1		1.8	• 1.3
G88 ^S	• 2.7	1.1		1.0	• NB
Q89	• 92	• >100	• 1.0	2.5	• NB
H90	• 1.0	1.2		1.7	• 1.8
I91	• 6.9	• 1.3	• 1.0	1.0	• 1.2
G92	0.9	1.4		1.2	• 8.5
E93	2.9	1.2		0.5	• 6.6
M94	1.0	1.1		2.8	• NB
V14	1.1	1.2		1.8	1.5
K16		• 0.8	• 1.8	1.1	1.2
F17	• >100	• >100	• 45	NB	1.2
M18	• 4.4	• >100	• 56	5.0	1.2
D19	1.0	• >100		0.6	0.7
Y21	• 33	• >100	• 123	2.8	2.6
Q22	• 7.2	• 0.5	• 11	2.2	1.4
R23	1.2	• 9.0		1.5	1.3
Y25	• 21	• >100	• 17	1.7	1.2
C26	nd	• nd			
H27 ^R	0.8	• 0.1			
C61	nd	• nd			
N62	0.5	• 1.7		2.0	2.2
D63	• 5.2	• 1.7	• 7.2	0.5	0.9
E64	• 2.4	1.7	5.0	8.5	1.7
G65 ^A	0.6	1.6	• 1.0	1.3	1.7
L66	• 0.7	• 1.8	• 47	0.3	1.6
N100 ^S	0.9	• 1.1			1.6
K101 ^R	1.1	• 1.0	1.0		
E103	0.7	• 1.0	• 1.0	1.1	2.3
C104	• nd	• nd	•		
R105	1.6	nd	• 1.0	0.8	1.4
P106	• 2.1	0.9	• 1.0		

FIGURE 1. Functional mapping of G6 and B20-4 epitopes on h-VEGF and comparison with other binding partners of VEGF. Selected residues of VEGF were mutated to alanine (ala mut), and the relative affinities (EC₅₀) of these mutants toward G6 and B20-4 were measured as described under "Materials and Methods." The functional binding epitopes of VEGF toward VEGFR1, VEGFR2, and A4.6.1 as determined previously (24, 29, 30) are listed for comparison. VEGF residues for this experiment were chosen based on previously reported crystal structures of VEGF. All selected residues are surface exposed and located close to one pole of the VEGF dimer. Amino acids in the top half of the figure stem from one VEGF monomer, and residues in the lower half originate from the second monomer to form one pole of the VEGF dimer. Relative affinities of >1 indicate a reduction in binding affinity when compared with VEGF wild type. Ratios of >4 are colored yellow, ratios of >10 are orange, and ratios of >20 are red. To verify the structural integrity of VEGF mutants with severe loss in binding affinity against G6 and B20-4, we retested the binding affinities of these mutants toward either A4.6.1 or the VEGFRs. Residues that differ between human and murine VEGF are colored green. The corresponding residues in the mouse sequence are shown in superscripts. Based on the structural data presented here and in previous publications we marked VEGF residues that form contacts (>2 Å² of buried surface) with one bullet point; residues that contribute >50 Å² of buried surface or have >50% of their exposed surface buried in the interface are marked with two bullet points; residues that contribute >50 Å² and have >80% of their exposed surface area buried in the interface are marked with three bullet points.

RESULTS

Functional Epitope of VEGF toward the G6- and B20-4-Fab—The G6-Fab binds m-VEGF and h-VEGF with a K_D of ~0.9 and 1.5 nM, respectively (20). We first mapped the functional binding epitope of VEGF toward the G6-Fab using alanine-scanning mutagenesis of solvent-accessible residues of h-VEGF (17) and identified 10 VEGF residues that result in >4-fold loss in binding affinity toward G6 when

replaced by alanine (Fig. 1). This functional epitope includes 5 residues (Phe-17, Met-18, Tyr-21, Gln-22, and Tyr-25) of the N-terminal helix, one residue from the 60s loop of one VEGF monomer, as well as 4 residues (Ile-83, His-86, Gln-89, Ile-91) from the 80s loop of the second monomer within the biologically active VEGF dimer. It matches the binding epitope of VEGF toward its receptor VEGFR1 very well as 7 of the 10 residues important for G6 binding are also important for the interactions with VEGFR1 (29, 30) (Fig. 1).

