Supplementary Information

Binding region and interaction properties of sulfoquinovosylacylglycerol (SQAG) with human vascular endothelial growth factor 165 revealed by biosensor based assays

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Experimental Procedures

Preparation of SQAG

βSQDG (**1**) and six different analogues **2-7** (**Fig. 1**) were synthesized as previously described[1] and subjected to structure-binding analysis. All the compounds were >95% pure as judged by analytical HPLC and ^{1}H NMR analyses. [1]

Proteins and reagents

Growth factors and receptors, which are shown in **Table S1**, were commercially obtained. T7select® system and expression vector were purchased from Novagen (Madison, HI).

Instruments

A 27-MHz QCM device, AffinixQ and ceramic sensor chip were purchased from Initium Inc. (Tokyo, Japan). PCR was performed using a PTC-200 (Peltier Thermal Cycler) (Bio-Rad, Hercules, CA). Sequencing analysis was carried out using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Centrifugation was performed using a MX-201 centrifuge (TOMY Industry, Tokyo, Japan). SPR analysis was carried out using Biacore® 3000 with a sensor chip CM5 (GE Healthcare, Piscataway, NJ).

Construction of T7 phage library displaying a fragment of hVEGF$_{165}$

The hVEGF$_{165}$-coding gene was partitioned into each domain, as shown in **Fig. 3**, and amplified by the polymerase chain reaction (PCR) using the primers shown in **Table S2**. The primers included recognition sites for *Eco*RI and *Hind*III to help facilitate the subsequent cloning steps. Each PCR product was subcloned into TA vector (Promega, Madison, WI) and the sequences were then

determined to ensure no mistakes had been introduced during amplification. The insert was double-digested with *Eco*RI and *Hind*III, and then inserted into the T7Select10-3b vector (Novagen), followed by *in vitro* packaging. The T7 phage (del 1-4) was amplified by host *E. coli* (BLT5615) to 1.0×10^{9} pfu ml^{-1}, respectively. These solutions were mixed together in a 1:1 ratio and used as a T7 phage library.

Procedure for the T7 phage display selection using a cuvette type QCM device

A 15 μl aliquot of βSQDG (**1**) (1 mM in DMSO) was dropped onto the gold electrode of the ceramic sensor chip that was immersed into bulk water and left for 5 min at room temperature (mean of immobilization amount: 1800 Hz (61.8 *p*mol). The sensor chip was setup for the QCM device with the cuvette containing 8 ml of buffer (10 mM Tris–HCl, pH 8.0, 200 mM NaCl), which was constantly stirred at 1000 rpm. The QCM sensor was then allowed to fully stabilize. An aliquot of 8 μl of the T7 phage library (1.0×10^{9} pfu ml^{-1}) was then injected into the cuvette (final 1.0×10^{6} pfu ml^{-1}). Frequency changes, caused by binding to the βSQDG immobilized on the gold electrode surface were then monitored for 10 min. For the recovery of bound phages, 20 μl of host *E. coli* culture was dropped onto the gold electrode and then incubated at 37 °C for 30 min. To the resulting solution was then added another 200 μl of LB medium. An aliquot of phage was then extracted from this solution and subjected to PCR analysis followed by DNA sequencing.

DNA sequencing

DNA sequencing was performed as described previously.[2]

Construction of bacterial expression plasmids and expression of recombinant proteins

As described above, the $hVEGF_{165}$ coding gene was partitioned into four fragments (del 1-4 **Fig. 2**).

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Each fragment was amplified by PCR using the primers shown in **Table S2**. The primers contained *Nde*I and *Xho*I reognition sites that were used in further cloning experiments. PCR products were double-digested with *Nde*I and *Xho*I and then inserted into the expression vector pET28a(+) (Novagen). For the expression of protein, *Escherichia coli* BL21 (DE3) (Novagen) was used.

Single colonies were incubated in 100 ml LB medium containing 1% glucose and 50 μg ml^{-1} of kanamycin, and cultured overnight at 37 °C. The cells were further cultured in 1000 ml of LB medium containing 1% glucose and 50 μg ml^{-1} of kanamycin for 3 h at 30 °C. After adding isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 1 mM, the cells were incubated for 3 h at 30 °C to induce heterologous gene expression. The cultured cells were harvested by centrifugation at 5000 *g* for 15 min at 4 °C.

