Antagonistic VEGF variants engineered to simultaneously bind to and inhibit VEGFR2 and $\alpha_v\beta_3$ integrin

Niv Papo, Adam P. Silverman, Jennifer L. Lahti, and Jennifer R. Cochran[1]

Department of Bioengineering, Cancer Institute, Bio-X Program, Stanford University, Stanford, CA 94305

Edited by Napoleone Ferrara, Genentech, Inc., South San Francisco, CA, and approved July 12, 2011 (received for review November 5, 2010)

Significant cross-talk exists between receptors that mediate angiogenesis, such as VEGF receptor-2 (VEGFR2) and $\alpha_v\beta_3$ integrin. Thus, agents that inhibit both receptors would have important therapeutic potential. Here, we used an antagonistic VEGF ligand as a molecular scaffold to engineer dual-specific proteins that bound to VEGFR2 and $\alpha_v\beta_3$ integrin with antibody-like affinities and inhibited angiogenic processes in vitro and in vivo. Mutations were introduced into a single-chain VEGF (scVEGF) ligand that retained VEGFR2 binding, but prevented receptor dimerization and activation. Yeast-displayed scVEGF mutant libraries were created and screened by high-throughput flow cytometric sorting to identify several variants that bound with high affinity to both VEGFR2 and $\alpha_v\beta_3$ integrin. These engineered scVEGF mutants were specific for $\alpha_v\beta_3$ integrin and did not bind to the related integrins $\alpha_v\beta_5$, $\alpha_{iib}\beta_3$, or $\alpha_5\beta_1$. In addition, surface plasmon resonance and cell binding assays showed that dual-specific scVEGF proteins can simultaneously engage both receptors. Compared to monospecific scVEGF mutants that bind VEGFR2 or $\alpha_v\beta_3$ integrin, dual-specific scVEGF proteins more strongly inhibited VEGF-mediated receptor phosphorylation, capillary tube formation, and proliferation of endothelial cells cultured on Matrigel or vitronectin-coated surfaces. Moreover, dual specificity conferred strong inhibition of VEGF-mediated blood vessel formation in Matrigel plugs in vivo, whereas monospecific scVEGF mutants that bind VEGFR2 or $\alpha_v\beta_3$ integrin were only marginally effective. Instead of relying on antibody associating domains or physical linkage, this work highlights an approach to creating dual-specific proteins where additional functionality is introduced into a protein ligand to complement its existing biological properties.

bispecific protein | directed evolution | protein engineering | yeast surface display

Growth factor receptors and adhesion molecules regulate biological functions through complex and overlapping cell signaling networks (1). Angiogenic growth factors such as VEGF bind to receptors and induce altered gene expression, endothelial cell proliferation, and cell migration, ultimately leading to the formation of new blood vessels (2, 3). Similar proliferative and proangiogenesis effects can be triggered by binding of endothelial cells to extracellular matrix ligands, most notably through adhesion receptors such as $\alpha_v\beta_3$ integrin (4, 5). Although many proangiogenic growth factors and receptors exist, there is compelling evidence for direct cross-talk between VEGF receptor-2 (VEGFR2) and $\alpha_v\beta_3$ integrin and their cell signaling pathways (6–13). For example, VEGFR2 phosphorylation and mitogenic activity are increased in endothelial cells cultured on a surface coated with vitronectin, a ligand for $\alpha_v\beta_3$ integrin, and these effects can be mitigated by integrin-specific antibodies (11). Likewise, inhibition of VEGF by an antibody or soluble VEGFR2 leads to a decrease in $\alpha_v\beta_3$-mediated tumor cell adhesion to a level similar to blocking $\alpha_v\beta_3$ integrin directly (6).

Angiogenesis is critical for potentiating biological processes in diseases such as cancer and macular degeneration (14). Inhibitors of angiogenesis, particularly the anti-VEGF monoclonal antibody bevacizumab (Avastin), have generated much therapeutic interest and have had clinical success (15–17). Inhibitors of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been shown to suppress tumor growth in preclinical models, although this has not translated to success in human subjects (18, 19). The reason for this lack of efficacy is unclear, but could be due to the highly complex in vivo regulation of VEGFR2 and integrins. For example, xenograft tumors expressing a mutant form of $\alpha_v\beta_3$ integrin that is constitutively inactive showed reduced integrin clustering, VEGF expression, and tumor neovascularization (7). In a contrasting study, β_3-integrin-null mice were shown to have enhanced VEGF-mediated angiogenesis (20). Recent work demonstrated that low concentrations of $\alpha_v\beta_3$ integrin inhibitors can increase tumor growth by promoting VEGF-stimulated angiogenesis and altering ligand recycling (21). The intertwined relationship between VEGFR2 and $\alpha_v\beta_3$ integrin suggests that a dual-specific agent capable of inhibiting both receptors would have great therapeutic potential.

The molecular interactions of VEGFR2 (22, 23) or $\alpha_v\beta_3$ integrin (24, 25) with their respective ligands have been well characterized. A VEGF homodimer binds to two molecules of VEGFR2 and dimerizes the receptor, resulting in autophosphorylation and activation of proangiogenic cell signaling pathways, including activation of mitogen-activated protein kinase, Src, Akt, and focal adhesion kinase (26). The $\alpha_v\beta_3$ integrin recognizes numerous ECM ligands, and binding leads to cell adhesion to the ECM and activation of similar growth and proliferation pathways (25, 27). In previous work, VEGF was converted to a receptor antagonist by introducing several point mutations into one of its VEGFR binding epitopes (28, 29). These mutations blocked one receptor binding site while leaving the other intact, preventing receptor dimerization. To facilitate recombinant production of such VEGF heterodimers, single-chain VEGF (scVEGF) variants were created by connecting the two VEGF subunits with a 14-amino acid linker (30) (Fig. 1*A*). Here, we constructed a scVEGF antagonist and engineered a high affinity $\alpha_v\beta_3$ integrin-binding epitope into its mutated VEGFR2-binding interface. Using this approach, we generated dual-specific scVEGF variants capable of simultaneously binding to and antagonizing the biological activity of both VEGFR2 and $\alpha_v\beta_3$ integrin in vitro and in vivo.