The B20-4-Fab binds m-VEGF and h-VEGF with a K_D of ~16 and 12 nM, respectively. Alanine-scanning mutagenesis reveals that fewer residues constitute the functionally important binding interface when compared with the G6 epitope, as only 7 residues lead to a >4-fold loss in binding affinity when exchanged to alanine. However, 6 of those exchanges severely impair binding and result in a loss of >100-fold in binding affinity. Of those 6 residues, 5 are located on the N-terminal helix of VEGF (Phe-17, Met-18, Asp-19, Tyr-21, Tyr-25), and 1 residue (Gln-89) is on the 80s loop. The functional binding epitopes for B20-4 and G6 as determined by alanine-scanning mutagenesis overlap very well, with 4 of the 5 biggest hits against G6 being also very important for B20-4 binding (Fig. 1).

Crystal Structures—To identify the structural binding interfaces and to compare binding modes of G6 and B20 to those of previously reported VEGF binding Fabs (24, 31), we determined the crystal structures of the G6-Fab and the B20-4-Fab in complex with the receptor binding domain of human VEGF (residues 8-109). The unexpected and non-canonical conformation of CDR-H2 in the G6-Fab-VEGF complex (see below) further prompted us to investigate the structure of the G6-Fab in its unbound form.

Crystals of the G6-Fab-VEGF and the B20-4-Fab-VEGF complex diffracted to maximum resolutions of 2.8 and 3.1 Å, respectively, and crystals for the G6-Fab in its unbound form diffracted to 2.65 Å (Table 1). All three structures were refined to R_{values} below 20% and R_{free} below 25% with good stereochemistries. The crystals of the G6-Fab-VEGF complex have one VEGF dimer bound to two Fabs in the asymmetric unit. The asymmetric unit of the B20-4-Fab-VEGF structure contains two VEGF monomers that form separate VEGF dimers after application of crystallographic symmetry elements. Each of these half complexes binds to one B20-Fab. The crystal structure of G6 in its unbound form contains 12 Fabs in the asymmetric unit that form a single dodecameric complex. Of the combined 6246 non-glycine and non-proline residues in the three crystal structures, 18 (0.3%) are located in the disallowed regions of the Ramachandran plot (32); all but 2 of these are due to Ala-51 in the light chain, which is in the disallowed region of the Ramachandran plot in all 16 copies in the three crystal structures presented here.

The structures of both VEGF-Fab complexes display VEGF in the same conformation as in previously reported structures (Fig. 2)⁵ (17-19, 24, 33). In brief, like other members of the cystine knot family (34), the receptor binding fragment of a VEGF monomer contains a 4-stranded antiparallel β -sheet with a cystine knot motif on one end and short loops on the other. Two VEGF monomers assemble in an anti-parallel fashion and are covalently linked by two disulfide bonds. The resulting biologically active homodimer is stabilized by the packing of the N-terminal helix of one monomer over the other and *vice versa*. This dimer has the shape of a curved sheet that is 60 Å long, 40 Å wide but only ~15 Å thick in its central part. In the biologically active signaling complex, each VEGF dimer binds two receptor molecules (35). The binding site on VEGF toward its two tyrosine kinase receptors has been determined

⁵ DeLano, W. L. (2002) www.pymol.org.