Procedure of recombinant $hVEGF_{165}$ polypeptide

Cultured cells expressing each fragment were resuspended in binding buffer (20 mM phosphate buffer, pH 7.4 containing 500 mM NaCl, 10 mM imidazole, 5 mM 2-mercaptoethanol, 0.1 mM leupeptin, 0.1 mM pepstatin and 1 mM PMSF). Following sonication and centrifugation, the supernatant was purified by Ni^{2+} chelation affinity chromatography. The recombinant protein obtained from this procedure was used in the kinetic analysis involving a surface plasmon resonance (SPR) biosensor.

SDS-PAGE, tricine-SDS-PAGE and Western blot analysis

The SDS-PAGE or tricine-SDS-PAGE (for recombinant del 4 polypeptide) analyses were performed using 10% or 15% SDS polyacrylamide gels with CBB staining. For Western blot analysis, purified proteins were first separated on each polyacrylamide gel and then transferred to PVDF membranes. The membranes were incubated with a polyhistidine (His×6) mouse monoclonal

antibody (SIGMA-ALDRICH). Anti-mouse IgG conjugated with alkaline phosphatase (AP) (SIGMA-ALDRICH) was used as the secondary antibody. Antigen and antibody binding was detected using BCIP/NBT solution.

SPR analysis

An analysis of binding between SQAGs and protein was performed using an SPR biosensor (Biacore®3000, GE healthcare). Protein was diluted 1:9 with 10 mM sodium acetate buffer at pH 4 or 5 and immobilized by an amine coupling reaction on a sensor chip CM5 (Biacore AB). The chip surface was activated by injecting a solution containing 200 mM *N*-ethyl-*N*'-dimethylaminopropyl carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) at a flow rate of 10 μl min^{-1} for 14 min. Protein was then injected and the coupling to the sensor surface was monitored. The surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 14 min. The amount of immobilized protein is given in **Table S1**. Various concentrations of analyte (**1**, **2**, **6**, **7**: 0, 0.125, 0.25, 0.5, 1 and 2 μM, **3-5**: 0, 1.25, 2.5, 5, 10 and 20 μM) in HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20)−8% DMSO were perfused at a flow rate of 20 μl min^{-1} at 25 °C. At least two replicate experiments were performed for each protein. Kinetic parameters were obtained by global fitting of the sensorgrams to a 1:1 drifted baseline model using BIAevaluation 3.2 software (Biacore AB).

Binding assay between T7 phage-displayed HBD and NRP1 using a cuvette type QCM device

A 15 μl aliquot of NRP1 (100 μg ml^{-1}) was dropped onto the gold electrode of the ceramic sensor chip that was immersed into bulk water and left for 30 min at room temperature (mean of immobilization amount: 600 Hz (200 *p*mol). The sensor chip was setup for the QCM device with the cuvette containing 8 ml of buffer (10 mM Tris–HCl, pH 8.0, 200 mM NaCl), which was

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constantly stirred at 1000 rpm. The QCM sensor was then allowed to fully stabilize. An aliquot of 8 μl of the T7 phage that displays HBD (1.0×10^{9} pfu ml^{-1}) was then injected into the cuvette (final 1.0×10^{6} pfu ml^{-1}). Frequency changes, caused by binding to the NRP1 immobilized on the gold electrode surface were then monitored for 10 min. The following process was carried out according to the procedure for the T7 phage display selection as described above.

Description of the kinetics

Saturation binding kinetics between SQAG and protein immobilized on a sensor chip CM5 can be described as i) Scatchard-plot or ii) Hill-plot represented by following equations:

i) $R_{eq} \cdot [SQAG]^{-1} = R_{max} \cdot K_D - R_{eq} \cdot (K_D)^{-1}$

ii) $\log [R_{eq} \cdot (R_{max} - R_{eq})^{-1}] = n \log [SQAG] + \log (K_D)^{-1}$

where [SQAG] is the concentration of SQAG; R_{eq} is the response (RU) on equilibrium between SQAG and immobilized VEGF on a sensor chip CM5, which was obtained from curve fitting; R_{max} is the maximum response (RU); K_D is the dissociation constant between SQAG and immobilized protein; n is the Hill coefficient.[3]

