

Supplementary Information for

Structural and Functional Analyses Reveal Promiscuous and Species-Specific Use of Ephrin Receptors by Cedar Virus

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Supplementary Information Text

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Receptor interaction analysis by surface plasmon resonance

Briefly, HBS-EP+ buffer (100 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20, pH 7.4) was used for all protein dilutions and as running buffer. To attach CedV sG to the sensor chip surface, the polyclonal anti-S-tag antibody (Bethyl Laboratories, Inc.; Montgomery, TX) was immobilized onto the chip surface using an amine coupling kit (GE Healthcare, Inc.). The anti-S-tag antibody (30 µg/ml) was diluted in 10 mM sodium acetate, pH 5.0 and immobilized on the CM5 chip surface. Next, the CedV sG was injected over the anti-S-tag antibody at 10 µL/min for 5 minutes to capture the CedV sG to the sensor chip surface. To monitor ephrin binding, different concentrations of the indicated recombinant soluble ephrin ligands (human ephrin-A1 Fc transiently expressed in 293 cells and purified; mouse ephrin-A1, mouse ephrin-A2, human ephrin-A5, mouse ephrin-B1, and mouse ephrin-B2 from the ephrin sampler pack 3, SMPK3, R&D Systems; Minneapolis, MN) were injected over the captured CedV sG at a flow rate of 25 µL/min. The soluble ephrin proteins were injected over the CM5 sensor chip surfaces either with or without captured CedV sG. Chip surfaces lacking captured CedV sG served as control surfaces for nonspecific ephrin binding. The antibody surface was regenerated by injection of 10 mM Glycine, pH 2.0 for 30 seconds. The curve-fitting function of the BIAevaluation 4.1 software was used to fit rate equations derived from the simple 1:1 Langmuir binding model to the experimental data. The equilibrium dissociation constant (Kd) was determined from the kinetic rate constants. All values are an average of two independent experiments with two technical replicates for each of the ephrin concentrations tested.

Cell-cell fusion assays

β- galactosidase cell-cell fusion reporter assay

Briefly, effector CHO745 cells transfected with a 1:1 ratio of henipavirus F and G expression plasmids were infected with recombinant vaccinia virus vTF7.3 encoding bacteriophage T7 RNA polymerase (MOI: 10). Target CHO745 cells transfected with expression plasmids encoding recombinant ephrin genes or the empty vector (mock), were infected with recombinant vaccinia virus vCB21R encoding the E.coli lacZ reporter gene under the control of the T7 promoter (MOI: 10). Cells were kept at 37°C with gentle rocking for 2.5 hours, washed with trypsin (0.25%)-EDTA (0.02%) (Quality Biological Inc.), collected, re-suspended in F12K-10 at a concentration of 2 × 10⁵ cells/mL in 50 mL conical tubes and incubated overnight at 31°C. The next day the cells were washed in F12K-10, counted and adjusted to 1 x 10⁶ cells/mL in F12K-10 supplemented with 0.04 mg/mL cytosine β -D-arabino-furanoside hydrochloride (Sigma-Aldrich). Various effector and target cell populations were mixed at a 1:1 ratio in 96-well plate format to yield 2 x 10⁵ total cells per well in a total volume of 0.2 mL. Nonidet P-40 Alternative (0.5%) (EMD Millipore; Billerica, MA) was added after 3 hours incubation at 37°C before the plates were frozen at -80°C overnight. Aliguots of the lysates were assayed for β -galactosidase activity at 37°C upon addition of the substrate chlorophenol red-D-galactopyranoside (CPRG) (Roche Applied Science; Indianapolis, IN). Assays were performed in technical duplicates; fusion results were collected with a VersaMAX microplate reader (Molecular Devices; Sunyvale, CA), calculated and expressed as rates of β -Gal activity (change in O.D. at 570 nm per minute x 1,000) (1).

Split-luciferase based cell-cell fusion kinetics assay

Briefly, CHO-K1 cells (1×10^4 cells/well in a clear bottom, black wall 96-well plate) were co-transfected with 60 ng of the expression plasmid for the indicated receptor and 60 ng of the expression plasmid for one half of a split-luciferase reporter protein (DSP1–7, a kind gift of Z.