TABLE 1

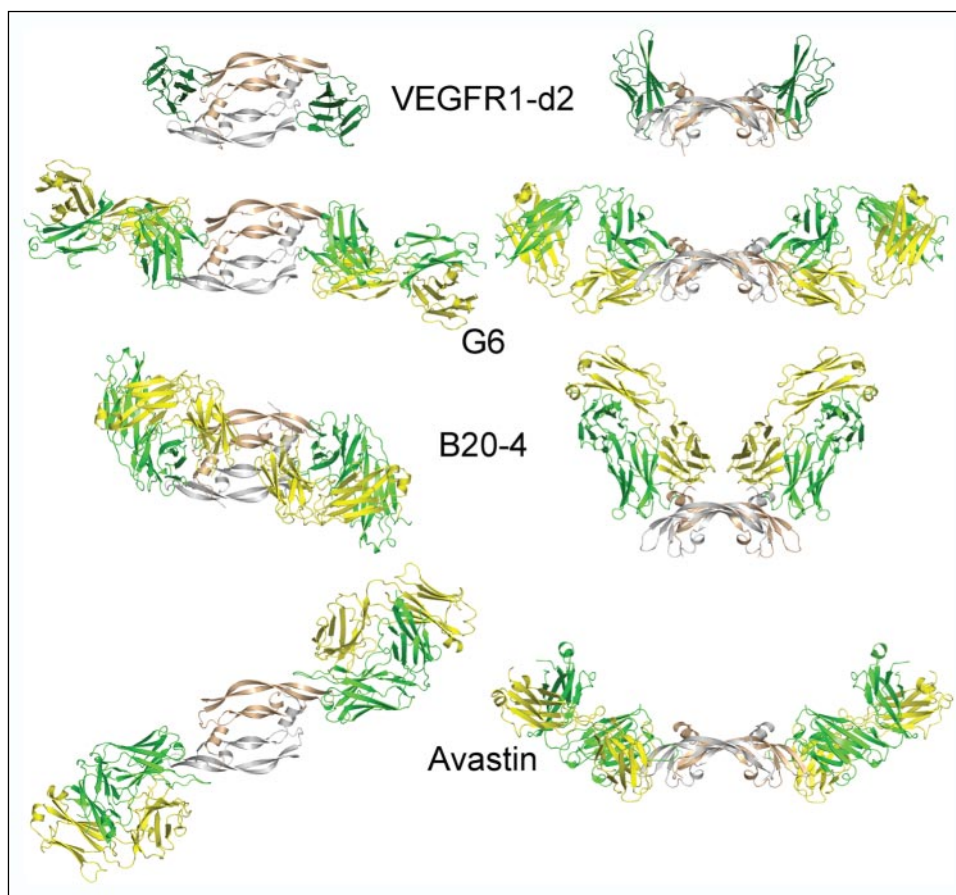
Data collection and refinement statistics

Numbers in parentheses refer to the highest resolution shell.

Data collection	G6	G6-VEGF complex	B20-VEGF complex
Space group	C2	P3 ₁ 21	P3 ₁ 12
Cell parameters	$a = 274.9, b = 192.2, c = 154.1 \beta = 117.0$	$a = 117.9, c = 212.6$	$a = 165.5, c = 150.5$
Resolution (Å)	50–2.65	50–2.8	50–3.1
R_{sym}^a	0.060 (0.52)	0.061 (0.51)	0.064 (0.59)
Number of observations	796044	209873	267422
Unique reflections	205393	42693	42755
Completeness (%)	100.0 (100.0)	99.7 (99.7)	99.9 (100.0)
Refinement			
Content of asymmetric unit	12 Fabs	1 VEGF dimer, 2 Fabs	2 VEGF monomers, 2 Fabs
Resolution (Å)	20–2.65	20–2.8	30–3.1
Number of reflections	200469	40389	39869
Final R^c , R_{free}^c ($F > 0$)	0.199, 0.248	0.198, 0.239	0.198, 0.239
Number of residues	5148	1047	1058
Number of water molecules	642	0	0
Number of ions	0	7 sulfates	0
Number of non-H atoms	39666	8068	8105
Root mean square deviation bonds (Å)	0.008	0.013	0.011
Root mean square deviation angles (°)	1.2	1.5	1.4

^a $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$. $\langle I \rangle$ is the average intensity of symmetry-related observations of a unique reflection.^b $r = \sum |F_o - F_c| / \sum F_o$. R_{free} is calculated as R except for 5% of the reflections excluded from all refinement.

