



Title	C-type lectins do not act as functional receptors for filovirus entry into cells
Author(s)	Matsuno, Keita; Nakayama, Eri; Noyori, Osamu; Marzi, Andrea; Ebihara, Hideki; Irimura, Tatsuro; Feldmann, Heinz; Takada, Ayato
Citation	Biochemical and Biophysical Research Communications, 403(1): 144-148
Issue Date	2010-12-03
Doc URL	http://hdl.handle.net/2115/44634
Type	article (author version)
File Information	BBRC403-1_144-148.pdf



[Instructions for use](#)

C-type lectins do not act as functional receptors for filovirus entry into cells

Keita Matsuno,^a Eri Nakayama,^a Osamu Noyori,^a Andrea Marzi,^b Hideki Ebihara,^b Tatsuro Irimura,^c Heinz Feldmann,^b and Ayato Takada^{a*}

^aDepartment of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

^bLaboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT, USA

^cGraduate School of Pharmaceutical Science, University of Tokyo, Tokyo, Japan

*Corresponding author:

Department of Global Epidemiology

Hokkaido University Research Center for Zoonosis Control

Kita-20, Nishi-10, Kita-ku

Sapporo 001-0020, Japan

Telephone: +81-11-706-9502

FAX: +81-11-706-7310

E-mail: atakada@czc.hokudai.ac.jp

Abstract

Cellular C-type lectins have been reported to facilitate filovirus infection by binding to glycans on filovirus glycoprotein (GP). However, it is not clearly known whether interaction between C-type lectins and GP mediates all the steps of virus entry (i.e., attachment, internalization, and membrane fusion). In this study, we generated vesicular stomatitis viruses pseudotyped with mutant GPs that have impaired structures of the putative receptor binding regions and thus reduced ability to infect the monkey kidney cells that are routinely used for virus propagation. We found that infectivities of viruses with the mutant GPs dropped in C-type lectin-expressing cells, parallel with those in the monkey kidney cells, whereas binding activities of these GPs to the C-type lectins were not correlated with the infectivities. These results suggest that C-type lectin-mediated entry of filoviruses requires other cellular molecule(s) that may be involved in virion internalization or membrane fusion.

Keywords; filovirus, virus entry, C-type lectin

1. Introduction

Ebola virus (EBOV) and Marburg virus (MARV) are enveloped negative-strand RNA viruses that constitute the family *Filoviridae*. Filovirus infection causes severe hemorrhagic fever in humans and non-human primates and mortality rates have ranged up to 90%. *Zaire ebolavirus* (ZEBOV) has caused multiple large outbreaks with the highest mortality rates (~90%) among EBOV species. Among MARVs, strain Angola (MARV-A) caused the largest outbreak in 2004-05 in Angola, with the highest mortality rate (90%) [1].

It has been shown that the filovirus entry into host cells depends on endosomal acidification [2,3] and proteolysis of the glycoprotein (GP) by endosomal cysteine proteases like cathepsin B and/or L [4]. Filovirus GP is the only spike protein on the surface of the virion, and therefore GP is responsible for both receptor binding and membrane fusion. GP is comprised of two molecules, GP1 and GP2, which are linked by a disulfide bond. GP1 contains a putative receptor binding region (RBR) [5,6] and a mucin-like region (MLR) that has a number of potential N- and O-linked glycosylation sites [7,8]. GP2 has a transmembrane domain, cytoplasmic tail and an internal fusion loop [1].

GP1, in particular MLR, is highly glycosylated by both N- and O-glycans, and these glycans are thought to be recognized by cellular C-type lectins such as liver-specific C-type lectin asialoglycoprotein receptor (ASGP-R) [9,10], dendritic cell- and liver/lymph node-specific ICAM-3-grabbing nonintegrin (DC-SIGN and L-SIGN) [10,11,12,13,14,15,16,17,18], human macrophage galactose-type C-type lectin (hMGL) [18,19], and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) [12,17]. Though these C-type lectins show different specificities, depending on the structures of target glycans, all have been reported to promote filovirus entry. Hepatocytes, dendritic cells, monocytes and macrophages are thought to be the preferred target cells of filoviruses, and infection of these cells is important for hemorrhagic manifestation and

immune disorders [20,21,22,23]. Thus, increased infection of these cells might be directly involved in the pathogenesis of filovirus infection [18,24].