Binding kinetic parameters can be written as the time-dependence of the response increase. The binding between SQAG and VEGF is as follows:

iii)
$[SQAG] + [VEGF] \rightleftarrows [SQAG \cdot VEGF]$

$k_a = [SQAG \cdot VEGF] \cdot \{[SQAG] \cdot [VEGF]\}^{-1}$, $k_d = \{[SQAG] \cdot [VEGF]\} \cdot [SQAG \cdot VEGF]^{-1}$,

$K_D = k_d \cdot (k_a)^{-1}$

iv) $[SQAG \cdot VEGF]_t = [SQAG \cdot VEGF]_\infty \cdot \{1 - \exp(-t \cdot \tau^{-1})\}$

v) $\tau^{-1} = k_a \cdot [SQAG] + k_d$

where [VEGF] is the concentration of VEGF; [SQAG・VEGF] is the concentration of complex of SQAG and VEGF; k_a is association rate constant between SQAG and VEGF; k_d is dissociation rate constant; τ is relaxation time, which can be obtained from non-linear curve fitting.[3]

Table S1 Growth factors and receptors used in this study.

Protein (human)		**MW (kDa)**	**Amino acid**	**Immobilized amount (RU)**	**R_{max} (RU)**[a]
$VEGF_{165}$	R&D Systems	19.1	A1−R165	9916	453
$VEGF_{121}$	R&D Systems	14	A1−R121	3836	239
del 1		12.7	A1−R110	4847	333
del 2		6.5	A111−R165	2844	382
del 3		3.7	A111−T142	860	203
del 4		2.8	C137−R165	2219	692
VEGFR1	R&D Systems	60.7	S27−I328	7000	101
VEGFR2	Acris antibodies	116	A20−E764	13600	102
NRP1	R&D Systems	90	M1−K644	9680	94
TGFβ2	Peprotech	25	A1−S112	4251	148
TGFβ RII	R&D Systems	18	M1−D159	10359	503
IGF-I	Peprotech	7.6	G49−A118	656	75
IGF-I R	R&D Systems	102.9	E31−N932	4724	40
FGF1	Peprotech	15.8	M1−D141	3040	168
FGF2	Peprotech	17.2	A1−S154	4116	209
FGF R2α	R&D Systems	66	R22−E378	6373	84
Ang-1	Acris antibodies	66	N21−F496	2942	39
Tie-2	R&D Systems	100	A23−K745	11267	98
EphB2[b]	R&D Systems	85.3	V27−K548	7474	77
EphB4	R&D Systems	57.9	L16−A539	2615	39
EGF	Peprotech	6.2	N1−R53	596	84
EGFR	R&D Systems	68.6	L25−S645	14622	186
PDGF-BB	Peprotech	12	S1−T110	3857	281
PDGFRB	Abnova Corp.	36.7[c]	L33−E133	5017	119
BMP-2	Peprotech	26	M1−R115	4084	137
BMP RII	R&D Systems	41.5	A26−I151	7245	152
sDLL-4	Peprotech	54	S1−P498	3781	61
Jagged-1	R&D Systems	180	S32−S1046	3187	15
Notch-1	R&D Systems	80.1	A19−Q526	3885	42
DNA polymerase λ (Cont.)		14.3	M1−R95	1614	100

[a]Calculated maximum response when 100% bound by βSQDG with 1:1 stoichiometry. R_{max} = (Immobilized amount of protein (RU)) / (MW of protein) × (MW of βSQDG). [b]The mouse PDGFRB was used. [c]This MW includes fusion GST tag. RU: resonance unit. 1RU = 1 *pg* mm^{-2}.

Table S2 Specific primers for construction of T7 phage and recombinant $hVEGF_{165}$ segment.

