A Potent D-Protein Antagonist of VEGF-A is Nonimmunogenic, Metabolically Stable, and Longer-Circulating in Vivo


ABSTRACT: Polypeptides composed entirely of D-amino acids and the achiral amino acid glycine (D-proteins) inherently have in vivo properties that are proposed to be near-optimal for a large molecule therapeutic agent. Specifically, D-proteins are resistant to degradation by proteases and are anticipated to be non-immunogenic. Furthermore, D-proteins are manufactured chemically and can be engineered to have other desirable properties, such as improved stability, affinity, and pharmacokinetics. Thus, a well-designed D-protein therapeutic would likely have significant advantages over L-protein drugs. Toward the goal of developing D-protein therapeutics, we previously generated RFX001.D, a D-protein antagonist of natural vascular endothelial growth factor A (VEGF-A) that inhibited binding to its receptor. However, RFX001.D is unstable at physiological temperatures ($T_m > 95^\circ C$), high affinity for VEGF-A ($K_d = 6 \text{ nM}$), and improved receptor blocking. Comparison of the two enantiomeric forms of RFX037 revealed that the D-protein is more stable in mouse, monkey, and human plasma and has a longer half-life in vivo in mice. Significantly, RFX037.D was nonimmunogenic in mice, whereas the L-enantiomer generated a strong immune response. These results confirm the potential utility of synthetic D-proteins as alternatives to therapeutic antibodies.

Monoclonal antibodies, molecules with intrinsic high specificities and affinities for their targets, have been adopted for clinical use as human therapeutics with great success. However, major drawbacks to this technology are immunogenicity and the limitations imposed by the large size and structural complexity of antibodies, which can impede the generation of new or improved molecules. Consequently, there is great interest in developing new strategies to efficiently create molecules that bind medically relevant targets with high affinity and specificity, and which can also obviate such issues. Several novel scaffolds have been developed to engineer proteins, but these have limited appeal for producing therapeutics as their products are often immunogenic and have short half-lives due to renal clearance and proteolytic degradation.

An underexplored but highly promising alternative approach is to engineer chemically synthesized D-proteins, comprised entirely of D-amino acids and the achiral amino acid glycine, to bind and modify the behavior of natural L-proteins. A D-amino acid is the enantiomer of a L-amino acid, and for a given sequence, the resulting D- and L-proteins have mirror image structures. It has long been recognized that natural L-protein receptors, enzymes, and especially proteases will not bind the mirror image D-protein form of their natural targets. This would permit the D-protein to resist proteolytic degradation and avoid triggering an immune response, and indeed, this has been observed in the case of rubredoxin. Resistance to proteolytic degradation, and the lack of immunogenicity, are likely intrinsic properties of D-proteins. Furthermore, D-proteins...
are accessible by chemical synthesis and can be engineered to improve stability, affinity, and pharmacokinetics. These molecules hold great potential as alternatives to conventional recombinant protein therapeutics.6–20

As a step toward generating D-protein therapeutics, we recently developed RFX001.D, a D-protein antagonist of angiogenic vascular endothelial growth factor A (VEGF-A), using a combination of total chemical protein synthesis and mirror image phage display of proteins.21 A phage library was created by designed mutagenesis of the 56-residue B1 domain of streptococcal protein G (GB1), and the library was screened against the mirror image protein D-VEGF-A.22,23 The lead molecule RFX001.L had good affinity for D-VEGF-A, and conversely, its synthesized D-form enantiomer RFX001.D bound specifically to natural VEGF-A, showing reciprocal chiral specificity. The X-ray crystal structure of the heterochiral complex of RFX001.D and L-VEGF-A revealed that the D-protein antagonist bound to the region of VEGF-A that interacts with the VEGF receptor-1 (VEGFR1). Despite these promising results, RFX001.D was unsuitable for further development because it is unstable at physiological temperatures (Table 1).

Here, we describe RFX035.D and RFX037.D, D-protein variants of RFX001.D engineered for greatly improved thermal stability and affinity. RFX037.D proved to be extremely stable ($T_m > 95 \degree C$), bound with high affinity to natural VEGF-A ($K_d = 6 \text{ nM}$) and antagonized binding to VEGFR1. Elucidation of the crystal structure of RFX037.D in complex with L-VEGF-A and comparison with the previously reported structure of the RFX001.D-L-VEGF-A complex revealed that the binding interfaces involve identical residues. However, compared with RFX001.D, RFX035.D and RFX037.D have additional residues at their N- and C-termini (Table 1). In the structure of RFX037.D, these extensions contribute to an extensive hydrogen bond network that likely confers the dramatic improvement in thermal stability.