Though the C-type lectins have been reported to enhance filovirus infection, DC-SIGN and L-SIGN did not confer susceptibility for EBOV to non-susceptible cells, i.e. CD4⁺ T-cells [11] and Ramos B cells [14]. In readily susceptible cells, it was reported that the internalization of DC-SIGN and L-SIGN themselves was not essential for trafficking EBOV into endosomal compartments [14]. These studies suggest that C-type lectins promote the filovirus entry by enhancing the virion attachment on the cell surface but not by enhancing the virion internalization. However, it has not been clarified yet whether C-type lectins independently act as a functional receptor mediating attachment, internalization, and membrane fusion. In the present study, to confirm the role of the C-type lectins in filovirus entry, we generated mutant GPs whose RBRs were impaired, and examined their abilities to infect C-type lectin-expressing cells without the interaction between RBR and its unknown putative counterpart(s).

2. Materials and methods

2.1. Cells

293T, Vero E6, and HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. HepG2 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. K562 cell clones expressing hMGL (K562/hMGL), DC-SIGN (K562/DC-SIGN), and mock transfected (K562/mock) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics.

2.2. Viruses

Construction of mutant GPs was done as previously described [18]. The modified GP genes were then ligated into pCAGGS and used to express GPs on 293T cells. Vesicular stomatitis virus expressing green fluorescent protein (GFP) (VSVΔG*) pseudotyped with GP was generated in 293T cells as previously described [2,18].

2.3. Western blot analysis

Anti-ZEBOV GP monoclonal antibody (MAb) 42/3.7 recognizing a linear epitope (amino acid positions 286-296) of ZEBOV GP [25], anti-MARV-A GP MAb 127-8 recognizing a linear epitope (amino acid positions 410-430) of MARV-A GP [25], and anti-VSV matrix protein (M) MAb 192/1 [18] were used for detection of the proteins. Peroxidase-conjugated AffiPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch) and Immobilon Western (Millipore) were used for visualization of the protein bands. Intensities of specific bands were measured with ImageJ [26].

2.4. Lectin-binding assay

VSVΔG* pseudotyped with GPs was purified by ultracentrifugation through a 25% sucrose cushion and diluted in phosphate-buffered saline (PBS). The GP amounts in the VLPs were quantified by Western blotting using MAb ZGP42/3.7 or AGP127-8, and standardized based on the band intensities. Enzyme-linked immunosorbent assay (ELISA) plates were coated with the diluted viruses (2mg/ml) and then blocked with 3% bovine serum albumin in PBS. After each well was washed with Dulbecco's Tris-buffered saline (dTBS), biotinylated soluble recombinant hMGL (hMGL ECD) or DC-SIGN (DC-SIGN ECD) [18] in dTBS was added. To detect C-type lectins bound to the viruses, horseradish peroxidase (HRP)-streptavidin (Jackson ImmunoResearch) and 3,3',5,5'-tetra-methylbenzidine (Sigma) were used.