	Name		Nt	Sequence
Phage	del 1	up	28	GAATTCTGCACCCATGGCAGAAGGAGGA
	del 1	down	29	AAGCTTTCATCTATCTTTCTTTGGTCTGC
	del 2, 3	up	27	GAATTCTGCAAGACAAGAAAATCCCTG
	del 2, 4	down	25	AAGCTTTCACCGCCTCGGCTTGTCA
	del 3	down	29	AAGCTTTCATGTGTTTTTGCAGGAACATT
	del 4	up	27	GAATTCTTCCTGCAAAAACACAGACTC
Protein	del 1	up	29	GGAATTCCATATGGCACCCATGGCAGAAG
	del 1	down	32	GGCCTCGAGTCATCTATCCTTCTTTGGTCTGC
	del 2, 3	up	33	GGAATTCCATATGGCAAGACAAGAAAATCCCTG
	del 2, 4	down	26	CCGCTCGAGTCACCGCCTCGGCTTGT
	del 3	down	29	CCGCTCGAGTCATGTGTTTTTGCAGGAAC
	del 4	up	29	CCCATATGTCCTGCAAAAACACAGACTCG

＊ Underlined nucleotide sequences represent the following recognition sites of restriction enzyme; **GAATTC**: *Eco*RI, **AAGCTT** : *Hind*III, **CATATG** : *Nde*I, **CTCGAG** : *Xho*I.

Table S3 Raw DNA sequence data of del 1-4 phage.

del	Sequence											
1	ATG	CTC	GGG	GAT	CCG	AAT	TCT	GCA	CCC	ATG	GCA	GAA
	GGA	GGA	GGG	CAG	AAT	CAT	CAC	GAA	GTG	GTG	AAG	TTC
	ATG	GAT	GTC	TAT	CAG	CGC	AGC	TAC	TGC	CAT	CCA	ATC
	GAG	ACC	CTG	GTG	GAC	ATC	TTC	CAG	GAG	TAC	CCT	GAT
	GAG	ATC	GAG	TAC	ATC	TTC	AAG	CCA	TCC	TGT	GTG	CCC
	CTG	ATG	CGA	TGC	GGG	GGC	TGC	TGC	AAT	GAC	GAG	GGC
	CTG	GAG	TGT	GTG	CCC	ACT	GAG	GAG	TCC	AAC	ATC	ACC
	ATG	CAG	ATT	ATG	CGG	ATC	AAA	CCT	CAC	CAA	GGC	CAG
	CAC	ATA	GGA	GAG	ATG	AGC	TTC	CTA	CAG	CAC	AAC	AAA
	TGT	GAA	TGC	AGA	CCA	AAG	AAA	GAT	AGA	**TGA**	AAG	CTT
	GCG	GCC	GCA	CTC	GAG	TAA						
2	ATG	CTC	GGG	GAT	CCG	AAT	TCT	GCA	AGA	CAA	GAA	AAT
	CCC	TGT	GGG	CCT	TGC	TCA	GAG	CGG	AGA	AAG	CAT	TTG
	TTT	GTA	CAA	GAT	CCG	CAG	ACG	TGT	AAA	TGT	TCC	TGC
	AAA	AAC	ACA	GAC	TCG	CGT	TGC	AAG	GCG	AGG	CAG	CTT
	GAG	TTA	AAC	GAA	CGT	ACT	TGC	AGA	TGT	GAC	AAG	CCG
	AGG	CGG	**TGA**	AAG	CTT	GCG	GCC	GCA	CTC	GAG	TAA	
3	ATG	CTC	GGG	GAT	CCG	AAT	TCT	GCA	AGA	CAA	GAA	AAT
	CCC	TGT	GGG	CCT	TGC	TCA	GAG	CGG	AGA	AAG	CAT	TTG
	TTT	GTA	CAA	GAT	CCG	CAG	ACG	TGT	AAA	TGT	TCC	TGC
	AAA	AAC	ACA	**TGA**	AAG	CTT	GCG	GCC	GCA	CTC	GAG	TAA
4	ATG	CTC	GGG	GAT	CCG	AAT	TCT	TCC	TGC	AAA	AAC	ACA
	GAC	TCG	CGT	TGC	AAG	GCG	AGG	CAG	CTT	GAG	TTA	AAC
	GAA	CGT	ACT	TGC	AGA	TGT	GAC	AAG	CCG	AGG	CGG	**TGA**
	AAG	CTT	GCG	GCC	GCA	CTC	GAG	TAA				

＊ Underlined nucleotide sequences represent the following recognition sites of restriction enzyme; **GAATTC**: *Eco*RI, **AAGCTT** : *Hind*III. **TGA**: stop codon.