FIGURE 2. **Ribbon diagrams of various VEGF complexes.** Shown are VEGF in complex with domain-2 of VEGFR1, G6-Fab, B20-4-Fab, and Avastin-Fab. VEGF is colored in gray and pink; domain-2 of the VEGFR1 is dark green; the light and heavy chains of the Fabs are yellow and light green, respectively. The left side shows VEGF in top-down. The right side depicts the same complexes rotated 90° around the y-axis. Note the different relative orientations of the Fab light and heavy chains with respect to VEGF in all three complexes. All figures were rendered with Pymol.



functionally by alanine scanning for VEGFR1 and VEGFR2 (18, 29, 30) and structurally for VEGFR1 (Fig. 2) (19). These studies show that both receptors bind similar epitopes of VEGF, which are located on the two opposite poles of the growth factor and span across the interface formed by the anti-parallel monomers within the VEGF homodimer.

The crystal structure of the VEGF-G6-Fab shows two G6-Fabs binding to the VEGF dimer at those same poles (Fig. 2). Both G6-Fabs adopt an extended conformation, which gives the complex a rod-like shape with the longest dimension exceeding 180 Å. A total amount of 1720 Å² of solvent-accessible surface is buried in the interface; thus, the size of

the binding epitope between G6 and VEGF is not unusual and well within the observed range seen in other Fab-antigen or protein-protein interactions (36). As observed in many other Fab-antigen complexes, >75% of the contacts are mediated by the CDRs of the heavy chain, whereas only ~200 Å² of the light chain is buried.

The crystal structure of the B20-4-Fab-VEGF complex shows the B20-4-Fab binding to an epitope that overlaps to a large extent with binding epitopes for the VEGFR1 and G6 (Fig. 2). However, B20-4 approaches the growth factor from a different angle compared with G6. The core of its epitope has shifted when compared with the G6 binding

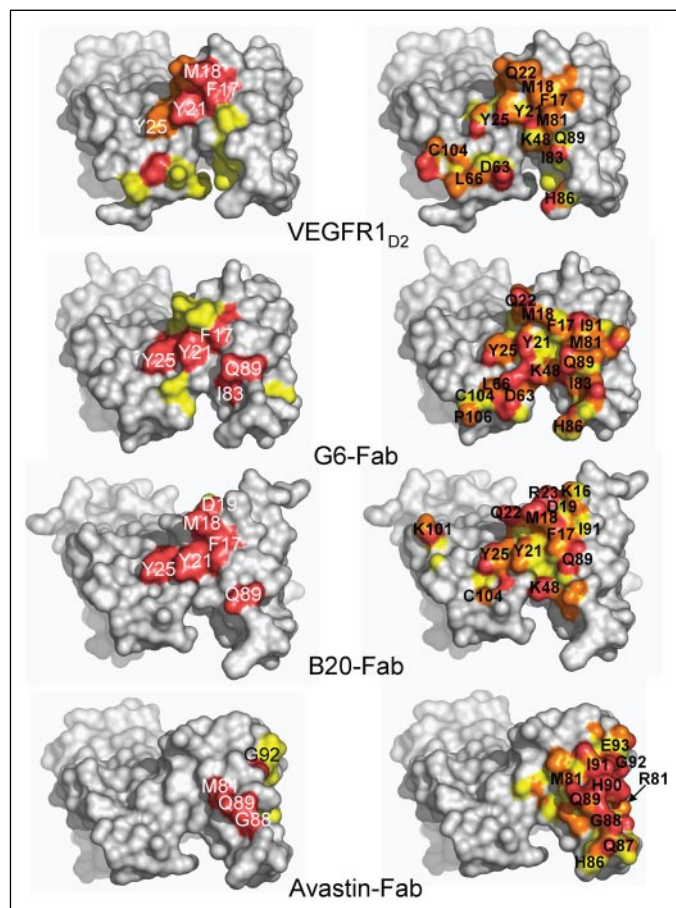


FIGURE 3. Functional and structural binding epitopes of VEGF. Binding epitopes for VEGFR1_{D2}, G6, B20–4, and Avastin. *Left side*, functional epitopes. Residues that bind >4-, 10-, and 20-fold weaker when mutated to alanine are colored yellow, orange, and red, respectively. *Right side*, structural epitopes. Atoms closer than 4.5, 4, and 3.5 Å to their respective binding partners are colored yellow, orange, and red, respectively. Residues that contribute significantly to the structural binding epitopes are labeled.