3. Results and discussion

We first constructed RBR-deletion mutant GPs of ZEBOV GP ($\Delta 54-149$, $\Delta 54-227$, $\Delta 54-313$, $\Delta 89-149$, $\Delta 89-227$, and $\Delta 89-313$) and MARV-A GP ($\Delta 38-188$, $\Delta 38-308$, $\Delta 60-188$, and $\Delta 60-308$) (Fig. 1A), and viruses pseudotyped with these mutant GPs were generated. Lysates of GP-expressing 293T cells and culture supernatants containing pseudotyped viruses were examined by SDS-PAGE and Western blot analysis to verify the expression and the virion incorporation of the GPs (Table 1). Though MAbs 42/3.7 and 127-8 failed to react with ZEBOV GP $\Delta 54-313$, ZEBOV GP $\Delta 89-313$, MARV-A GP $\Delta 38-308$, and MARV-A GP $\Delta 60-308$, the other mutant GPs were detected by these antibodies. Although ZEBOV GP $\Delta 54-149$, ZEBOV GP $\Delta 89-149$, MARV-A GP $\Delta 38-188$, and MARV-A GP $\Delta 60-188$ showed significantly lower band intensities than wild-type GP, the expression on 293T cells and incorporation into the virion of these mutant GPs were verified. We then tested the infectivity of VSV ΔG^* pseudotyped with GPs in the various cell lines (Fig. 1B). The infectivity of VSV ΔG^* bearing the RBR-deletion mutant GPs was undetectable or significantly lower than VSV ΔG^* bearing wild-type GPs in all the cells tested, including the C-type lectin-expressing cells. These results indicated that GPs lacking RBR did not confer the sufficient infectivity to VSV ΔG^* , even when the C-type lectins existed on the target cell surface.

However, deletion of entire RBR polypeptides might cause not only a defect of binding ability to the putative functional receptor but also defects in other essential functions such as membrane fusion. Therefore we constructed mutant ZEBOV GPs with single amino acid substitutions in RBR, which were reported to impact the receptor binding capacity, leading to reduced infectivity [27,28,29]. Based on the amino acid sequence alignment between ZEBOV and MARV-A GPs, MARV-A mutant GPs that had corresponding mutations were also constructed (Table 1). The expression and virion incorporation of each mutant GP were compared with those of wild-type GPs by Western blot analysis (Table 1). Consistent with previous studies [27,28,29], all

the mutant GPs were expressed and incorporated into the virion except MARV-A GP Y146A. The infectivity of the VSVΔG* pseudotyped with mutant GPs was tested in the same cell lines used in Fig. 1 (Fig. 2). As expected, almost all mutant ZEBOV GPs conferred lower infectivity to VSVΔG* in Vero E6, 293, and K562/mock cells than wild-type ZEBOV GP. Similarly, mutations in MARV-A GP (L41A and G127A) significantly reduced the infectivities of the viruses. In the C-type lectin-expressing cells (HepG2, K562/hMGL, and K562/DC-SIGN), the infectivities of the viruses bearing the mutant GPs were also lower than those of the viruses with wild-type GPs, and were likely reduced parallel to the infectivities in Vero E6, 293, and K562/mock cells. These results suggested that the reduced infectivity caused by the mutations in RBR could not be complemented by the interaction between the glycans on GP and C-type lectins.

In a lectin-binding assay using pseudotyped viruses and soluble recombinant hMGL (hMGL ECD) and DC-SIGN (DC-SIGN ECD), we further confirmed that the binding capacities of GPs to these lectins were not significantly reduced by the mutations that gave the lowest infectivities to VSVΔG* in K562/hMGL and K562/DC-SIGN (i.e., F88A and L41A of ZEBOV and MARV-A GPs, respectively)(Fig. 3). This finding indicated that there was no remarkable correlation between GP binding capacity to C-type lectins and reduced infectivity of the viruses with the mutant GPs, and suggested a limited contribution of the interaction between C-type lectin and GP to the subsequent steps in filovirus entry.

In the present study, we demonstrated that the structure of RBR was essential for the entry of filoviruses even when C-type lectins existed on the cell surface, suggesting that the C-type lectins were not independently able to mediate filovirus entry into cells. Therefore, we conclude that C-type lectin-mediated entry of filoviruses requires other cellular molecule(s) that may be critical for virion internalization and/or membrane fusion. Identification of the unknown ubiquitous receptor(s) or coreceptor(s) is essential for further understanding of the molecular mechanisms of filovirus

cellular entry and may provide information on the link to the tropism and pathogenesis of filovirus infection.