RFX037 was further characterized for in vivo stability and immunogenicity. RFX037.D was metabolically stable in plasma, whereas RFX037.L was degraded rapidly. Furthermore, whether dosed subcutaneously or intravenously in mice, RFX037.D had a longer serum half-life than RFX037.L. Significantly, RFX037.D was nonimmunogenic in contrast to RFX037.L. These results confirm the utility of synthetic D-proteins as viable alternatives to therapeutic antibodies and i-proteins and support the continued development of RFX037.D as a therapeutic antagonist of angiogenesis.

**RESULTS AND DISCUSSION**

In an effort to improve stability, two D-protein variants of RFX001 were designed to contain additional amino acid sequences at their N- and C-termini (Table 1). This strategy was based on the observation that a bacterially expressed variant of RFX001 (RFX035.L) had higher affinity for D-VEGF-A than chemically synthesized RFX001.L ($K_d = 95 \text{ nM}$) and contained an N-terminal His10 tag attached to a three-residue linker plus five extra residues at its C-terminus. Thus, RFX035.D was designed as the D-enantiomer of RFX035.L. In addition, RFX037.D was designed to examine the effect of a shorter N-terminal Arg5 tag in place of the His10 tag of RFX035.D.

**Protein Synthesis.** A novel one-pot native chemical ligation and desulfurization method was devised to synthesize the full-length polypeptides RFX035.L, RFX035.D, RFX037.L, and RFX037.D. For each protein, Ala33–Ala34 of the RFX001 core peptide sequence was used as the ligation site for joining...
the two synthetic peptide segments (bold and italic in Table 1).

To support native chemical ligation, Ala34 was initially replaced by Cys34, which was subsequently desulfurized to regenerate native Ala34. The two peptide segments were synthesized using manual in situ neutralization Boc chemistry solid phase peptide synthesis protocols. After assembly of the resin-bound peptide chains, the peptides were cleaved and simultaneously deprotected by treatment with anhydrous hydrogen fluoride, recovered by ether precipitation after removal of hydrogen fluoride, then purified by reverse phase high performance liquid chromatography (HPLC). Native chemical ligation of the peptide segments was carried out in phosphate buffer (pH 7.0) containing 6 M guanidium chloride (GnHCl) and 4-mercaptophenylacetic acid as the thiol catalyst. After completion of ligation, the reaction mixture was treated with freshly prepared nickel boride to effect quantitative desulfurization of Cys34 to yield Ala34. Full-length polypeptide was purified from the crude mixture by treatment with anhydrous hydrogen fluoride, recovered by ether precipitation after removal of hydrogen fluoride, then purified by reverse phase high performance liquid chromatography (HPLC). Native chemical ligation of the peptide segments was carried out in phosphate buffer (pH 7.0) containing 6 M guanidium chloride (GnHCl) and 4-mercaptophenylacetic acid as the thiol catalyst. After completion of ligation, the reaction mixture was treated with freshly prepared nickel boride to effect quantitative desulfurization of Cys34 to yield Ala34. Full-length polypeptide was purified from the crude mixture by treatment with anhydrous hydrogen fluoride, recovered by ether precipitation after removal of hydrogen fluoride, then purified by reverse phase high performance liquid chromatography (HPLC). Native chemical ligation of the peptide segments was carried out in phosphate buffer (pH 7.0) containing 6 M guanidium chloride (GnHCl) and 4-mercaptophenylacetic acid as the thiol catalyst. After completion of ligation, the reaction mixture was treated with freshly prepared nickel boride to effect quantitative desulfurization of Cys34 to yield Ala34. Full-length polypeptide was purified from the crude mixture by treatment with anhydrous hydrogen fluoride, recovered by ether precipitation after removal of hydrogen fluoride, then purified by reverse phase high performance liquid chromatography (HPLC).