Results

scVEGF as a Scaffold for Engineering Molecular Recognition Properties. A single-chain version of VEGF (scVEGFwt), in which the homodimeric chains of VEGF are connected by a 14-amino

Author contributions: N.P., A.P.S., J.L.L., and J.R.C. designed research; N.P., A.P.S., and J.L.L. performed research; N.P., A.P.S., J.L.L., and J.R.C. analyzed data; and N.P., A.P.S., J.L.L., and J.R.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: jennifer.cochran@stanford.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1016635108/-/DCSupplemental.

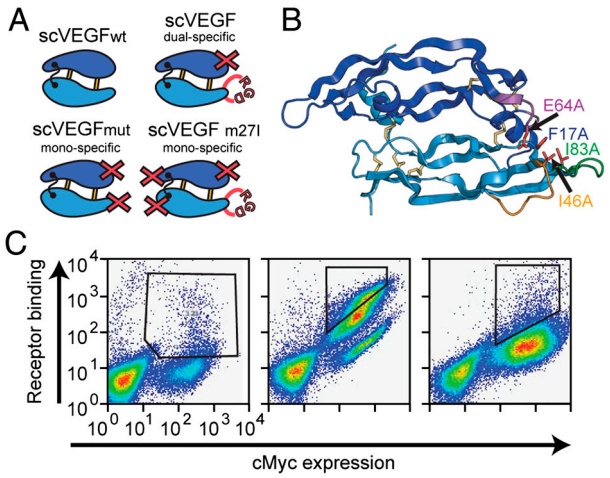

Fig. 1. Protein engineering strategy and screening of scVEGF loop libraries. (*A*) Single-chain wild-type VEGF agonist (scVEGFwt), where chains 1 and 2 of the VEGF homodimer are connected through a flexible linker. The single-chain VEGF antagonist (scVEGFmut) has one VEGFR2 binding site mutated, thereby blocking activation. Engineered dual-specific scVEGF variants are capable of binding to both VEGFR2 and $\alpha_v\beta_3$ integrin. The scVEGF m27I variant can bind $\alpha_v\beta_3$ integrin but not VEGFR2. (*B*) Structure of wild-type VEGF homodimer [Protein Data Bank (PDB) ID code 2VPF]. VEGF chain 1: dark blue; VEGF chain 2: light blue. Loop 1: purple; loop 2: orange; loop 3: green. Location of point mutations introduced into scVEGFmut are indicated in red. (*C*) FACS density dot plots of scVEGF loop libraries. Sort round 1, 250 nM $\alpha_v\beta_3$ integrin (*Left*); sort round 4, 100 nM VEGFR2-Fc (*Center*); sort round 7, 25 nM $\alpha_v\beta_3$ integrin (*Right*). X axis indicates cMyc expression and y axis indicates receptor binding. Polygons indicate sort gates used to collect yeast cells.

acid linker (GSTSGSGKSSEGKG), was displayed on the surface of yeast as a fusion to the agglutinin mating proteins (Fig. S1*A*). The construct contained a C-terminal cMyc epitope tag that allowed quantification of yeast cell surface expression levels. An antagonist variant (scVEGFmut) was also created that contained four amino acid mutations corresponding to residues critical for VEGFR recognition at one pole of the molecule: chain 1 F17A, E64A; chain 2 I46A, I83A (Fig. 1 *A* and *B* and Table S1) (28–30). Both scVEGFwt and scVEGFmut were well expressed on the yeast cell surface and bound to soluble VEGFR2 extracellular domain (Fig. S1*B*). To engineer a molecular recognition site for $\alpha_v\beta_3$ integrin into the mutated pole of scVEGF, we first determined if loops within this region were amenable to substitution without disrupting VEGFR2 binding. We prepared 32 different libraries of three such loops (Table S1), in which five to eight amino acids were removed in different registers and replaced with randomized sequences of six to nine amino acids. We displayed each library on the yeast cell surface and measured its expression level and relative binding to 50 nM VEGFR2 (Fig. S2*A*). Nearly all VEGFR2 binding was abolished for substitutions in loop 1 (Fig. 1*B*, purple), whereas substitutions in loop 2 (Fig. 1*B*, orange) varied and bound VEGFR2 at levels ranging from 20–80% of scVEGFmut, or in a few cases was unchanged. In contrast, all registers and loop lengths tested for loop 3 (Fig. 1*B*, green) were well expressed and bound VEGFR2 at levels comparable to scVEGFmut, indicating that loop 3 could potentially be replaced and engineered to bind a different target, such as $\alpha_v\beta_3$ integrin.