Matsuda). As a control, CHO-K1 cells were only transfected with DSP1–7. Concurrently, CHO-K1 cells (5×10^5 cells/well in a 6-well plate) were transfected with 500ng of the other dual-split-reporter expression plasmid (DSP8 –11), and 500 ng each of the untagged CedV F- and G-expression plasmids. Thirty-six hours post-transfection, Versene (0.48 mM EDTA in PBS) (Thermo Fisher Scientific) was used to gently detach the CHO-K1 cells from the 6-well plate and 2×10^4 cells/well overlaid on the receptor-expressing CHO-K1 cells in the 96-well plate. EnduRen (Promega; WI, USA) was added as the substrate to the culture medium (DMEM, 10% FBS) according to the manufacturer's instructions. Content mixing between CHO-K1 cells expressing the two different halves of the luciferase protein as a result of fusion driven by interactions between cells expressing the CedV fusion apparatus and the receptor-expressing CHO-K1 cells was monitored at the indicated times using an Infinite M200 Pro microplate reader (Tecan; Switzerland).

Multiplex microsphere immunoassay

CedV, HeV and NiV soluble receptor-binding proteins (sG) were each coupled to specific magnetic microspheres following a standard manufacturer protocol (Bio-Rad) and mixed together. Henipavirus sG coupled microspheres were incubated with serial diluted human monoclonal antibody, m102.4, at indicated concentrations and incubated at room temperature with agitation. Wells were washed with PBS-Tween (0.05%) then incubated with biotinylated anti-human IgG (1:10,000), agitated, washed and finally incubated with streptavidin-phycoerythrin (PE). The PE signal for each sG-coupled microsphere – hm102.4 complex was detected as a median fluorescence intensity (MFI) by a Bio-Plex 200 machine (Bio-Rad). The graph is a representation of two independent experiments performed in technical triplicates.

References

1. Nussbaum O, Broder CC, & Berger EA (1994) Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virusbased assay quantitating cell fusion-dependent reporter gene activation. *Journal of virology* 68(9):5411-5422.



Supplementary Figure 1. CHO-K1 cells are refractory to Cedar virus infection. HeLa-CCL2, HeLa-USU and CHO-K1 were infected with rCedV-GFP (MOI: 1.0); fluorescent images were collected 48 hpi using a Zeiss Axio Observer A1 inverted microscope using a 5X objective.