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Table S4 Raw DNA sequence data of del 1-4 protein.

del	Sequence											
1	ATG	GGC	AGC	AGC	CAT	CAT	CAT	CAT	CAT	CAC	AGC	AGC
	GGC	CTG	GTG	CCG	CGC	GGC	AGC	CAT	ATG	GCA	CCC	ATG
	GCA	GAA	GGA	GGA	GGG	CAG	AAT	CAT	CAC	GAA	GTG	GTG
	AAG	TTC	ATG	GAT	GTC	TAT	CAG	CGC	AGC	TAC	TGC	CAT
	CCA	ATC	GAG	ACC	CTG	GTG	GAC	ATC	TTC	CAG	GAG	TAC
	CCT	GAT	GAG	ATC	GAG	TAC	ATC	TTC	AAG	CCA	TCC	TGT
	GTG	CCC	CTG	ATG	CGA	TGC	GGG	GGC	TGC	TGC	AAT	GAC
	GAG	GGC	CTG	GAG	TGT	GTG	CCC	ACT	GAG	GAG	TCC	AAC
	ATC	ACC	ATG	CAG	ATT	ATG	CGG	ATC	AAA	CCT	CAC	CAA
	GGC	CAG	CAC	ATA	GGA	GAG	ATG	AGC	TTC	CTA	CAG	CAC
	AAC	AAA	TGT	GAA	TGC	AGA	CCA	AAG	AAA	GAT	AGA	TGA
	CTC	GAG	CAC	CAC	CAC	CAC	CAC	CAC	TGA	GAT	CCG	GCT
	GCT	AA										
2	ATG	GGC	AGC	AGC	CAT	CAT	CAT	CAT	CAT	CAC	AGC	AGC
	GGC	CTG	GTG	CCG	CGC	GGC	AGC	CAT	ATG	GCA	AGA	CAA
	GAA	AAT	CCC	TGT	GGG	CCT	TGC	TCA	GAG	CGG	AGA	AAG
	CAT	TTG	TTT	GTA	CAA	GAT	CCG	CAG	ACG	TGT	AAA	TGT
	TCC	TGC	AAA	AAC	ACA	GAC	TCG	CGT	TGC	AAG	GCG	AGG
	CAG	CTT	GAG	TTA	AAC	GAA	CGT	ACT	TGC	AGA	TGT	GAC
	AAG	CCG	AGG	CGG	TGA	CTC	GAG	CAC	CAC	CAC	CAC	CAC
	CAC	TGA	GAT	CCG	GCT	GCT	AA					
3	ATG	GGC	AGC	AGC	CAT	CAT	CAT	CAT	CAT	CAC	AGC	AGC
	GGC	CTG	GTG	CCG	CGC	GGC	AGC	CAT	ATG	GCA	AGA	CAA
	GAA	AAT	CCC	TGT	GGG	CCT	TGC	TCA	GAG	CGG	AGA	AAG
	CAT	TTG	TTT	GTA	CAA	GAT	CCG	CAG	ACG	TGT	AAA	TGT
	TCC	TGC	AAA	AAC	ACA	TGA	CTC	GAG	CAC	CAC	CAC	CAC
	CAC	CAC	TGA	GAT	CCG	GCT	GCT	AA				
4	ATG	GGC	AGC	AGC	CAT	CAT	CAT	CAT	CAT	CAC	AGC	AGC
	GGC	CTG	GTG	CCG	CGC	GGC	AGC	CAT	ATG	TCC	TGC	AAA
	AAC	ACA	GAC	TCG	CGT	TGC	AAG	GCG	AGG	CAG	CTT	GAG
	TTA	AAC	GAA	CGT	ACT	TGC	AGA	TGT	GAC	AAG	CCG	AGG
	CGG	TGA	CTC	GAG	CAC	CAC	CAC	CAC	CAC	CAC	TGA	GAT
	CCG	GCT	GCT	AA								

∗ **CATATG** : *Nde*I, **CTCGAG** : *Xho*I. **TGA**: stop codon.