Acknowledgments

We thank Hiroko Miyamoto, Ayaka Yokoyama, Teiji Murakami, and Aiko Ohnuma for technical assistance and Kim Barrymore for editing the manuscript. This work was supported by Research Fellowships for Young Scientists from the Japan Society for the Promotion of Science, the Takeda Science Foundation, a Grant-in-Aid for Scientific Research on Priority Areas (19041001), and in part, by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases (05021011) and Global COE Program "Establishment of International Collaboration Centers for Zoonosis Control" (F-001) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (<http://www.mext.go.jp/english/index.htm>).

References

- [1] A. Sanchez, T. Geisbert, H. Feldmann, *Filoviridae: Marburg and Ebola Viruses*, in: D.M. Knipe, P.M. Howley, D.E. Griffin, R.A. Lamb, M.A. Martin, B. Roizman, S.E. Straus (Eds.), *Field's Virology*, Lippincott Williams & Wilkins, Philadelphia, PA, 2007, pp. 1409-1448.
- [2] A. Takada, C. Robison, H. Goto, A. Sanchez, K.G. Murti, M.A. Whitt, Y. Kawaoka, A system for functional analysis of Ebola virus glycoprotein, *Proceedings of the National Academy of Sciences of the United States of America* 94 (1997) 14764-14769.
- [3] R. Wool-Lewis, P. Bates, Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines., *J Virol* 72 (1998) 3155-3160.
- [4] K. Chandran, N.J. Sullivan, U. Felbor, S.P. Whelan, J.M. Cunningham, Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection, *Science (New York, NY)* 308 (2005) 1643-1645.
- [5] D. Dube, M.B. Brecher, S.E. Delos, S.C. Rose, E.W. Park, K.L. Schornberg, J.H. Kuhn, J.M. White, The Primed Ebolavirus Glycoprotein ('19 kDa' GP1,2): Sequence and Residues Critical for Host Cell Binding, *Journal of virology* (2009).
- [6] J.H. Kuhn, S.R. Radoshitzky, A.C. Guth, K.L. Warfield, W. Li, M.J. Vincent, J.S. Towner, S.T. Nichol, S. Bavari, H. Choe, M.J. Aman, M. Farzan, Conserved receptor-binding domains of Lake Victoria marburgvirus and Zaire ebolavirus bind a common receptor, *The Journal of biological chemistry* 281 (2006) 15951-15958.
- [7] H. Geyer, C. Will, H. Feldmann, H.D. Klenk, R. Geyer, Carbohydrate structure of Marburg virus glycoprotein, *Glycobiology* 2 (1992) 299-312.
- [8] H. Feldmann, S.T. Nichol, H.D. Klenk, C.J. Peters, A. Sanchez, Characterization of filoviruses based on differences in structure and antigenicity of the virion glycoprotein, *Virology* 199 (1994) 469-473.
- [9] S. Becker, M. Spiess, H.D. Klenk, The asialoglycoprotein receptor is a potential liver-specific receptor for Marburg virus, *The Journal of general virology* 76 (Pt 2) (1995) 393-399.
- [10] A. Marzi, T. Gramberg, G. Simmons, P. Möller, A.J. Rennekamp, M. Krumbiegel, M. Geier, J. Eisemann, N. Turza, B. Saunier, A. Steinkasserer, S. Becker, P. Bates, H. Hofmann, S. Pöhlmann, DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus, *Journal of virology* 78 (2004) 12090-12095.
- [11] G. Simmons, J.D. Reeves, C.C. Grogan, L.H. Vandenberghe, F. Baribaud, J.C. Whitbeck, E. Burke, M.J. Buchmeier, E.J. Soilleux, J.L. Riley, R.W. Doms, P. Bates, S. Pöhlmann, DC-SIGN and DC-SIGNR bind ebola glycoproteins and enhance infection of macrophages and endothelial cells, *Virology* 305 (2003) 115-123.
- [12] T. Gramberg, H. Hofmann, P. Möller, P.F. Lalor, A. Marzi, M. Geier, M. Krumbiegel, T. Winkler, F. Kirchhoff, D.H. Adams, S. Becker, J. Münch, S. Pöhlmann, LSECtin interacts with filovirus glycoproteins and the spike protein of SARS coronavirus, *Virology* 340 (2005) 224-236.