Construction and Screening of scVEGF Libraries for Mutants That Bind Both VEGFR2 and $\alpha_v\beta_3$ Integrin. Substitution of scVEGFmut loop 3 (IKPHQGQ) with an Arg-Gly-Asp (RGD)-containing sequence derived from fibronectin (TGRGDSPAS) resulted in a mutant that was well expressed on yeast, but did not significantly bind $\alpha_v\beta_3$ integrin at concentrations up to 500 nM (Fig. S2*B*). Thus, to optimize this loop sequence for high affinity integrin binding, we created three yeast-displayed libraries (approximately 2×10^7 transformants each) in which loop 3 of scVEGFmut was substituted with the sequences XXRGDXXXX, XXXRGDXXX, or XXXXRGDXX, where X corresponds to any amino acid. The individual libraries contained relatively few clones that bound to 250 nM $\alpha_v\beta_3$ integrin (Fig. S2*C*). To enrich for integrin binders, the pooled libraries were incubated with detergent-solubilized $\alpha_v\beta_3$ integrin and an anti-cMyc antibody, followed by fluorescently labeled secondary antibodies. Yeast with the highest levels of receptor binding relative to protein expression were isolated by FACS, propagated, and the process was repeated for multiple sort rounds (Fig. 1*C*). Seven sort rounds were performed using decreasing concentrations of $\alpha_v\beta_3$ integrin in each round (ranging from 250 nM in round 1 to 25 nM in round 7). In an intermediate sort round (round 4), yeast were screened against VEGFR2 to remove a population of proteins with weaker receptor binding (Fig. 1*C*). Sixteen clones were sequenced after the final sort round, and seven unique sequences were obtained (Table S1). We chose several yeast-displayed variants that bound with the highest affinities to VEGFR2 and $\alpha_v\beta_3$ integrin for further characterization. scVEGFwt, scVEGFmut, and the dual-specific mutants 7H, 7I, and 7P were expressed in *Pichia pastoris* and purified and characterized as described (*SI Materials and Methods* and Fig. S3). To measure the effects of integrin binding alone we also expressed a control protein, scVEGF m27I (Fig. 1*A*), which contains the integrin-binding loop from mutant 7I and the amino acid mutations F17A, E64A, I46A, and I83A in both chain 1 and chain 2 of the scVEGF scaffold. Based on these mutations, scVEGF m27I was expected not to recognize VEGFR2 at either pole of the molecule, but to bind $\alpha_v\beta_3$ integrin with high affinity.

Binding of scVEGF Proteins to VEGFR2 and Integrin Receptors. Binding affinities of scVEGF proteins were measured against VEGFR2 and integrins expressed on the surface of several cell lines. The receptor expression in each cell line was measured by flow cytometry (Fig. S4). The dual-specific mutants 7H, 7I, and 7P bound with similar apparent K_D values of approximately 35 nM to K562 human leukemia cells stably transfected to express $\alpha_v\beta_3$ integrin (Fig. 2*A* and Table 1). As these cells do not express VEGFR2, binding was not observed for scVEGFwt and scVEGFmut. The monospecific mutant scVEGF m27I bound to K562-$\alpha_v\beta_3$ cells with comparable affinity to the dual-specific mutant 7I, indicating that introduction of additional mutations used to create m27I did

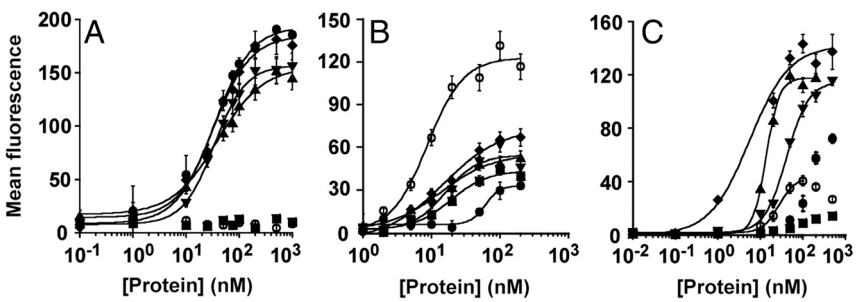

Fig. 2. Binding titrations against mammalian cell lines. (*A*) *K*562-$\alpha_v\beta_3$, (*B*) PAE/KDR, and (*C*) HUVEC. Mean fluorescence values of scVEGFwt (○), scVEGFmut (■), scVEGF m27I (•), scVEGF 7H (▲), scVEGF 7I (◆), and scVEGF 7P (▼) were determined by flow cytometry using a fluorescently labeled antibody against a hexahistidine or FLAG epitope tag. Data shown are the average of triplicate experiments and error bars represent standard deviations.

Table 1. Equilibrium binding affinities and kinetic rate constants of VEGF and scVEGF variants to cell surface receptors or immobilized VEGFR2

Variant	Cell lines, apparent K_D, nM				SPR (immobilized VEGFR2)		
	K562-$\alpha_v\beta_3$	PAE/KDR	HUVEC	SVR	K_D, nM	k_{on} (M^{-1} s^{-1}) × 10^5	k_{off} (s^{-1}) × 10^{-3}
$VEGF_{121}$	*	*	*	*	3.1 ± 0.4	3.3 ± 0.2	1.0 ± 0.1
scVEGFwt	†	9 ± 2	28 ± 2	‡	2.5 ± 0.4	2.4 ± 0.4	0.7 ± 0.1
scVEGFmut	†	19 ± 4	‡	†	17.1 ± 0.4	0.9 ± 0.1	1.5 ± 0.1
scVEGF m27I	36 ± 4	60 ± 8 §	‡	‡	*	*	*
scVEGF 7H	39 ± 5	14 ± 3 §	14 ± 1	48 ± 6	43 ± 9	0.4 ± 0.1	1.8 ± 0.1
scVEGF 7I	32 ± 3	15 ± 3 §	6 ± 2	25 ± 3	50 ± 10	0.4 ± 0.1	1.8 ± 0.3
scVEGF 7P	34 ± 2	16 ± 5 §	42 ± 3	43 ± 5	50 ± 10	0.3 ± 0.1	1.7 ± 0.2

Cell binding data were fitted to sigmoidal curves to calculate apparent dissociation constants. SPR sensorgram curves were globally fit with a 1:1 Langmuir binding model. Binding experiments were conducted in triplicate and errors denote standard deviations.

*Not measured.

†No binding was observed at the highest concentration tested (1 μM).

‡K_D value could not be accurately determined.

§scVEGF protein also binds to untransfected PAE cells, presumably through porcine integrins (Fig. S6*A*).

not affect integrin binding. None of the scVEGF proteins bound to wild-type K562 cells, which naturally express $\alpha_5\beta_1$ integrin, or to K562 cells transfected with $\alpha_v\beta_5$ or $\alpha_{IIb}\beta_3$ integrins (Fig. S5).