**Thermal Stability.** To determine whether the N- and C-terminal extensions improved conformational stability, thermal denaturation experiments were conducted in phosphate buffered saline (pH 7.4) and monitored by circular dichroism (CD) at 222 nm. As expected, RFX001.D was unstable at physiological temperatures with a Tm of 33 °C (Figure 1A). In contrast, thermal melting of the variants occurred at higher temperatures. RFX035.D proved to be very stable with a Tm of 82 °C (Figure 1A). Surprisingly, the loss of molar ellipticity classically seen upon protein denaturation was not seen for RFX037.D, suggesting that this protein did not denature at 95 °C. To confirm the highly stable nature of RFX037.D, thermal denaturation was recorded in the presence of the chaotrope GnHCl. Even with the addition of 250 mM GnHCl, an extremely high Tm value of 93 °C was recorded for RFX037.D (Figure 1A).

**Binding Kinetics and Affinity.** The binding kinetics of RFX037.D for l-VEGF-A were measured by surface plasmon resonance (Figure 1B), and the affinity was calculated to be in the single-digit nanomolar range (Kd = 6 nM), which was significantly tighter than the previously reported value for RFX001.D (Kd = 85 nM). RFX037.D significantly inhibited VEGF-A binding to VEGFR1 as measured by biolayer interferometry (Figure S3), an in vitro assay in which the extracellular domain of VEGFR1 was immobilized on a sensor tip and VEGF-A binding to the sensor tip was measured after exposure to different concentrations of inhibitors.

**Structural Analysis.** RFX037.D bound to l-VEGF-A with high affinity and was the most thermally stable variant, suggesting that the N-terminal Arg5 moiety may participate in stabilizing the tertiary structure or may be a component of the binding interface with l-VEGF-A. To investigate the precise role of the Arg5 moiety, we solved the crystal structure of the heterochiral VEGF-A:RFX037 complex at 2.1 Å resolution by using racemic protein crystallography (Table S1). The structure shows that two RFX037.L proteins bind to opposite poles of a o-VEGF-A homodimer and two RFX037.D proteins bind to the analogous epitopes of a l-VEGF-A homodimer (Figure 2A). Residues 9 through 64 of RFX037 superposed with the corresponding residues 1 through 56 of RFX001 with a very low C-alpha root-mean-square deviation of 0.49 Å2 (Figure 2).
showing that the structures of the main chains are essentially identical in these regions. Superposition of the RFX001.D: L-VEGF-A and RFX037.D: L-VEGF-A complex structures revealed that the residues involved in binding L-VEGF-A are identical for RFX001.D and RFX037.D. In total, 17 residues on RFX037.D make contact with L-VEGF-A and contribute to a large contact surface area of 1350 Å² at the binding interface (Figure 2C). On L-VEGF-A, the contact surfaces with RFX037.D and VEGFR1 overlap significantly (Figure 2D), consistent with the VEGFR1 antagonist activity of RFX037.D (Figure S3).

The structure also provides insights into how the N- and C-terminal extensions (Figure S4A) may contribute to the enhanced stability of RFX037.D compared with RFX001.D. Both termini of RFX037 project away from the binding interface, and thus do not interact directly with VEGF-A. The eight-residue N-terminal extensions of the two independent RFX037.D molecules in the asymmetric unit present somewhat different main chain conformations (Figure S4B), but in both cases the extension doubles back to form a salt bridge between the side chains of Arg1 and Asp32 (Figure 2E). The N-terminal salt bridge initiates an extensive hydrogen bond network that spans the width of the GB1 domain to complete an indirectly bonded cyclical peptide loop of the N-terminal extension. Specifically, Arg1 and Asp32 participate in additional hydrogen bonds with the carbonyl of Glu26 or with the amide nitrogen of Val28, respectively. These flanking hydrogen bonds position the amide and carbonyl of Ala27 to form hydrogen bonds with the amide and carbonyl of Ser8. Furthermore, the C-terminal Gly65 and Gly66 residues form hydrogen bonds that are mediated through the main chain or side chain of Asp47, respectively. These additional hydrogen bond networks likely stabilize the protein tertiary structure to confer the high thermal stability and improved affinity of RFX037.D.