CedV	1	MLSQLQKNYLDNSNQQGDKMNNPDKKLSVNFNPLELDKGQKDLNK-SYYVKNKNYNVSNLLNESLHDIKFC	70
NiV	1	IPSKVIK-SYYGTMDIKKINEGLLDSKI-	46
HeV	1	DQGKVIK-NYYGTMDIKKINDGLLDSKI-	46
GhV	1	FALL CALE CALE CALE CALE CALE CALE CALE C	65
MojV	1	K <mark>EK</mark> VADSISGNKV-	41
CedV	71	IYCIFSLL-IIITII-NITI-SIVITRLKVHEENNGMESPNLOSIODSLSSITNMINTEITERIGILVTATSVILSSINYV	150
NiV	47	-LSAFNTVIALLGSI-VIIVMNIMIIONYTRSTDNOAVIKDALQGIOOQIKGLADKIGTEIGPKVSLIDTSSTITIPANIGLL	127
HeV	47	-LGAFNTVIALLGSI-II VMNIMI IONYTRTTDNOALIKESLOSVOOOIKALTDKIGTEIGEKVSLIDISSTITIPANIGLL	127
GhV	66	ILEILVVLGIMFNLIVL-TMVYYONDNINORMAELTSNITVLNLNLNOLTNKIOREIIPRITLIDTATTITIPSAITYI	143
MojV	42	-FILMNTLLILTGAI-ITHTLNITNLTAAKSQQNMLKIIQDDVNAKLEMFVNLDQLVKGEIKEKVSLINHAVSVSIPGQISNL	122
CedV	151	GTETNOLVNELKDYTTKSCGEKVPELKLHECNISCADPKISKSAMYSTNAVAFILACPPKIFCKSVSKDPDFRLKOTDY	228
NiV	128	GSKISOSTASINENVNEKOKETI. PPLKIHECNISCENEL PEREYRPOTEGVSNI. VGLENNICLOKTSNOILKEKISY	205
HeV	128	GSKISOSTSSINENVNDKCKFTLPPLKTHECNISCENPLPFREYRPTSOGVSDLVGLPNOICLOKTTSTILKPRLISY	205
GhV	144	LATLITRISELLPSINOKCEFKTPTLVLNDCRINCTPPLNPSDGVKMSSLATNLVAHGPSPCRNFSSVPTIYYYRIPG	221
MojV	123	QTKFLQKYVYLEESITKQCTCNPLSGIFPTSGPTYPPTDKPDDDTTDDDKVDTTIKPIEYPKPDGCNRTGDHFTMEPGANFYTVPN	208
		2 2' 3 40	
CedV	229	VIPVQQDRSICMNNELLDISDGFFTYIHYEGINSCKKSDSFKVLLSH <mark>CEIVDRGDYR</mark> ESLYLLSSHYHPYSMQVINCVPVTCNQSS	314
NiV	206	TLPVVGQSGTCITDELLAMDEGYFAYSHLERIGSCSRGVSKQRIIGVCEVLDRGDEVESLFMTNVWTPPNPNTVYHCSAVYNNEFY	291
HeV	206	TLPINTREGVCITDELLAVDNGFFAYSHLEKIGSCTRGIAKQRIIGVCEVLDRGDKVSSMFMTNVWTPPNPSTIHHCSSTYHEDFY	291
GhV	222	LYNRTALDERCILNERITISSTKFAYVHSEYDKNCTRGFKYYELMTFCEILEGPEKE RMFSRSFYSPTNAVNYHSCTPIVTVNEG	307
MojV	209	LGPASSNSDECYTN SFSIGSSIYMFSQEIRKTDCTAGEILSIQIVLCRIVDKGQQGCQASPLLVWAVPNPKIINSCAVAAGDEMG	294
Codi	21 5		202
Vill	313	FUE ALSONIATIONS DESTITITING DEPAITEPINNMIADIR THEFTS GG VOLGEEFTIPUTVINIDV	393
HOV	292	IVDERVSIVGDFINSTIWSGSIMTIRER - VREASNGGGINGLARE SIERGENVMFVGESEIRGODIEFFAVGFUNRIV	374
ChV	308		386
MojV	295	THE RECEVENT AS A SEPTEMBER OF VETEX - DEPENDENT OF STATISTICS OF TAXABLE CONTRACT OF TAXAB	373
110 5 V	200		515
CedV	394	5 06 5′ **0 0 *	477
NiV	375	FKYNDSNO PITKOVSKPENCRI SMGTRPNSHY I LESGL KYNLSDGENPKVVFTET SDORLST SPSK TYDSLGOPVEYOA	456
HeV	375	FOYNDSNOPITHCKYSKAENCRI, SMGVNSKSHYTLRSGLLKYNLSLGGDTTLOFTETADNRLTTCS PSKTYNSLGOPVEYOA	456
GhV	387	VTHPLCKKSNCSRTDDESCLKSYYNOGSPOHOVVNCLIRIRNAORDNPTWDVITVDLTNTYPGSRSRIFGSFSKPMLYOS	466
MojV	374	SFKALCEHGSCLGTGGGGYQVLCDRAVMSFCSEESLITNAYLKVNDLASGKPVIIGQTFPPSDSYKGSNGRMYTIGDKYGLYLA	457
		* 7 6′ 7′*	
CedV	478	SMSWDTYLKAGFVEKWKPFTPNAMNNTVISRPNQGNGPRYHK <mark>CFB</mark> ICYGGTYNDIAPLDLGKDMYVSVILDSDQLAFNPEITVF	561
NiV	457	SFSWDTMIKFGDVLTVNPLVVNMRNNTVISRPGQSQCPRFNTCPEIGWEGVYNDAFLIDRINWISAGVFLDSNQTAENPVFTVF	540
HeV	457	SYSWDTMIKLGDVDTVDPLRVQWRNNSVISRPGQSQCPRFNVCPEVOWEGTYNDAFLIDRLNWVSAGVYLNSNQTAENPVFAVF	540
GhV	467	SVSWHTLLQVAEITDLDKYQLDDLDTPYISRPGGSECPFGNYCFTVCWEGTYNDVYSLTPNNDLFVTVYIKSEQVAENPYFAIF	550
MojV	458	P <mark>SSM</mark> NRYLRF G ITPDISVRSTT <mark>M</mark> LKSQDPIMKIL <mark>S</mark> TGTNTDRDM <mark>CFBICN</mark> TRG <mark>NQB</mark> IFPLSEDSEYYTYIGITPNNGC <mark>H</mark> KNFVAVR	543
G. JV	E CO		600
Ceav	562	NSTITETREKVSKUPURIKSTTISCELFLUBPUCISVLIPINKFNGSTRPEITSYKIPKY	622
NIV	541 541		604
ChV	551		630
MoiV	544	ONCE THE ADDRESS OF A DEPARTMENT OF	625