Similar relative binding affinities were measured for scVEGFwt, scVEGFmut, and scVEGF 7H, 7I, and 7P to porcine endothelial cells stably transfected to express human VEGFR2 (PAE/KDR) (Fig. 2*B* and Table 1). However, the maximum level of cell binding was significantly higher for scVEGFwt, likely due to differences in apparent affinity where scVEGF variants with monovalent VEGFR2 binding reach equilibrium at a lower bound level compared to scVEGFwt. In contrast, compared to scVEGFwt, scVEGFmut, and scVEGF m27I, the dual-specific mutants 7H, 7I, and 7P exhibited a substantial increase in maximum levels of binding to human umbilical vein endothelial cells (HUVECs) that express both human VEGFR2 and $\alpha_v\beta_3$ integrin, demonstrating their ability to effectively bind to both receptors (Fig. 2*C* and Table 1). The monospecific mutant scVEGF m27I exhibited binding to PAE/KDR cells, which do not express human $\alpha_v\beta_3$ integrin (Fig. 2*B* and Fig. S4). However, both scVEGF m27I and the scVEGF 7I variant from which it was derived bound with similar apparent affinity to untransfected parental PAE cells, which express porcine $\alpha_v\beta_3$ integrin (31) (Fig. S6*A*). In addition, scVEGF mutants 7H and 7P, but not scVEGFwt or scVEGFmut, bound to the parental PAE cells, indicating cross-reactivity of the mutants to both human and porcine integrin receptors.

Sequence alignments showed that human and murine α_v and β_3 are approximately 90% homologous, whereas the extracellular domains of human and murine VEGFR2 share 85% sequence homology (32). To test cross-reactivity with murine receptors for in vivo experiments, we measured binding to angiosarcoma endothelial cells (SVR), which express both murine VEGFR2 and $\alpha_v\beta_3$ integrin (Fig. S4). The dual-specific mutants 7H, 7I, and 7P bound to SVR cells with apparent K_D values of 25–50 nM (Table 1 and Fig. S6*B*). In comparison, scVEGF m27I and scVEGFwt exhibited weaker binding to these cells, whereas scVEGFmut did not bind. The dual-specific mutants were created by introducing an integrin-binding loop into scVEGFmut without directly altering VEGF/VEGFR2 interactions. However, the higher binding levels of the dual-specific variants compared to their monospecific counterparts show that both receptors contribute to these interactions. We used a blocking experiment to further test if binding of scVEGF mutant 7I to SVR cells was mediated by both VEGFR2 and $\alpha_v\beta_3$ integrin. Incubation of SVR cells with an excess of integrin competitor completely blocked binding of scVEGF 7I (Fig. S6*C*). In addition, significant inhibition of scVEGF 7I binding was observed after addition of an excess of VEGFR2 competitor, indicating that integrin engagement is necessary for cell surface binding of scVEGF 7I, and appears to increase the binding to murine VEGFR2. Binding of scVEGF m27I to SVR cells was blocked by an excess of integrin competitor, but not VEGFR2 competitor, further confirming that scVEGF m27I binds monospecifically to $\alpha_v\beta_3$ integrin and does not bind VEGFR2 (Fig. S6*C*).

The binding kinetics of scVEGF proteins to VEGFR2 were determined by surface plasmon resonance (Fig. S7*A*). In comparison, the measured kinetic rate constants of $VEGF_{121}$ to immobilized VEGFR2 were in overall agreement with those previously reported (33) (Table 1). The dual-specific mutants 7H, 7I, and 7P had approximately 20-fold weaker affinity for VEGFR2 compared to scVEGFwt and $VEGF_{121}$, and 2-fold weaker affinity than scVEGFmut (Table 1). The weaker binding affinity of these mutants compared to scVEGFmut primarily result from slower kinetic on-rates. Similar binding experiments were attempted with immobilized $\alpha_v\beta_3$ integrin; however, we were unable to find suitable surface regeneration conditions after exhaustive efforts.

Simultaneous Binding of scVEGF Proteins to VEGFR2 and $\alpha_v\beta_3$ Integrin. While engaged with integrin receptors on *K*562-$\alpha_v\beta_3$ cells, the dual-specific scVEGF mutants were able to bind to soluble recombinant human VEGFR2 extracellular domain (Fig. S6*D*). These results were confirmed with surface plasmon resonance by flowing scVEGF proteins over a surface of immobilized VEGFR2 and then monitoring the increase in binding response upon addition of soluble recombinant human $\alpha_v\beta_3$ integrin (Fig. 3). Alternatively, scVEGF proteins were flowed over a surface of immobilized $\alpha_v\beta_3$ integrin, and the binding increase was measured in response to the addition of soluble VEGFR2 (Fig. S7*B*). In all of these experiments, scVEGF mutants 7H, 7I, and 7P simultaneously bound to both VEGFR2 and $\alpha_v\beta_3$ integrin, whereas scVEGFmut did not. No binding interactions were detected between immobilized VEGFR2 and soluble $\alpha_v\beta_3$ integrin or immobilized $\alpha_v\beta_3$ integrin and soluble VEGFR2.

scVEGF Proteins with Dual Receptor Binding Strongly Inhibit Ligand-Mediated Activity in Endothelial Cells. In the absence of vitronectin, the dual-specific mutants 7H, 7I, and 7P inhibited VEGF-stimulated VEGFR2 phosphorylation in HUVECs at similar levels compared to scVEGFmut and scVEGF m27I (Fig. 4*A*, *Left*). However, when HUVECs were grown on vitronectin-coated surfaces, scVEGF 7I strongly inhibited VEGF-stimulated VEGFR2 phosphorylation compared to its monospecific counterparts scVEGFmut and scVEGF m27I (Fig. 4*A*, *Right*). scVEGF mutants 7H and 7P inhibited greater levels of VEGFR2-phosphorylation than the monospecific variant scVEGFmut and were slightly improved over scVEGF m27I. Next, scVEGF mutants 7H, 7I, and 7P all strongly inhibited VEGF-stimulated prolifera-