- [13] A. Marzi, A. Akhavan, G. Simmons, T. Gramberg, H. Hofmann, P. Bates, V. Lingappa, S. Pöhlmann, The signal peptide of the ebolavirus glycoprotein influences interaction with the cellular lectins DC-SIGN and DC-SIGNR., *J Virol* 80 (2006) 6305-6317.
- [14] A. Marzi, P. Möller, S. Hanna, T. Harrer, J. Eisemann, A. Steinkasserer, S. Becker, F. Baribaud, S. Pöhlmann, Analysis of the interaction of Ebola virus glycoprotein with DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) and its homologue DC-SIGNR., *J Infect Dis* 196 Suppl 2 (2007) S237-246.
- [15] C.P. Alvarez, F. Lasala, J. Carrillo, O. Muñiz, A.L. Corbí, R. Delgado, C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans, *Journal of virology* 76 (2002) 6841-6844.
- [16] F. Baribaud, S. Pöhlmann, G. Leslie, F. Mortari, R.W. Doms, Quantitative expression and virus transmission analysis of DC-SIGN on monocyte-derived dendritic cells, *Journal of virology* 76 (2002) 9135-9142.
- [17] T. Gramberg, E. Soilleux, T. Fisch, P.F. Lalor, H. Hofmann, S. Wheeldon, A. Cotterill, A. Wegele, T. Winkler, D.H. Adams, S. Pöhlmann, Interactions of LSECTin and DC-SIGN/DC-SIGNR with viral ligands: Differential pH dependence, internalization and virion binding, *Virology* 373 (2008) 189-201.
- [18] K. Matsuno, N. Kishida, K. Usami, M. Igarashi, R. Yoshida, E. Nakayama, M. Shimojima, H. Feldmann, T. Irimura, Y. Kawaoka, A. Takada, Different potential of C-type lectin-mediated entry between Marburg virus strains., *J Virol* 84 (2010) 5140-5147.
- [19] A. Takada, K. Fujioka, M. Tsuiji, A. Morikawa, N. Higashi, H. Ebihara, D. Kobasa, H. Feldmann, T. Irimura, Y. Kawaoka, Human macrophage C-type lectin specific for galactose and N-acetylgalactosamine promotes filovirus entry, *Journal of virology* 78 (2004) 2943-2947.
- [20] K.J. Davis, A.O. Anderson, T.W. Geisbert, K.E. Steele, J.B. Geisbert, P. Vogel, B.M. Connolly, J.W. Huggins, P.B. Jahrling, N.K. Jaax, Pathology of experimental Ebola virus infection in African green monkeys. Involvement of fibroblastic reticular cells, *Archives of pathology & laboratory medicine* 121 (1997) 805-819.
- [21] H. Feldmann, H. Bugany, F. Mahner, H.D. Klenk, D. Drenckhahn, H.J. Schnittler, Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages, *Journal of virology* 70 (1996) 2208-2214.
- [22] T.W. Geisbert, P.B. Jahrling, M.A. Hanes, P.M. Zack, Association of Ebola-related Reston virus particles and antigen with tissue lesions of monkeys imported to the United States, *Journal of comparative pathology* 106 (1992) 137-152.
- [23] H.J. Schnittler, H. Feldmann, Molecular pathogenesis of filovirus infections: role of macrophages and endothelial cells, *Current topics in microbiology and immunology* 235 (1999) 175-204.
- [24] T.W. Geisbert, L.E. Hensley, Ebola virus: new insights into disease aetiopathology and possible therapeutic interventions, *Expert reviews in molecular medicine* 6 (2004) 1-24.
- [25] E. Nakayama, A. Yokoyama, H. Miyamoto, M. Igarashi, N. Kishida, K. Matsuno, A. Marzi, H. Feldmann, K. Ito, M. Saijo, A. Takada, Enzyme-linked immunosorbent assay for the