epitope and is centered on the N-terminal helix of VEGF. Consequently, the two B20-Fabs bind to the “top” face of VEGF; the resulting complex has a V-shaped form with the C termini of the two B20–4-Fabs separated by ~120 Å. The binding interface between the B20–4-Fab and VEGF is slightly larger than the interface in the VEGF-G6-Fab complex. Overall, a total 1990 Å² of surface area is buried in the interface, and ~70% of the 1045 Å² of buried surface on the VEGF side is sequestered from solvent by the heavy chain of B20–4.

Structural Binding Epitopes of B20–4 and G6—The epitope recognized by the G6-Fab in the crystal structure is in excellent agreement with the functional epitope determined by alanine-scanning mutagenesis: all 10 VEGF residues that show >4-fold loss in binding affinity toward G6-Fab when exchanged to alanine have a significant portion (>50%) of their solvent-accessible surface buried in the interface with the antibody (Figs. 1 and 3). This structural epitope of G6 is remarkably similar to the epitope responsible for VEGFR1_{D2} binding and includes residues from the N-terminal helix, the 60s loop, and the C-terminal tail of one VEGF monomer as well as residues from the 40s loop and the 80s loop of the second monomer. In Fig. 3 the aromatic residues Phe-17, Tyr-21, and Tyr-25 from the N-terminal helix of VEGF together with residues Lys-48, Ile-83, and Gln-89 can be identified as the center of the structural binding epitope for G6. Similar comparisons for the B20–4 Fab reveal that, again, structural and functional binding epitopes of VEGF are in excellent agreement (Fig. 3). All 7 functionally important

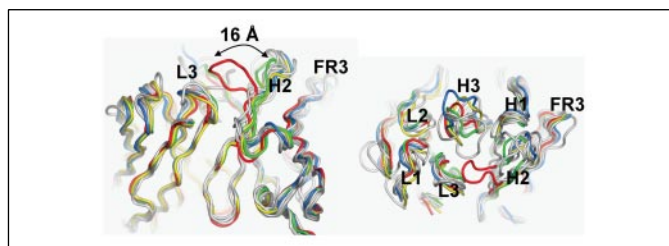


FIGURE 4. G6 bound to VEGF (shown in red) and G6 in its unbound form (green) superimposed on a number of Fab fragments in two different views. Shown are B20–4 (yellow), the Avastin-Fab (blue), and 4D5 (Protein Data Bank code 1FVC, in white), which adopt canonical conformations, as well as a selection of Fabs that have non-canonical conformations in CDR-H2 (Protein Data Bank codes 1IND, 1BBJ, 1FIG, 6FAB, all shown in white). Note the significant conformational shift of the G6 CDR-H2 upon VEGF binding.

VEGF residues are either completely buried in the interface or contribute >50 Å² of buried surface to the interface (Figs. 1 and 3).

The epitopes of VEGF toward B20–4 and G6 overlap to a large extent, but there are some important differences. The contribution of the N-terminal VEGF helix is significant in both complexes; however, residues from this helix dominate the interaction between B20–4 and VEGF. Alanine-scanning mutagenesis reveals that with one exception all residues that weaken the affinity of VEGF toward B20–4 by 100-fold or more are located on the N-terminal helix of VEGF (Figs. 1 and 3). These residues, Phe-17, Met-18, Asp-19, Tyr-21, Gln-22, and Tyr-25 of VEGF, are almost completely buried in the interface and account for more than half of the entire surface buried in the B20–4-Fab-VEGF complex. The most prominent residue in this binding event is Met-18; its side-chain adopts an extended conformation in the complex and is deeply buried in a hydrophobic cavity formed by residues from CDR-H1, CDR-H2, CDR-H3, and CDR-L3. Consistent with the involvement of Met-18 in the structural interface, the substitution of Met-18 with alanine reduces the binding affinity of VEGF toward B20 by >100-fold (Fig. 1).