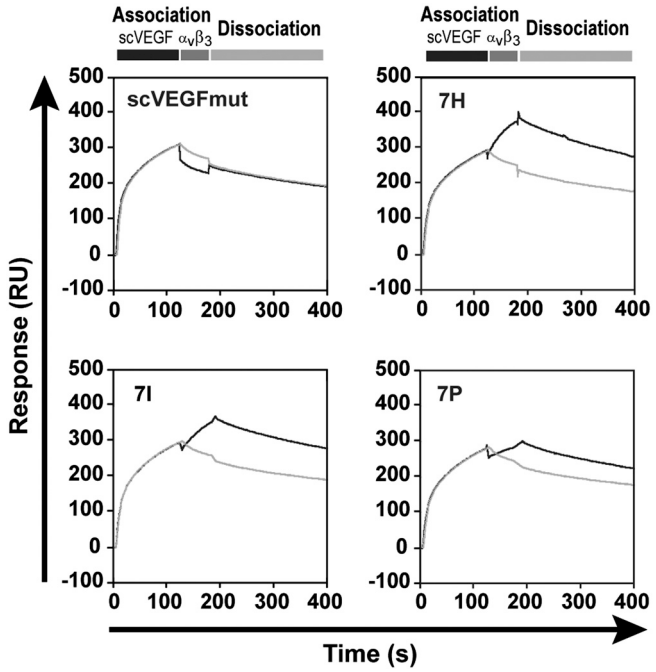

Fig. 3. Dual binding of scVEGF proteins to recombinant VEGFR2 and $\alpha_v\beta_3$ integrin. Representative surface plasmon resonance sensorgram traces of scVEGF proteins flowed over a VEGFR2-immobilized dextran sensor chip. After scVEGF was bound to the chip, a subsequent injection was made of buffer alone (gray) or $\alpha_v\beta_3$ integrin (black). scVEGF variants 7H, 7I, and 7P, but not scVEGFmut, showed an increase in response units after injection of soluble $\alpha_v\beta_3$ integrin, indicating simultaneous binding to both receptors.

tion compared to the monospecific variants scVEGFmut and scVEGF m27I when HUVECs were grown on vitronectin-coated surfaces (Fig. 4*B*, *Right*). In contrast, the dual-specific and monospecific variants inhibited proliferation at similar levels in the absence of vitronectin (Fig. 4*B*, *Left*). Addition of the scVEGFwt agonist to HUVECs caused an increase in cell proliferation that was further potentiated in the presence of vitronectin. Finally, scVEGF mutants 7H, 7I, and 7P all strongly inhibited HUVEC adhesion mediated to vitronectin-coated surfaces with IC_{50} values <20 nM (Fig. 4*C*). scVEGF m27I inhibited similar levels of cell adhesion, indicating that the integrin-binding loops of 7I and m27I are functionally equivalent. scVEGFmut did not inhibit cell adhesion at any concentration tested, and scVEGFwt conversely promoted cell adhesion to vitronectin. These results support a model where a VEGF agonist mediates VEGFR2-$\alpha_v\beta_3$ integrin cross-talk upon binding VEGFR2, stimulating cell adhesion via $\alpha_v\beta_3$ integrin.

scVEGF Proteins with Dual Receptor Binding Strongly Inhibit Angiogenic Processes In Vitro and In Vivo. scVEGF mutants 7H and 7I inhibited VEGF-induced capillary tube formation by HUVECs grown on Matrigel, an extracellular basement membrane matrix (34), with IC_{50} values of approximately 10 nM (Fig. 5 *A* and *B*). scVEGF mutant 7P inhibited capillary tube formation to a lesser extent, similar to inhibition observed with scVEGFmut and scVEGF m27I. We next measured the ability of the scVEGF proteins to block angiogenesis in vivo using a Matrigel plug assay (35). We chose scVEGF mutant 7I for this study, as it had the strongest binding to HUVECs and SVR cells and was the most effective at inhibiting VEGFR2 phosphorylation and capillary tube formation. C3H/HeN mice were subcutaneously injected with Matrigel alone, a $VEGF_{165}$/heparin positive control, or $VEGF_{165}$/heparin along with 20 nM scVEGF proteins. After 10 d, the Matrigel plugs were removed from the animals and processed for microscopy and hemoglobin quantification. Strikingly, the dual-specific mutant 7I inhibited angiogenesis to levels approaching that of the negative control (Fig. 5 *C* and *D*). The monospecific variants scVEGFmut and scVEGF m27I were significantly less effective at inhibiting angiogenesis compared to scVEGF mutant 7I, indicating that both receptor pathways play a role in these biological processes (Fig. 5 *C* and *D*). H&E staining of Matrigel sections showed the presence of red