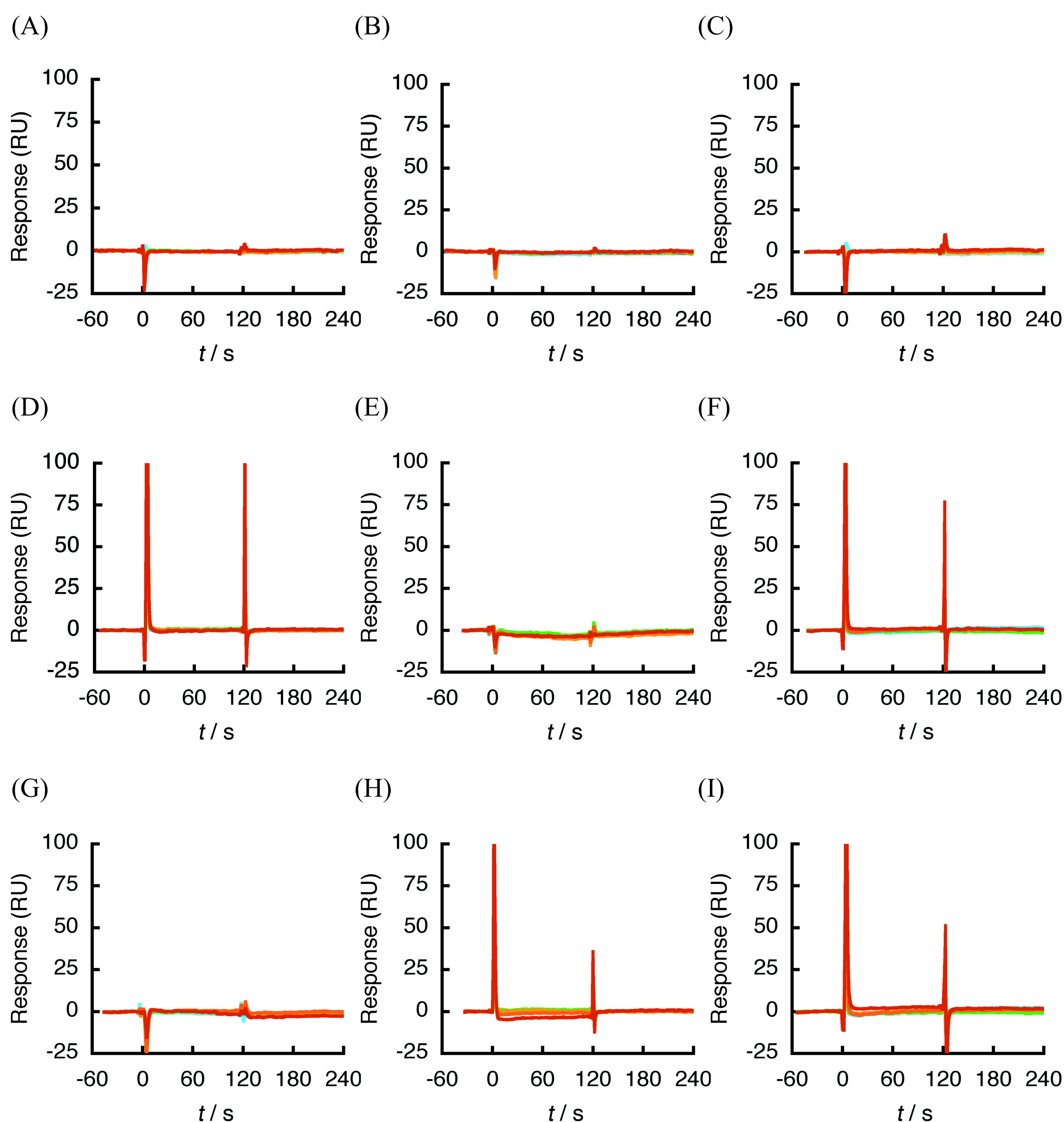
Table S5 QCM-based affinity selection of the T7 phage-displayed defined segment of $hVEGF_{165}$.