G6 Has an Unusual Conformation when Bound to VEGF—The B20–4-Fab and the G6-Fab bind VEGF at similar epitopes, yet they approach the growth factor from different angles. In addition, there is another important difference in the binding mode of both Fabs. B20–4 bound to VEGF represents a typical antibody-antigen complex with all CDRs of B20–4 adopting canonical conformations. In contrast, CDR-H2 of G6, when bound to VEGF, adopts a highly unusual and non-canonical conformation.

In published Fab structures CDR-H2 packs tightly against CDR-H1 and a section of the antibody that is part of the framework and termed Framework-3 (FR-3) (37). Surprisingly, when bound to VEGF, CDR-H2 of G6 is in a very different position and packs against CDR-L3 and CDR-H3 (Fig. 4). At the same time the FR-3 loop does not change its position and remains fixed in its commonly observed location tightly packing against CDR-H1. The extent of this difference in CDR-H2 conformations becomes clear when superimposing the VEGF-bound form of G6 onto a number of unrelated Fabs with canonical or even non-canonical conformations in their CDR-H2 (37) as shown in Fig. 4.

We were interested to learn whether this highly unusual conformation of G6 is a result of VEGF binding or whether it represents an inherent property of the Fab. To address this question, we crystallized the G6-Fab in its unliganded form. The resulting structure contains 12 copies of the G6-Fab in the asymmetric unit with each Fab having all CDRs in canonical conformations. In particular, CDR-H2 adopts the commonly observed canonical conformation 3A according to the nomenclature in Al-Lazikani *et al.* (37) (Fig. 3 and supplemental Fig. S1). Superposition of the G6-Fab in its liganded and unliganded form shows

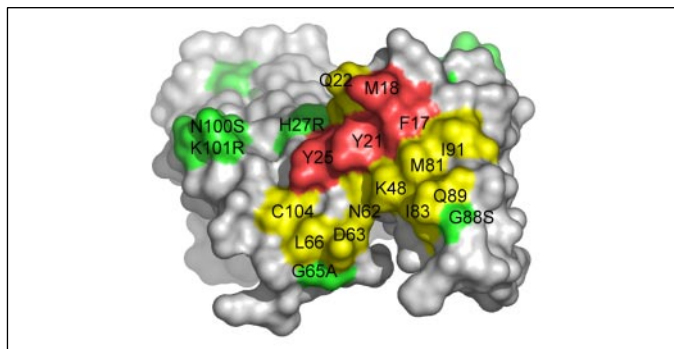


FIGURE 5. **Common hot spot of VEGF for binding of VEGFR1, G6, and B20-4.** Depicted is the surface of VEGF. All residues that form contacts with VEGFR1, G6, and B20-4 are colored yellow, and all residues that when exchanged to alanine cause a loss in binding affinity of >4-fold in all three complexes are colored red. All residues that differ in the sequence between mouse and human VEGF are green. Only 5 of the 10 residues that differ in the receptor binding domain of human and murine VEGF are labeled; the remaining 5 residues are disordered in the crystal structure of the VEGF or more distant from the binding interface of the molecules investigated here.

that residues Gly-50 to Tyr-57 of CDR-H2 change their position in the bound form, with the biggest movement occurring in the tip of the loop where the C α of Ala-53 shifts >15 Å (Fig. 4). This conformational change in CDR-H2 appears to be required for VEGF binding, as the canonical CDR-H2 observed in the unliganded G6-Fab causes steric clashes in the VEGF complex. Residues of the CDR-H2 form contact with VEGF and contribute ~20% of the buried surface, indicating the functional importance of this structural anomaly.