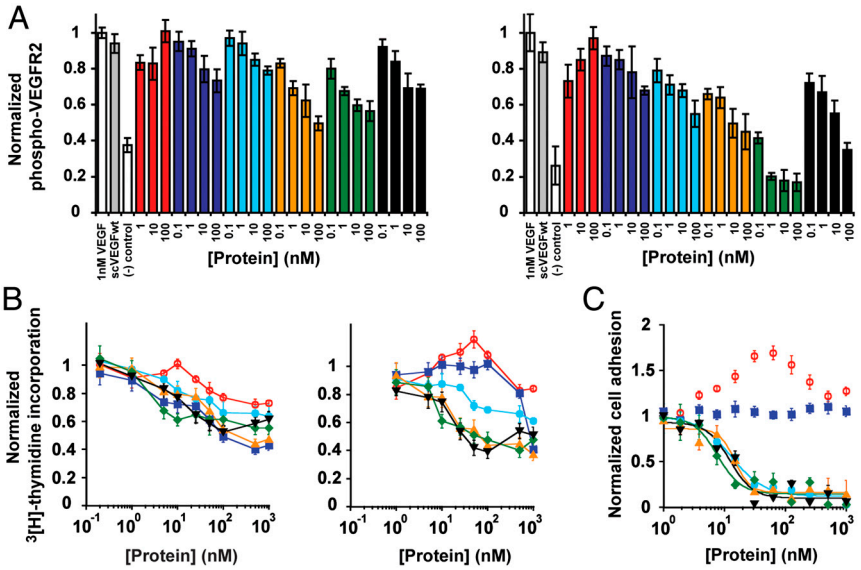

Fig. 4. Antagonistic activity of scVEGF proteins in cell culture assays. (*A*) Effect of scVEGF proteins on VEGF-mediated VEGFR2 phosphorylation in HUVECs in the absence (left bar graph) or presence (right bar graph) of vitronectin. Colored bars indicate phospho-VEGFR2 levels observed upon coincubation of 1 nM $VEGF_{121}$ with varying concentrations of scVEGFwt (red), scVEGFmut (blue), scVEGF m27I (cyan), scVEGF 7H (orange), scVEGF 7I (green), and scVEGF 7P (black). White bars represent unstimulated cells (– control) or cells treated with 1 nM $VEGF_{121}$ alone. Compared to 1 nM $VEGF_{121}$, similar levels of agonistic activity were seen with cells treated with 1 nM scVEGFwt agonist alone (gray bars). (*B*) Effect of scVEGF proteins on VEGF-mediated HUVEC proliferation in the absence (left line graph) or presence (right line graph) of vitronectin. As above, 1 nM $VEGF_{121}$ was coincubated with varying concentrations of scVEGF proteins. (*C*) Inhibition or promotion of HUVEC adhesion to vitronectin-coated wells mediated by scVEGF proteins. Symbols: scVEGFwt (red ○), scVEGFmut (blue ■), scVEGF m27I (cyan •), scVEGF 7H (orange ▲), scVEGF 7I (green ◆), and scVEGF 7P (black ▼). Data shown are the average of triplicate values and error bars represent standard deviations.

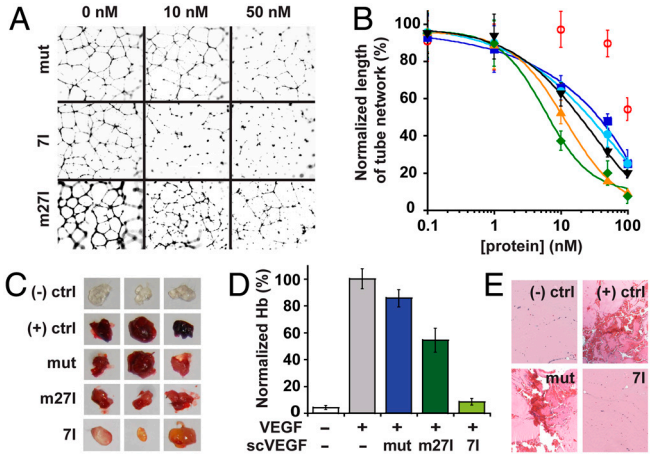

Fig. 5. Inhibition of angiogenesis by scVEGF proteins. (*A* and *B*) Matrigel-induced capillary tube formation of HUVECs treated with 10 nM $VEGF_{121}$ alone or with various concentrations of scVEGF proteins. (*A*) After 20 h, the vital dye calcein-AM was added and tube formation was visualized by microscopy (50×). Representative images are shown. (*B*) Quantification expressed as the percent length of tube formation compared to 10 nM $VEGF_{121}$. Symbols: scVEGFwt (red ○), scVEGFmut (blue ■), scVEGF m27I (cyan •), scVEGF 7H (orange ▲), scVEGF 7I (green ◆), and scVEGF 7P (black ▼). (*C–E*) Inhibition of in vivo angiogenesis in Matrigel plugs implanted in C3H/HeN mice. Matrigel plugs contained phosphate buffered saline (– ctrl), $VEGF_{165}$/heparin (+ ctrl), or $VEGF_{165}$/heparin + 20 nM scVEGF proteins. (*C*) Plugs were removed from mice and photographed after 10 d. (*D*) Hemoglobin (Hb) content within Matrigel plugs was quantified and depicted as the percent of Hb compared to $VEGF_{165}$/heparin (gray bar). (*E*) H&E staining of 5-μm Matrigel sections for blood vessel formation. Error bars represent standard deviations of experiments performed in triplicate.

blood cells and blood vessel formation in the positive control and scVEGFmut-treated samples, which were completely absent in the negative control and scVEGF 7I-treated samples (Fig. 5*E*). Immunohistochemistry confirmed that these blood cells, which arose from the mouse, expressed murine VEGFR2 and $\alpha_v\beta_3$ integrin, and expressed the endothelial cell marker CD34, which colocalized with VEGFR2 expression (Fig. S8).

Discussion

The biopharmaceutical industry has been rapidly moving toward the development of multispecific proteins that can bind to and modulate the activity of more than one clinical target (36). Such agents can potentially increase binding affinity, avidity, potency, and selectivity compared to protein therapeutics that target a single cell surface receptor. The vast majority of current bispecific protein therapeutics are antibodies or antibody fragments that are assembled through associating domains, or by physically tethering two protein domains through a flexible linker (37, 38). We created a dual-specific therapeutic protein that does not rely on associating domains or physical linkage, but rather is based on a naturally occurring ligand into which an additional high affinity receptor binding epitope has been introduced without disrupting the original function.

Extracellular matrix proteins bind to integrin receptors through an RGD motif, which must be presented in a particular conformation for integrin binding (24, 39). Hence, simple substitution of scVEGFmut loop 3 with an RGD-containing sequence grafted from the integrin-binding domain of fibronectin (TGRGDSPAS) did not confer binding to $\alpha_v\beta_3$ integrin (Fig. S2*B*). Similarly, our initial RGD-loop libraries contained very few integrin binders, which were enriched over multiple rounds of sorting. In our library-isolated clones, the RGD motif was found in the center of the loop, and there was little consensus among the flanking residues except for the presence of a proline in the first loop position for five out of the seven sequences. We were surprised to find that scVEGF mutant 7I contained an 11-amino acid loop, two residues longer than the 9-amino acid RGD loop used for the library construction. Interestingly, this mutant contained the sequence SPAS immediately following the RGD tripeptide motif, similar to the RGDSPAS sequence found in fibronectin. As expected, yeast-displayed scVEGFwt and scVEGFmut bound with high affinity to VEGFR1, consistent with previous studies on wild-type VEGF and similar protein mutants (29, 40). VEGFR1 is thought to modulate the activity of VEGFR2 and also plays a role in a number of human diseases (2); thus it will be interesting in future studies to explore the biological effects of known point mutations that diminish VEGFR1 binding.