Clone	Parent library	Selection 1	Selection 2	Selection 3
1	del 1	del 2	del 4	del 4
2	del 4	del 4	del 3	del 4
3	del 3	del 3	del 4	del 4
4	del 3	del 1	del 3	del 4
5	del 3	del 4	del 4	del 4
6	del 4	del 4	del 4	del 4
7	del 4	del 3	del 4	del 4
8	del 3	del 1	del 3	del 4
9	del 2	del 2	del 2	del 4
10	del 3	del 3	del 2	del 4
11	del 1	del 4	del 2	del 4
12	del 1	del 3	del 2	del 4
13	del 2	del 3	del 2	del 4
14	del 4	del 4	del 4	del 4
15	del 3	del 3	del 3	del 4
16	del 4	del 2	del 2	del 4
17	del 4	del 4	del 1	del 4
18	del 4	del 2	del 1	del 4
19	del 3	del 4	del 4	del 4
20	del 3	del 4	del 2	del 4
21	del 1	del 4	del 3	del 4
22	del 3	del 2	del 4	del 4
23	del 4	del 4	del 3	del 4
24	del 4	del 3	del 2	del 2
del 1 (*n*)	4	2	2	0
del 1 (%)	16.7	8.3	8.3	0
(*vs*. parent library)	(1)	(0.5)	(0.5)	(0)
del 2 (*n*)	2	5	8	1
del 2 (%)	8.3	20.8	33.3	4.2
(*vs*. parent library)	(1)	(2.5)	(4.0)	(0.5)
del 3 (*n*)	9	7	6	0
del 3 (%)	37.5	29.2	25.0	0
(*vs*. parent library)	(1)	(0.78)	(0.67)	(0)
del 4 (*n*)	9	10	8	23
del 4 (%)	37.5	41.7	33.3	95.8
(*vs*. parent library)	(1)	(1.11)	(0.89)	(2.56)

Table S6 Phage titer data from the binding assay between NRP1 and T7 phage-displayed HBD.

	Rescued del 2 phage titer (pfu ml^{-1})		
	Vehicle (DMSO)	**1** (1 μM βSQDG)	**4** (10 μM αSQMG)
	42500	9300	270
	76000	21900	3420
	49500	10000	2765
Means	56000	13733	2152
% (vehicle *vs*. compound)	100	24.5	3.8
S.D.	18.2	7.3	1.7

Figure S1 SPR sensorgram between βSQDG (**1**) and human growth factors and cognate receptors. Each protein was immobilized on sensor chip CM5 by an amine coupling reaction and various concentrations of **1** (0.125-2 μM) were injected. Association: 120 sec, dissociation: 120 sec. Response curves were generated by subtraction of the background signals generated simultaneously on the control flow cell (protein non-immobilized cell), the injection of vehicle, and bulk response by DMSO. (A) VEGFR1. (B) VEGFR2. (C) NRP1. (D) TGFβ2. (E) TGFβ RII. (F) IGF-1. (G) IGF-1 R. (H) FGF1. (I) FGF2. (J) FGF R2α. (K) Ang-1. (L) Tie-2. (M) EphB2. (N) EphB4. (O) EGF. (P) EGFR. (Q) PDGF-BB. (R) Mouse PDGFRB. (S) BMP-2. (T) BMP RII. (U) sDLL-4. (V) Jagged-1. (W) Notch-1. (X) DNA polymerase λ fragment as a control. RU: resonance unit. 1 RU = 1*pg* mm^{-2}.