**Protease Resistance and Plasma Stability.** D-Proteins are expected to have inherent resistance to proteolytic degradation. However, in addition to D-amino acids, RFX037.D contains the achiral amino acid glycine, and therefore the resistance of RFX037.D to proteases was examined and compared with that of RFX037.L by incubating each synthetic protein with papain, a protease widely distributed in nature, and Pronase, which contains a mixture of proteases. The progress of protein degradation was monitored by HPLC (Figure S5). After 16 h, RFX037.L was nearly completely degraded, while RFX037.D remained undigested, demonstrating that RFX037.D is resistant to proteolysis. This also suggested that RFX037.D should be non- or minimally immunogenic, as protein processing is necessary to elicit a strong MHC-assisted immune response. Additionally, D-peptides can be differentially presented by MHC class II receptors compared to L-enantiomers, which often reduces their immunogenicity.

The stabilities of the L- and D-enantiomers of RFX037 in plasma were assessed by measuring the degradation of the proteins by LC-MS/MS following incubation in mouse, monkey, or human plasma (Figure 3A). RFX037.L was degraded in all three plasma types with the degradation in mouse plasma occurring more quickly than in monkey or human plasma, which were indistinguishable from each other. In contrast, RFX037.D showed no appreciable degradation in any of the plasma types.

**In Vivo Stability and Clearance.** LC-MS/MS analysis was used to assess the in vivo stability and clearance of RFX037.L and RFX037.D after intravenous or subcutaneous administration in male CD-1 mice. After intravenous infusion,

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**Figure 3.** Plasma stability, pharmacokinetics, and immunogenicity of RFX037.L (solid symbols, solid lines) and RFX037.D (open symbols, dashed lines). (A) Stability in mouse (triangles), cynomolgus monkey (squares), or human (diamonds) plasma. (B) Serum levels following intravenous injection (10 mg/kg) in mice. (C) Serum levels following subcutaneous injection (10 mg/kg) in mice. (D) ELISA for measurement of serum antibody binding to antigen immobilized at varying concentrations. Sera were collected from BALB/c mice given three subcutaneous injections with 5 (circles), 20 (triangles), or 50 (squares) µg/injection of RFX037.L or RFX037.D emulsified in adjuvant. The mean absorbance value for five animals ± SEM is shown. See “Methods” for further details.

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RFX037.D reached an approximately 4-fold higher serum concentration than RFX037.L at the first measured time point (30 min) and was cleared from the serum after 4 h (Figure 3B). In contrast, the L-enantiomer was essentially cleared from the serum after 2 h. The disappearance of the D-protein from the serum after 4 h is consistent with a renal clearance mechanism, which was expected based on the small size of the protein. The more rapid clearance of RFX037.L was likely due primarily to proteolysis and metabolism rather than renal clearance. After subcutaneous administration, RFX037.D reached a maximum serum concentration at the first measured time point (30 min) and was cleared by 6 h (Figure 3C). In contrast, RFX037.L never reached measurable levels in the serum after subcutaneous administration.

**Immunogenicity.** Previous studies using linear and multichain polypeptides composed entirely of D-amino acids showed that, when injected at low doses, these polypeptides can induce an immune response.

This response could not be boosted with subsequent injections and repeated low dose injections, or exposure to high doses led to tolerance against these antigens. In general, immune responses to the D-peptide antigens was thymus-independent, as these peptides were immune responses seen with multichain D-amino acid polymers.

The objective of some of the above-described studies was to make an effective immunogen as opposed to avoiding immune response to a potential therapeutic molecule. Moreover, all of these studies involved small linear peptides that do not require processing instead of well-folded D-proteins. Therefore, we thought it possible that folded D-proteins may be non-immunogenic, as reported by Dintzis et al. Because the immune responses seen with multichain D-amino acid polymers were dose-dependent, we decided it would be prudent to study the effect of dosage on the immune response.

To assess the in vivo immunogenicity of the L- and D-proteins, BALB/c mice were immunized with three injections of low (5 μg), medium (20 μg), or high (50 μg) doses of antigen in combination with a potent adjuvant (Figure 3D). Sera were collected after 42 days, and the presence of anti-RFX037.L or anti-RFX037.D circulating antibodies was measured by ELISA. Sera from all mice immunized with RFX037.L showed strong binding to immobilized RFL037.L by ELISA, and binding activity was dependent on the coating concentration of antigen. The highest binding activity was observed in sera from mice immunized with the high dose followed by the medium and low dose sera. In contrast, sera from all mice immunized with RFX037.D showed no binding to RFX037.D at all coated antigen concentrations. RFX037.L or RFX037.D immune sera did not exhibit binding activity to the opposite enantiomeric form of the antigen (data not shown).