- detection of filovirus species-specific antibodies., *Clin Vaccine Immunol* (2010).
- [26] M.D. Abramoff, P.J. Magelhaes, S.J. Ram, Image Processing with ImageJ, *Biophotonics International* 11 (2004) 36-42.
- [27] B. Manicassamy, J. Wang, H. Jiang, L. Rong, Comprehensive analysis of ebola virus GP1 in viral entry., *J Virol* 79 (2005) 4793-4805.
- [28] O. Mpanju, J. Towner, J. Dover, S. Nichol, C. Wilson, Identification of two amino acid residues on Ebola virus glycoprotein 1 critical for cell entry., *Virus Res* 121 (2006) 205-214.
- [29] M. Brindley, L. Hughes, A. Ruiz, P.J. McCray, A. Sanchez, D. Sanders, W. Maury, Ebola virus glycoprotein 1: identification of residues important for binding and postbinding events., *J Virol* 81 (2007) 7702-7709.

Figure legends

Figure 1. Infectivity of VSVΔG* pseudotyped with GPARBR. Functional domains and putative regions of ZEBOV GP and A-MARV GP are represented in schematic forms (A) (SP; signal peptide, RBR; receptor binding region, MLR; mucin-like region, IFL; internal fusion loop, and TM; transmembrane domain). Infectivities of the viruses in Vero E6, 293, HepG2, K562/mock, K562/hMGL, and K562/DC-SIGN were determined by counting GFP-positive cells and the infectious units (IUs) are indicated on the vertical lines (B). All experiments were done at least three times and averages and standard deviations are shown.

Figure 2. Infectivity of VSVΔG* pseudotyped with mutant GPs having single amino acid substitutions. The infectious units determined for each virus in Vero E6, 293, HepG2, K562/mock, K562/hMGL, and K562/DC-SIGN are indicated on the vertical lines. All experiments were done at least three times and averages and standard deviations are shown.

Figure 3. Binding capacity of the C-type lectins to VSVΔG* pseudotyped with MARV GPs. ELISA plates were coated with purified VSVΔG* bearing mutant GPs. Biotinylated recombinant soluble hMGL ECD (2.5mg/ml) and DC-SIGN ECD (2.5mg/ml) were incubated with the viruses and visualized as described in Materials and Methods. All experiments were done in triplicate, and average results and standard deviations are shown.

Tables

Table 1. Characteristics of entry deficient mutant GPs

	Protein expression [†]	Virion incorporation [‡]	Reference
ZEBOV GP	++++	++++	
ZEBOV GPΔ54-149	++	+	
ZEBOV GPΔ54-227	++++	+++	
ZEBOV GPΔ54-313	ND	ND	
ZEBOV GPΔ89-149	++	+	
ZEBOV GPΔ89-227	++++	++++	
ZEBOV GPΔ89-313	ND	ND	
D55A	++++	++++	[27]
L57A	++++	++++	[27]
L57I	++++	++++	[27]
L57F	++++	++++	[27]
L57K	++++	++++	[27]
L63A	++++	+++	[27]
R64E	++++	++++	[27]
F88A	++++	++	[27,28]
K95A	++++	+++	[27]
R134A	++++	++	[29]
K140A	++++	+++	[29]
G143A	++++	+++	[29]
I170A	++++	+++	[27]
MARV-A GP	++++	++++	
MARV-A GPΔ38-188	+	++++	
MARV-A GPΔ38-308	ND	ND	
MARV-A GPΔ60-188	+	++++	
MARV-A GPΔ60-308	ND	ND	
L41A	++++	++++	
K79A	++++	++++	
K118A	++++	++++	
G127A	++++	++++	
Y146A	ND	ND	