Supplementary Figure 2. Alignment of henipavirus G protein sequences. The alignment was done with Clustal Omega. The disulfide bonds are numbered sequentially, the N-linked sites are indicated by a red hexagon, the ephrin-binding residues mentioned in the text are boxed and those that differ between CedV and HeV/NiV are indicated by a star. The coloring denotes residue identity.

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Salt Bridges

CedV G	Dist. [Å]	Ephrin-B2	
GLU 522 [OE2]	2.95	LYS 103 [NZ]	
GLU 576 [OE2]	2.72	LYS 113 [NZ]	
GLU 554 [OE1]	2.81	LYS 113 [NZ]	
GLU 554 [OE2]	3.80	LYS 113 [NZ]	

Hydrogen bonds

CedV G	Dist. [Å]	Ephrin-B2
THR 408 [OG1]	3.17	TYR 75 [OH]
PRO 423 [O]	3.01	THR 96 [OG1]
ASN 513 [OD1]	2.92	ASN 100 [N]
GLN 407 [OE1]	3.57	ASN 100 [ND2]
SER 405 [O]	3.10	LYS 103 [NZ]
GLN 551 [OE1]	2.67	THR 111 [N]
GLU 576 [OE2]	2.72	LYS 113 [NZ]
GLU 554 [OE1]	2.81	LYS 113 [NZ]
GLU 554 [OE1]	3.67	GLN 115 [NE2]
ASN 578 [OD1]	3.73	GLU 116 [N]
ASN 578 [OD1]	3.41	PHE 117 [N]
ASN 261 [O]	3.00	GLN 127 [NE2]
ASN 513 [ND2]	2.81	ASN 100 [O]
THR 408 [OG1]	3.48	ALA 102 [O]
SER 405 [OG]	2.97	ASP 105 [OD2]
GLN 551 [NE2]	2.86	LYS 109 [O]
GLN 511 [NE2]	2.97	THR 111 [O]
ASN 578 [ND2]	3.51	PHE 117 [O]
GLY 527 [N]	2.82	PRO 119 [O]

Supplementary Figure 3. Interface analysis of CedV G and ephrin-B2 complex. (a) Interaction network between CedV G and ephrin-B2 residues. (b) List of salt-bridges. (c) List of

hydrogen bonds.

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Hydrogen bonds				
CedV G	Dist. [Å]	Ephrin-B1		
GLU 576 [OE2]	3.89	LYS 59 [NZ]		
GLU 554 [OE2]	3.01	LYS 59 [NZ]		
THR 408 [OG1]	3.32	TYR 75 [OH]		
ASN 513 [OD1]	2.99	THR 100 [N]		
GLU 522 [OE1]	3.65	ASN 102 [ND2]		
GLU 576 [OE1]	3.60	LYS 113 [NZ]		
GLN 511 [OE1]	2.98	SER 118 [OG]		
ASN 261 [O]	2.42	LYS 127 [NZ]		
LYS 325 [NZ]	3.20	ASP 94 [OD2]		
GLN 511 [NE2]	2.83	THR 111 [O]		
GLN 551 [NE2]	3.88	THR 111 [O]		
ASN 578 [ND2]	2.34	GLN 115 [OE1]		
GLY 527 [N]	3.07	PRO 119 [O]		
CYS 263 [N]	2.67	GLU 125 [OE1]		
GLU 576 [OE2]	3.89	LYS 59 [NZ]		
GLU 554 [OE2]	3.01	LYS 59 [NZ]		
THR 408 [OG1]	3.32	TYR 75 [OH]		
ASN 513 [OD1]	2.99	THR 100 [N]		
GLU 522 [OE1]	3.65	ASN 102 [ND2]		

Supplementary Figure 4. Interface analysis of CedV G and ephrin-B1 complex. (a) Interaction network between CedV G and ephrin-B1 residues. (b) List of salt-bridges. (c) List of hydrogen bonds.