We showed by surface plasmon resonance and cell surface staining that dual-specific scVEGF variants can simultaneously bind to both VEGFR and $\alpha_v\beta_3$ integrin, leading to antagonism of immediate signaling events (VEGFR2 phosphorylation) and downstream processes (proliferation), particularly in the presence of vitronectin. In contrast, the scVEGFwt agonist, which can presumably bind to and dimerize two VEGFR2 molecules, exhibited bell-shaped curves in many of the binding and biological assays, suggesting less receptor cross-linking or receptor internalization and autoinhibition of signaling at higher concentrations. This reduction in activity at high ligand concentrations has also been observed with wild-type VEGF (28, 41) and other growth factors (42). The scVEGFwt agonist strongly promoted cell adhesion to vitronectin, providing further evidence of cross-talk between VEGFR and $\alpha_v\beta_3$ integrin, and highlights the potential importance of blocking both receptors for more effective inhibition of angiogenesis.

The scVEGF mutant 7I exhibited increased binding to HUVECs and was a more potent inhibitor of biological activity compared to its monospecific counterparts, highlighting the effects of the dual functionality we engineered into this protein. In addition, mutant 7I was more effective than 7H and 7P at inhibiting ligand-mediated VEGFR2 phosphorylation in HUVECs, in agreement with its increased binding to these cells at lower concentrations. However, scVEGF 7I was similar to mutants 7H and 7P in its ability to inhibit ligand-mediated cell proliferation, which measures a biological response over longer time periods after initial stimulation. These results suggest that in cell culture models mutant 7I might be a more potent inhibitor of early activation events, which are more closely coupled to receptor binding interactions. scVEGF mutant 7P showed decreased ability to simultaneously engage VEGFR2 and $\alpha_v\beta_3$ integrin compared to mutants 7H and 7I (Fig. 3 and Fig. S6*D*), perhaps due to reduced flexibility within its integrin-binding loop, which contains a higher percentage of proline residues compared to the other engineered variants (Table S1).

Remarkably, the dual-specific mutant 7I, but not the monospecific variants scVEGFmut or scVEGF m27I, exhibited nearly complete inhibition of VEGF-mediated murine blood vessel formation within implanted Matrigel plugs. This inhibition could be due to biological effects mediated by blocking both VEGFR2 and $\alpha_v\beta_3$ integrin, or through blocking receptor cross-talk. These results indicate promise for further clinical development of dual-specific VEGFR2/$\alpha_v\beta_3$ integrin antagonists. Currently, drug cocktails are used to obtain additive or synergistic therapeutic effects. For example, combination of etaracizumab (anti-$\alpha_v\beta_3$ integrin) and bevacizumab (anti-VEGF) was superior to single agent therapy in orthotopic mouse models of ovarian cancer (8). In another study, combination therapy with the cyclic RGD peptide EMD270179 and the small molecule VEGFR2 antagonist SU5416 exhibited greater inhibition of functional vessel density and subcutaneous tumor growth and metastasis compared to single agent treatment (13). Dual-specific proteins could substantially reduce costs associated with drug development, production, clinical testing, and regulatory approval compared with separate

agents used in combination therapies. Moreover, agents that have the potential to modulate multiple receptors would be beneficial due to differential expression of disease markers among patients and the ability of this expression to change over time. The pharmacokinetics and therapeutic efficacy of dual-specific and monospecific scVEGF mutants will be interesting to explore in future studies. Compared to monoclonal antibodies (approximately 150 kDa), the engineered scVEGF proteins (approximately 25 kDa) are expected to have faster blood clearance, but their circulation half-life could be increased for therapeutic applications through the addition of polyethylene glycol or through fusion to an antibody Fc domain.

In summary, we demonstrate application of a growth factor as a molecular scaffold for engineering dual-specific proteins that bind to and inhibit the biological activity of two clinically relevant receptors. We envision this approach as a general strategy that can be applied to other ligands to develop multifunctional proteins against additional biomedical targets.

Material and Methods

For materials and methods, see *SI Materials and Methods*

ACKNOWLEDGMENTS. We thank Scott Blystone (State University of New York Upstate Medical University, Syracuse, NY) for providing the integrin-transfected K562 cell lines, and Juergen Willmann (Stanford University, Stanford, CA) for providing the PAE/KDR and SVR cells. We also acknowledge the Stanford Protein and Nucleic Acid Facility for assistance with surface plasmon resonance experiments, Roy Wollman for assistance with fluorescence microscopy, Frezghi Habte for assistance with Matrigel sprout image analysis, and Kira Foygel for assistance with immunohistochemistry. This work was funded by an American Cancer Society Research Scholars Grant (to J.R.C). N.P. was supported by a European Molecular Biology Organization Fellowship, A.P.S. was supported by Cancer Biology Training Grant PHS NRSA 5T32 CA09302, and J.L.L. was supported by California Breast Cancer Research Program Grant 13GB-0161 and a Gerald J. Lieberman Fellowship.