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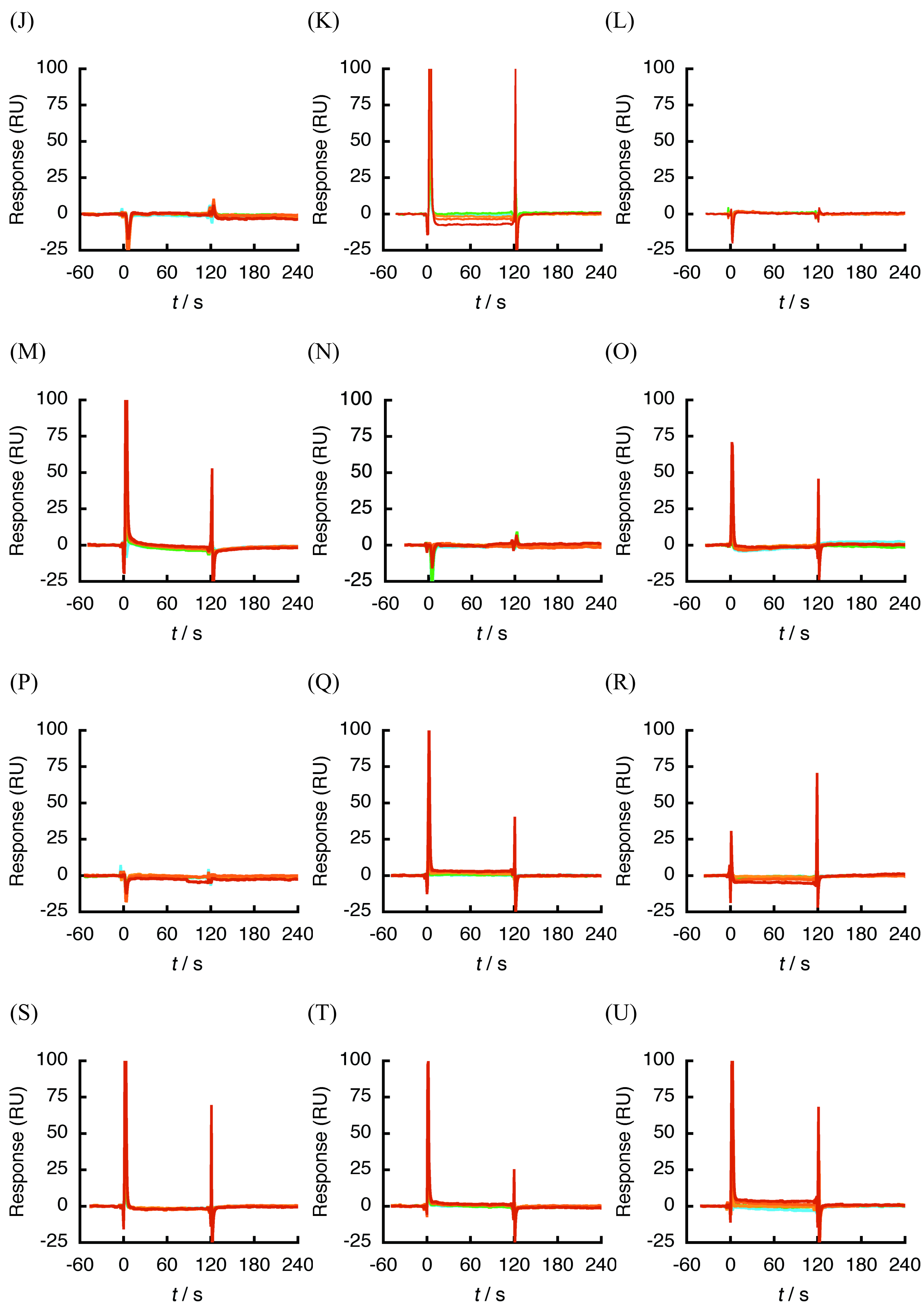

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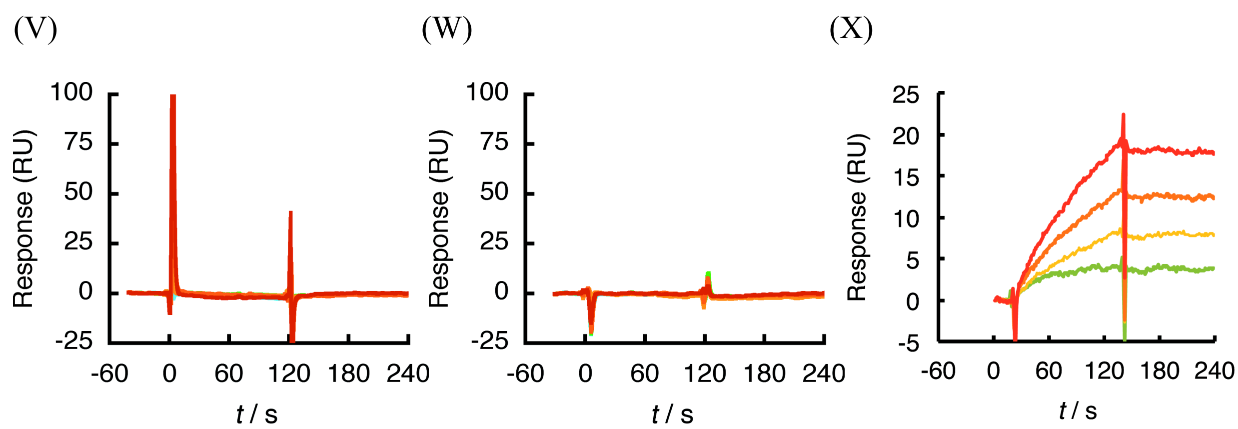

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