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Salt Bridges

CedV G	Dist. [Å]	Ephrin-B1	
GLU 576 [OE2]	3.89	LYS 59 [NZ]	
GLU 554 [OE2]	3.01	LYS 59 [NZ]	
GLU 576 [OE1]	3.60	LYS 113 [NZ]	
GLU 554 [OE1]	3.59	LYS 113 [NZ]	



Supplementary Figure 5. Structural comparison of CedV G and ephrins in various states

(a) Superimposition of CedV G global domain in different states. CedV G shown as ribbons. (b) Comparison of ephrin engagement in different G protein complex structures. Complexes were aligned using G proteins as reference. G proteins presented as semi-transparent grey surface and ephrins shown as cartoon in different colors, as indicated.

A1	106	EKFQRFTPFTLGKEFK 121
A2	128	EKFQLFTPFSLGFEFR 143
A3	139	EKFQRYSAFSLGYEFH 154
A4	125	EKIQRFTPFSLGFEFL 140
A5	116	EKFQLFTPFSLGFEFR 131
B1	112	I K F Q E F S P N Y M G L E F K 127
B2	112	I K F Q E F S P N L WG L E F Q 127
B3	115	K F Q E Y S P N L WG H E F R 130

Supplementary Figure 6. Sequence alignment of human ephrins. Key contact residues in the G-H loop responsible for interaction with henipavirus G (e.g. HeV/NiV and ephrin-B2/B3) are indicated. Numbers indicate position of first and last amino acid for each ephrin in alignment. Alignment and image were generated by Geneious version 11.1 (Biomatters; New Zealand); colors indicate hydrophobicity.



Supplementary Figure 7. CedV sG is not bound by a HeV and NiV sG reactive monoclonal antibody. HeV, NiV and CedV sG were coupled to magnetic microspheres and tested for reactivity with a human monoclonal antibody (m102.4) that is strongly reactive with HeV and NiV G. Microspheres coupled with sG binding to hm102.4 was tested by a Bio-Plex 200 (Bio-Rad; Hercules, CA) machine using Luminex xMAP technology.

	CedV G	CedV G-eprhinB1	CedV G-ephrinB2
Wavelength (Å)	0.9792	0.9792	0.9792
Resolution range (Å)	50 - 3.3 (3.42 - 3.3)	50 - 3.5 (3.63 - 3.5)	50 – 2.85 (2.95 – 2.85)
Space group	P 65	P 65	P 65
Unit cell	211.675 211.675 113.194	207.57 207.57 119.377	207.021 207.021 120.65
	90 90 120	90 90 120	90 90 120
Total reflections	320698	414726	505212
Unique reflections	43911	37272	68991
Multiplicity	7.3 (6.9)	11.1 (11.1)	7.3 (6.9)
Completeness (%)	99.16 (92.57)	99.71 (97.17)	99.79 (98.54)
Mean I/sigma(I)	15.36 (1.34)	10.00 (1.24)	15.77 (1.37)
Wilson B-factor	108.94	132.65	82.90
<i>R</i> -merge	0.105 (0.574)	0.158 (0.507)	0.121 (0.511)
<i>R</i> -work	0.21 (0.32)	0.20 (0.33)	0.21 (0.32)
<i>R</i> -free	0.23 (0.37)	0.23 (0.35)	0.24 (0.34)
Number of atoms	7197	9114	9852
macromolecules	6820	8879	9073
ligands	377	235	618
water	0	0	161
Protein residues	880	1124	1183
RMS(bonds)	0.010	0.011	0.010
RMS(angles)	1.39	1.51	1.35
Ramachandran favored (%)	96	96	97
Ramachandran outliers (%)	0	0	0
Clashscore	11.49	16.52	10.04
Average B-factor	62.20	49.90	66.00
macromolecules	60.10	49.30	63.70
ligands	98.70	71.40	103.60
solvent			50.30

Supplementary Table 1. Crystallographic data collection and refinement statistics.

Statistics for the highest-resolution shell are shown in parentheses