These results are consistent with the hypothesis that proteins composed of D-amino acids and achiral glycine are non-immunogenic, even when immunized with a strong adjuvant. The absence of any immune response when the D-proteins were immunized at low, medium, or high doses contrasts with earlier unexplained results that some D-amino acid polymers were only immunogenic at low doses.

**Conclusions.** Protein therapeutics benefit from their inherent large protein–protein interaction surfaces, maximizing specificity and affinity toward their targets while minimizing off-target effects. However, the half-life of proteins can be short due to their in vivo proteolysis and metabolism, limiting their potency and increasing their dosing frequency. In addition, repeated dosing can often lead to immunogenicity issues, limiting their long-term use. Here, we address these issues by demonstrating dramatic differences between otherwise identical L- and D-proteins with respect to their resistance to proteolytic degradation, plasma stability, in vivo half-life, and immunogenicity.

In contrast to the L-protein RFX037.L, the D-protein RFX037.D was completely resistant to proteolysis and metabolism in mouse, monkey, and human plasma; had a longer in vivo half-life when dosed by intravenous infusion or subcutaneous injection; and had a complete lack of immunogenicity, even when dosed in combination with a strong adjuvant. Moreover, RFX037.D bound to natural VEGF-A with an affinity comparable to those of antibodies and proved to be even more stable than therapeutic antibodies. Together, these observations suggest that D-proteins have inherent properties that could make them an important new class of pharmaceutical products, and our results demonstrate that stereochemistry is a fundamental property of proteins that regulates immunogenicity and metabolic stability.

**METHODS**

**Chemical Protein Synthesis.** The D-proteins were prepared by total chemical synthesis. Full details are provided in the Supporting Information.

**Thermal Denaturation.** The thermal denaturation measurements of RFX001.D and RFX035.D were recorded using an AVIV-202 CD spectrophotometer (Aviv Biomedical, Lakewood, NJ, USA), while those of RFX037.D were recorded using a JASCO J-1500 spectrometer (Jasco Instruments, Easton, MD, USA). Each protein sample (0.4 mg mL⁻¹) was monitored at 222 nm in a 0.1 cm path length CD cuvette over the temperature range 10–95 °C in increments of 2.5 °C, with 90 s equilibration time and averaging over 5 s. RFX001.D and RFX035.D were assayed in phosphate-buffered saline, pH 7.4 (PBS). RFX037.D was assayed in PBS with 250 mM GlnHCl.

**Surface Plasmon Resonance.** Synthetic L-VEGF-A (0.25 mg mL⁻¹) and n-VEGF-A (0.25 mg mL⁻¹) were immobilized in separate flow cells of a GLM sensor chip in a ProteOn Protein Interaction Array System (Bio-Rad) using an amine coupling kit (EDC/NHS). Sensorsgrams were generated by injecting several concentrations (12.5–100 nM) of protein ligand over the chip. The interactions were analyzed by global kinetic analysis using nonlinear regression fits (Langmuir model).

**Biolayer Interferometry.** Measurements were carried out using a ForteBio Octet instrument. A VEGFR1 sensor was prepared using antihuman IgG(Fc) sensors (ForteBio). The sensor tips were incubated with rhVEGFR1/hIgGFc (R&D Systems) followed by a quenching incubation with 1 μM human IgG (Jackson Immunoresearch). Wavelength shifts for VEGF165 binding to the VEGFR1 sensor at each inhibitor concentration were normalized by subtracting the wavelength shift of the inhibitor alone. Percent inhibition was calculated using the wavelength shift for VEGF165 binding in the absence of inhibitor as 0% and the wavelength shift for VEGF165 binding with a nonreactive sensor as 100%.