[†]: intensities of GP specific bands in the lysate of 293T cells

[‡]: the ratio between intensities of GP and the VSV M specific band in the supernatant of 293T cells

++++: >75% of wild-type GP

+++ : 50-75% of wild-type GP

++ : 25-50% of wild-type GP

+ : <25% of wild-type GP

ND: GP specific bands not detected

Figure1
[Click here to download high resolution image](#)

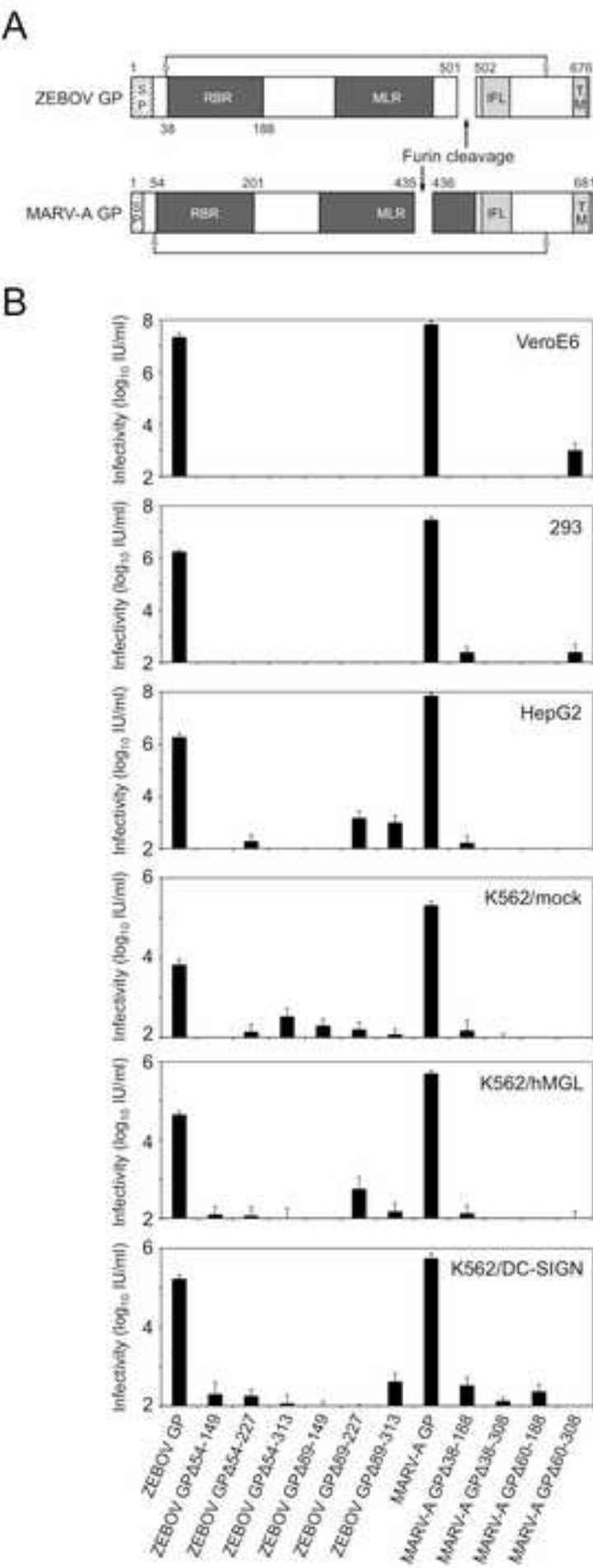


Figure2

[Click here to download high resolution image](#)