1. Somanath PR, Ciocea A, Byzova TV (2009) Integrin and growth factor receptor alliance in angiogenesis. *Cell Biochem Biophys* 53:53–64.
2. Ferrara N (2004) Vascular endothelial growth factor: Basic science and clinical progress. *Endocr Rev* 25:581–611.
3. Adams RH, Alitalo K (2007) Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 8:464–478.
4. Brooks PC, Clark RA, Cheresh DA (1994) Requirement of vascular integrin alphav beta3 for angiogenesis. *Science* 264:569–571.
5. Stupack DG, Cheresh DA (2004) Integrins and angiogenesis. *Curr Top Dev Biol* 64:207–238.
6. Byzova TV, et al. (2000) A mechanism for modulation of cellular responses to VEGF: Activation of the integrins. *Mol Cell* 6:851–860.
7. De S, et al. (2005) VEGF-integrin interplay controls tumor growth and vascularization. *Proc Natl Acad Sci USA* 102:7589–7594.
8. Kim TJ, et al. (2009) Combined anti-angiogenic therapy against VEGF and integrin alphav beta3 in an orthotopic model of ovarian cancer. *Cancer Biol Ther* 8:2263–2272.
9. Mahabeleshwar GH, Feng W, Reddy K, Plow EF, Byzova TV (2007) Mechanisms of integrin-vascular endothelial growth factor receptor cross-activation in angiogenesis. *Circ Res* 101:570–580.
10. Serini G, Napione L, Arese M, Bussolino F (2008) Besides adhesion: New perspectives of integrin functions in angiogenesis. *Cardiovasc Res* 78:213–222.
11. Soldi R, et al. (1999) Role of alphav beta3 integrin in the activation of vascular endothelial growth factor receptor-2. *EMBO J* 18:882–892.
12. Somanath PR, Malinin NL, Byzova TV (2009) Cooperation between integrin $\alpha_v\beta_3$ and VEGFR2 in angiogenesis. *Angiogenesis* 12:177–185.
13. Strieth S, et al. (2006) Antiangiogenic combination tumor therapy blocking α_v-integrins and VEGF-receptor-2 increases therapeutic effects *in vivo*. *Int J Cancer* 119:423–431.
14. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1:27–31.
15. Folkman J (2007) Angiogenesis: An organizing principle for drug discovery? *Nat Rev Drug Discov* 6:273–286.
16. Eskens FA (2004) Angiogenesis inhibitors in clinical development; where are we now and where are we going? *Br J Cancer* 90:1–7.
17. Ferrara N, Mass RD, Campa C, Kim R (2007) Targeting VEGF-A to treat cancer and age-related macular degeneration. *Annu Rev Med* 58:491–504.
18. Tucker GC (2006) Integrins: Molecular targets in cancer therapy. *Curr Oncol Rep* 8:96–103.
19. Stupp R, Ruegg C (2007) Integrin inhibitors reaching the clinic. *J Clin Oncol* 25:1637–1638.
20. Reynolds AR, et al. (2004) Elevated Flk1 (vascular endothelial growth factor receptor 2) signaling mediates enhanced angiogenesis in beta3-integrin-deficient mice. *Cancer Res* 64:8643–8650.
21. Reynolds AR, et al. (2009) Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. *Nat Med* 15:392–400.
22. Muller YA, et al. (1997) Vascular endothelial growth factor: Crystal structure and functional mapping of the kinase domain receptor binding site. *Proc Natl Acad Sci USA* 94:7192–7197.
23. Wiesmann C, et al. (1997) Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. *Cell* 91:695–704.
24. Xiong JP, Stehle T, Goodman SL, Arnaout MA (2003) Integrins, cations and ligands: Making the connection. *J Thromb Haemost* 1:1642–1654.
25. Luo BH, Carman CV, Springer TA (2007) Structural basis of integrin regulation and signaling. *Annu Rev Immunol* 25:619–647.
26. Kowanetz M, Ferrara N (2006) Vascular endothelial growth factor signaling pathways: Therapeutic perspective. *Clin Cancer Res* 12:5018–5022.
27. Hynes RO (2002) Integrins: Bidirectional, allosteric signaling machines. *Cell* 110:673–687.
28. Fuh G, Li B, Crowley C, Cunningham B, Wells JA (1998) Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. *J Biol Chem* 273:11197–11204.
29. Siemeister G, et al. (1998) An antagonistic vascular endothelial growth factor (VEGF) variant inhibits VEGF-stimulated receptor autophosphorylation and proliferation of human endothelial cells. *Proc Natl Acad Sci USA* 95:4625–4629.
30. Boesen TP, Soni B, Schwartz TW, Halkier T (2002) Single-chain vascular endothelial growth factor variant with antagonist activity. *J Biol Chem* 277:40335–40341.
31. Maeshima Y, Colorado PC, Kalluri R (2000) Two RGD-independent alphav beta3 integrin binding sites on tumstatin regulate distinct anti-tumor properties. *J Biol Chem* 275:23745–23750.
32. Popkov M, et al. (2004) Human/mouse cross-reactive anti-VEGF receptor 2 recombinant antibodies selected from an immune b9 allotype rabbit antibody library. *J Immunol Methods* 288:149–164.
33. Huang X, Gottstein C, Brekken RA, Thorpe PE (1998) Expression of soluble VEGF receptor 2 and characterization of its binding by surface plasmon resonance. *Biochem Biophys Res Commun* 252:643–648.
34. Kleinman HK, et al. (1986) Basement membrane complexes with biological activity. *Biochemistry* 25:312–318.
35. Passaniti A, et al. (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 67:519–528.
36. Cochran JR (2010) Engineered proteins pull double duty. *Sci Transl Med* 2:17ps15.
37. Fischer N, Leger O (2007) Bispecific antibodies: Molecules that enable novel therapeutic strategies. *Pathobiology* 74:3–14.
38. Kufer P, Lutterbuse R, Baeuerle PA (2004) A revival of bispecific antibodies. *Trends Biotechnol* 22:238–244.
39. Haas TA, Plow EF (1994) Integrin-ligand interactions: A year in review. *Curr Opin Cell Biol* 6:656–662.
40. Keyt BA, et al. (1996) Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *J Biol Chem* 271:5638–5646.
41. Gerber HP, et al. (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273:30336–30343.
42. Blechman JM, et al. (1995) The fourth immunoglobulin domain of the stem cell factor receptor couples ligand binding to signal transduction. *Cell* 80:103–113.