**Crystallization, Structure Determination, and Refinement.** For crystallization, the racemic protein solution was prepared by mixing 1 equiv of L-VEGF-A (2.72 mg mL⁻¹), 1 equiv of n-VEGF-A (2.72 mg mL⁻¹), 2 equiv of RFX037.D (0.89 mg mL⁻¹), and 2 equiv of RFX037.L (0.89 mg mL⁻¹) in aqueous buffer containing 10 mM HEPES, at pH 7.0. Racemic crystals of the VEGF-A:RFX037 complex were grown at 19 °C using the hanging drop vapor diffusion technique
with 1 μL of protein solution and 1 μL of reservoir solution placed over 1 mL of reservoir solution containing 0.1 M MgCl₂, 0.1 M HEPES (pH 7.0), and PEG3350 (11% v/v; Pro-complex suit, catalogue number 13071S). Crystals appeared within 7 days. Selected crystals were flash frozen in liquid nitrogen after a brief wash in the cryoprotectant (reservoir solution plus 20% v/v glycerol). A crystal data set to 2.1 Å was collected at 100 K using 0.97 Å wavelength synchrotron radiation at the Argonne National Laboratory (Advanced Photon Source, beamline 24-ID C, equipped with ADSC Q315 CCD detector).

Crystal diffraction images were indexed, integrated, scaled, and merged with HKL2000. Examination of the diffraction intensity statistics revealed that the protein complex racemate was crystallized in a centrosymmetric space group P21/n. Cell content analysis suggested that the crystal most likely contained one VEGF-A homodimer and two RFX037 molecules in the asymmetric unit. The structure was solved by molecular replacement with the program PHASER using the previously reported X-ray structure of the RFX010-D3:VEGF-A complex (PDB accession code 4GLN) as a search model. The resulting protein complex model was refined with PHENIX:REFINE using a maximum likelihood target function. After each refinement step, the model was visually inspected in COOT and F₂−F₁ and F₁−F₀ difference maps. The final model had an R-factor (Rfree) of 26.1% (30.7%). The structure was also solved in the lower symmetry space group P21 using the noninverted and inverted coordinates of the structure solved in P21/n. The final model refined in space group P21 had an Rfactor (Rfree) of 22.2% (28.2%). X-ray crystal diffraction data and refinement statistics of the heterochiral protein complex racemate in both the P21 and P21/n space groups are listed in Table S1. Figures were rendered in PYMOL, and buried surfaces were calculated using PISA.

**Proteolysis Assays.** RFX037.L or RFX037.D (25 μM) was incubated with papain or Pronase (2.5 μM) in PBS at RT for 16 h, and the solutions were analyzed by HPLC.

**Preparation of Calibration Standards for Plasma and Serum Analysis.** Stock solutions of peptides (1 mg mL⁻¹) were prepared in PBS and stored at 2–8 °C. Serum calibration standards were prepared by serial dilution in mouse serum. The peptide calibration concentrations were 50, 100, 250, 500, 1000, 2500, 5000 and 10 000 ng/mL.

**Preparation of Samples for in Vitro Plasma Stability Analysis.** Peptide stock solutions (1 mg mL⁻¹) were used to prepare 20 μg/mL of peptide working solutions in 10:90 (v/v) methanol/water, and these were stored in a refrigerator at 2–8 °C. Plasma stability samples were prepared by adding 225 μL plasma into vials containing 25 μL of 20 μg/mL peptide working solutions at each time point (0, 1, 3, 5, and 7 h) for incubation at 37 °C.

**Biological Sample Extraction by Solid Phase Extraction (SPE).** Plasma and serum samples were prepared using SPE to enrich the peptides and remove the endogenous components such as serum proteins, lipids, and salts. Oasis HLB μElution 96-well SPE plates were used and conditioned by sequential addition of 400 μL methanol and water under a vacuum. A 25 μL plasma or serum sample was aliquoted into the appropriate well of a 96-well plate, followed by the addition of 50 μL of 0.1 M NaOH and, after mixing, 300 μL of 10% MeOH. The prefiltered samples were transferred to the conditioned 96-well SPE plate and drawn through with a vacuum. The plate was washed sequentially with 100 μL of water, then 10% MeOH with 2% NH₄OH, and finally water again. The final extract was eluted into a 96-well plate with 30 μL of methanol/water (90:10, v/v) containing 0.1% formic acid, and the eluate was diluted with 30 μL of water. The 96-well plate was capped with a polypropylene cover and transferred to the autosampler. Samples were injected onto the LC-MS/MS system for analysis.