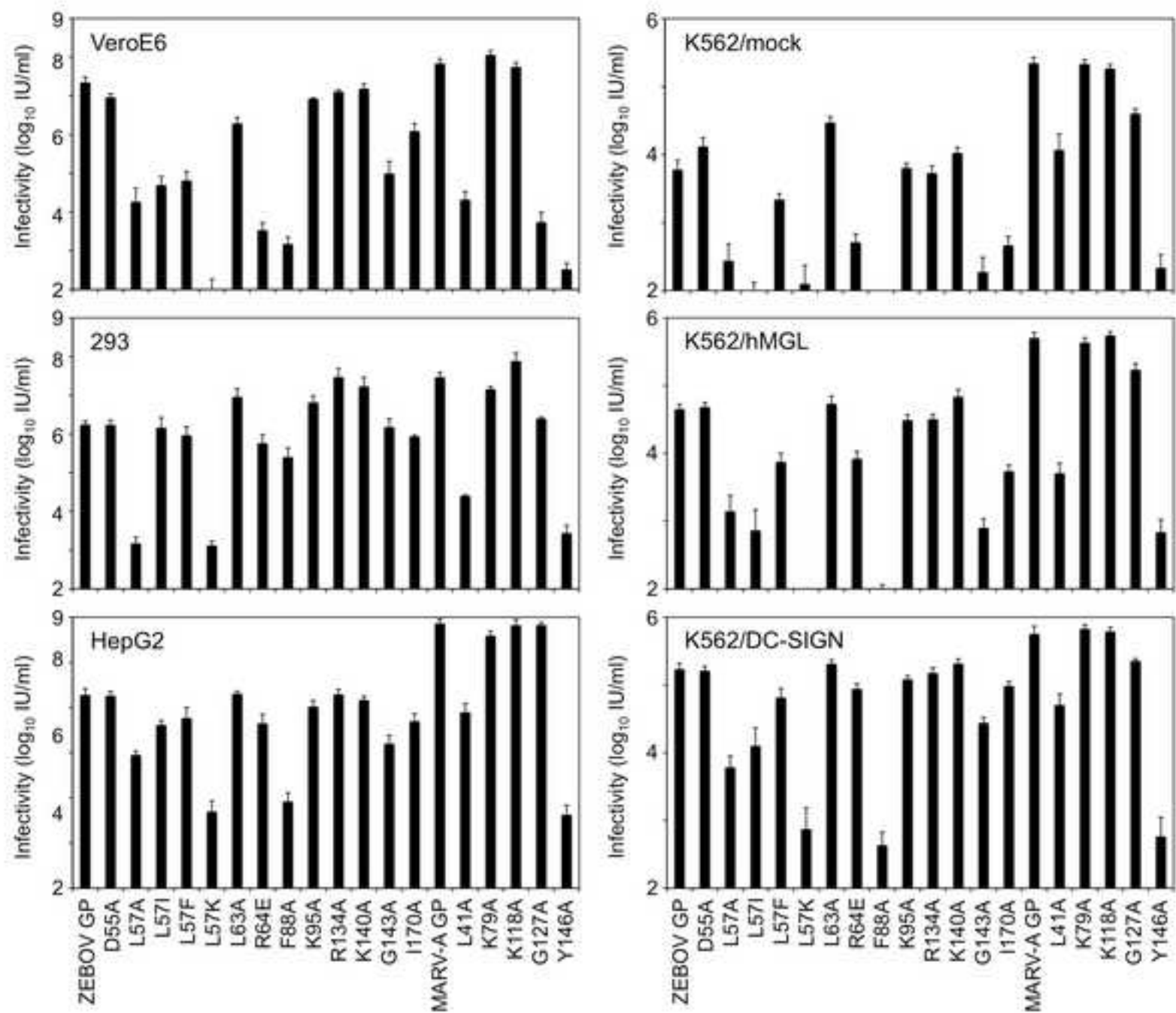


Figure3
[Click here to download high resolution image](#)