The G6 antibody was isolated from an antibody library designed to mimic the sequences in natural antibodies; accordingly, the sequence of its CDR-H2 is not unusual. In the crystal structures, all residues (50–57) undergoing conformational changes upon VEGF binding are in allowed regions of the Ramachandran plot in both the VEGF-bound and the unliganded conformation. Among these residues are three glycines at positions 50, 54, and 55, which are likely to lend CDR-H2 the flexibility required to undergo such a drastic conformational change. Interestingly, glycine is the most common amino acid for positions 54 (~40%) and 55 (~65%) and quite common in position 50 (~10%) for human antibodies (38), suggesting that this type of conformational flexibility of CDR-H2 could represent a possible binding mode that is utilized by other antibodies as well. The conformational changes observed in the bound *versus* free form of G6 demonstrate that the plasticity of the binding surfaces might be an alternative way for antibodies to achieve specificity and, at times, multi-specificities as described for other antibodies that adopt various conformations (39).

The VEGF Hot Spot—Proteins often energetically engage their binding partners with a relatively small set of contact residues or so-called hot spots. These hot spots were defined originally as a set of amino acids that when mutated into alanine result in a severe loss in binding affinity toward their binding partner (40). Some proteins have also been shown to utilize a common hot spot to interact with multiple binding partners (41). The exact properties of hot spots are still not well defined, but it appears that they are generally highly accessible, relatively hydrophobic, densely packed, adaptive, and in respect to sequence relatively well conserved (41) (42). Comparison of the functional and structural VEGF epitopes toward G6 and B20-4 show convincingly that a small but common set of residues on the VEGF surface is responsible for binding of both Fabs. Most of these residues are also important for binding to VEGFR1 (Fig. 3). In particular, the importance of the aromatic residues Phe-17, Tyr-21, and Tyr-25 and a few neighboring residues that stem from the N-terminal helix of VEGF in the complexes with VEGFR1, G6-Fab, and B20-4-Fab identifies this area as a central hot spot neces-

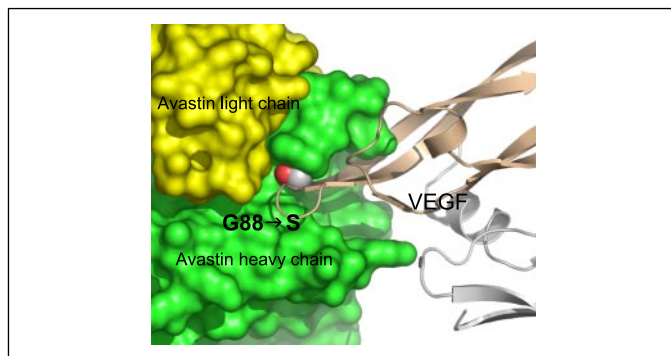


FIGURE 6. **Model of murine VEGF in complex with Avastin.** The surface of Avastin is shown in yellow (light chain) and green (heavy chain). The ribbon diagram depicts human VEGF with the exchange Gly-88 → Ser present in the mouse sequence. The C β and O γ of the modeled serine side-chain are shown as spheres. The side-chain of the serine in mouse VEGF is responsible for the weak affinity of mouse VEGF toward Avastin, as there is no room in the interface for them to be accommodated.

sary for binding all three molecules. This hot spot of VEGF is depicted in Fig. 5; it is a contiguous surface with the functionally important residues located in the center of the structural binding epitope. Although there are no structural data available on the complex between VEGF and VEGFR2, the complete disruption of binding to this receptor when Phe-17 is replaced by alanine (18) indicates that the N-terminal helix in the center of this hot spot is important for binding to this receptor as well.