**Chromatography and Mass Spectrometry for Plasma and Serum Analysis.** The LC-MS/MS consisted of an Acquity UPLC system (Waters, Milford, MA) coupled to a 6500 QTRAP mass spectrometer (AB Sciex, Toronto, Canada) with a Turbo IonSpray ionization source operated in the positive ion mode. The analytical column was an Aeries C4 2.1 × 100 mm (Phenomenex, Torrance, CA). A 0.2 μm precolumn filter unit was used to protect the analytical column. The mobile phases of 0.1% formic acid in acetonitrile/water (5:95, v/v, mobile phase A) and 0.1% formic acid in acetonitrile/water (95:5, v/v, mobile phase B) were delivered under gradient programs for 5 min. The LC gradients in minutes per percent of mobile phase B were 0.0/0.15, 0.15/3.0, 3.0/3.15, 3.15/4.5, 4.5/6.15, and 5.0/5.0 for ubiquitin as control peptides and 0.0/25, 0.25/3.0, 3.0/3.15, 3.15/4.5, 4.5/6.25, and 5.0/25 for RFX037.L and RFX037.D. The flow rate was 0.5 mL/min with a column temperature of 70 °C. MRM parameters were optimized by direct infusion of 10 μg/mL of peptide tuning solutions. The ESI spray voltage was set at 5000 V. The source temperature was 500 °C. The curtain gas (CUR) was 30. The nebulizer gas setting (GS1) was 40, and the auxiliary gas setting (GS2) was 50 (all arbitrary units). The ion transitions for MS/MS detection were m/z 754.0 → 817.3 for ubiquitin and m/z 867.9 → 915.8 for RFX037.L and RFX037.D.

**Data Analysis for Plasma and Serum Analysis.** Data were collected and processed using AB Sciex Analyst software (version 1.6.2). The stability of the tested peptides was derived from the peak area at each time point corresponding to peptides obtained from the LC-MS/MS analysis. All data were normalized to the value at the 0 h time point as the percentage of intact peptide and plotted against incubation time for the stability profile of each peptide. For pharmacokinetic study samples, the calibration curve was derived using 1/x² weighted linear regression of the peak area versus the concentration of the corresponding standard. The regression equation from the calibration standards was used to back-calculate the measured concentration for each standard, control, and unknown sample.

**Pharmacokinetic Studies.** Serum samples were collected from male CD-1 mice after dosing subcutaneously or by intravenous bolus administration with each peptide at 10 mg/kg according to a protocol approved by the Institutional Animal Care and Use Committee of Amgen, Inc. The samples were frozen and stored at −70 °C until LC-MS/MS analysis.

**Immunization and Immunogenicity Assays.** Five BALB/c mice per group were given three subcutaneous injections in the back of the neck on days 0, 21, and 35 with 5, 20, or 50 μg injection of RFX037.D or RFX037.L antigen emulsified in Alhydrogel/MurNac-c-Ala-n-isogln (ALD/MDP) as described. Immune sera were collected on day 42.

**ELISAs** were performed in EIA/RIA 96-well microtiter plates (Corning) using 50 μl incubation volumes. Between each incubation step, wells were washed four times with 200 μl of PBS with 0.05% Tween 20. Wells were coated overnight at 4 °C with either RFX037.D or RFX037.L in PBS. After coating, wells were emptied and blocked with PBS-Casein (Surmodics) for 1 h at ambient temperature. Serum from each mouse was diluted 1:1000 in PBS-Casein and incubated in the wells for 1.5 h. Bound antibody was detected using Goat Anti-Mouse IgG (Fcγ fragment specific)—HRP conjugate (Jackson ImmunoResearch) and High Kinetic TMB (Moss Substrates). After the addition of stop solution (1.0 M H3PO4), absorbance was measured at 450 nm.

### ASSOCIATED CONTENT

#### Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b01006.

Detailed methods for peptide synthesis, LCMS data for synthesized peptides, data collection and refinement statistics for crystallography, additional data on inhibition of VEGFA—VEGFR1 interaction, and the role of tags in stabilization (PDF)

### Accession Codes
Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org. PDB
Joshua Lowitz, the other authors of this paper own equity in
exception of Dong Jun Lee, Hongan Li, Les Miranda, and
Riche of Chicago and the University of Toronto as part of a research
inhibitors that target the gp41 coiled-coil pocket.

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