DISCUSSION

Cross-reactivity of G6, B20-4, and Avastin for Human and Murine VEGF—The sequences of the human and mouse VEGF receptor binding domain (residues 8–109) differ in 10 positions. These differences are not only distributed over the entire primary sequence, they are also scattered spatially over most parts of the VEGF surface (Fig. 5). However, all residues that form the core of the binding interface toward the receptor VEGFR1 and the synthetically derived antibodies G6 and B20-4 are conserved between human and mouse. Only 3 residues at the periphery of the hot spot differ between the two species (Fig. 5). These three exchanges, Gly-65 → Ala, Gly-88 → Ser, and His-27 → Arg, represent rather conservative sequence modifications and cause minor alterations in the periphery of the binding interface that can be accommodated by G6, B20-4, and VEGFR1. Because the core of this common hot spot is completely conserved between mouse and human, these three molecules not only bind human VEGF but are also capable of binding murine VEGF with high affinity. The situation is significantly different for the Avastin antibody. The binding epitope for Avastin has been determined structurally and is shown in Fig. 3 to contrast the epitopes for G6, B20-4, and the VEGFRs. Avastin binding centers on Gly-88 and not on the conserved N terminus utilized by VEGFR1, G6, and B20-4. Gly-88 represents one of the 3 residues at the periphery of the receptor binding epitope that differ in the sequence between human and mouse VEGF; it is replaced with a serine residue in mouse. This exchange adds two non-hydrogen atoms, the side-chain of serine, into the core of the interface. The additional crowding caused by this exchange in the very tightly packed interface between the Fab and human VEGF apparently cannot be accommodated by the antibody; thus, Avastin and/or its non-humanized version A.4.6.1 is unable to bind m-VEGF with high affinity (Figs. 1 and 6). In fact, adding just one methyl group at the position of Gly-88, as introduced in the mutation G88A, into h-VEGF completely disrupts binding of the A.4.6.1 (Fig. 1). *Vice versa*, the substitution of the equivalent Ser-87 in m-VEGF with

glycine is sufficient to generate a VEGF that binds A.4.6.1 with high affinity (data not shown).

Advantage of Raising Fabs using Phage Display—Unlike B20–4 and G6, which were obtained using a synthetic antibody phage library, A.4.6.1, the parent antibody of Avastin, is a product of immune response against h-VEGF in mouse. The discovery of A.4.6.1 thus relied on sequence differences between human and murine VEGF. The hot spot of VEGF, responsible for binding its receptor tyrosine kinases and the two synthetic antibodies G6 and B20–4, is conserved in the sequence of mouse and human; therefore, antibodies that target the exact receptor binding epitope cannot be produced using the traditional method of raising antibodies in mice.

In general, amino acids that form hot spots and areas that are involved in protein-protein interaction surfaces tend to be better conserved across species than other solvent-exposed residues (22, 42–44). Therefore, traditional methods of raising antibodies may bias toward the production of tightly binding antibodies that are unable to block the protein function as they depend on the presence of sequence differences in the protein of host and the target organisms. In contrast, hot spots that are important for protein binding to biologically relevant binding partners most likely also possess all the properties that make them attractive as binding epitopes for antibodies. The generation of antibodies using phage display does not rely on sequence differences between host and target proteins but depends only on the property of the protein surface. Therefore, this new technique may bias the selection of antibodies toward high affinity binders that block protein function, as they are more likely to target surface areas that represent important protein-protein interaction sites. The two synthetically derived anti-VEGF antibodies analyzed here are important reagents to study the exact role of VEGF in mouse models,⁴ and because they block the epitopes that are important for biological function of VEGF, they represent novel potential drug candidates.

In conclusion, we have identified a hot spot on the surface of VEGF that is responsible for binding of VEGFR1 and two unrelated phage-derived antibodies. This hot spot is completely conserved between murine and human VEGF. Generation of antibodies using the phage display approach is independent of the degree of sequence conservation of the target protein. Therefore, this study emphasizes a clear advantage of generating antibodies using phage display over the traditional approach of raising antibodies using the hybridoma technology. We have further shown that Avastin binds to a non-conserved epitope adjacent to the conserved hot spot of VEGF and explained why Avastin is incapable of binding murine VEGF.

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