

## Functional studies of host-specific ephrin-B ligands as Henipavirus receptors

Katharine N. Bossart<sup>a,\*</sup>, Mary Tachedjian<sup>a,1</sup>, Jennifer A. McEachern<sup>a</sup>, Gary Crameri<sup>a</sup>,  
Zhongyu Zhu<sup>b,c</sup>, Dimiter S. Dimitrov<sup>c</sup>, Christopher C. Broder<sup>d</sup>, Lin-Fa Wang<sup>a</sup>

<sup>a</sup> CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria 3220, Australia

<sup>b</sup> BRP, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD 21702, USA

<sup>c</sup> Protein Interactions Group, CCRNP, CCR, NCI-Frederick, NIH, Frederick, MD 21702, USA

<sup>d</sup> Uniformed Services University, Bethesda, MD 20814, USA

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### Abstract

Hendra virus (HeV) and Nipah virus (NiV) are closely related paramyxoviruses that infect and cause disease in a wide range of mammalian hosts. To determine whether host receptor molecules play a role in species-specific and/or virus-specific infection we have cloned and characterized ephrin-B2 and ephrin-B3 ligands from a range of species, including human, horse, pig, cat, dog, bats (*Pteropus alecto* and *Pteropus vampyrus*) and mouse. HeV and NiV were both able to infect cells expressing any of the ephrin-B2 and ephrin-B3 molecules. There did not appear to be significant differences in receptor function from different species or receptor usage by HeV and NiV. Soluble ephrin ligands, their receptors and G-specific human monoclonal antibodies differentially blocked henipavirus infections suggesting different receptor affinities, overlapping receptor binding domains of the henipavirus attachment glycoprotein (G) and that the functional domains of the ephrin ligands may be important for henipavirus binding. © 2007 Elsevier Inc. All rights reserved.

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### Introduction

Hendra virus (HeV) and Nipah virus (NiV) are closely related, highly pathogenic paramyxoviruses that have independently emerged in the past 15 years and continue to re-emerge in new locations. Flying foxes in the genus *Pteropus* are considered to be the natural reservoir for both viruses and indeed their geographic range encompasses all locations where HeV and NiV have emerged (Chua et al., 2002; Halpin et al., 2000). HeV has appeared sporadically in Australia since 1994 where infection has been transmitted from flying foxes to horses to

humans (reviewed in Eaton et al., 2006). Most fatalities have occurred in horses where disease presented as a severe respiratory infection. To date, there have been two human mortalities; one presented with severe respiratory disease and another died of encephalitis 13 months post-exposure. Recent outbreaks of HeV where horse fatalities have been documented include 1999, 2004, 2006 and 2007 and one mildly ill, seroconverting, human case was reported in 2004 (Anonymous, 2006, 2007; Hanna et al., 2006; Westbury, 2000). NiV first appeared in Malaysia and Singapore in 1998–1999 and the majority of infections first occurred in pigs and were then transmitted to humans (reviewed in Chua, 2003; Tan and Wong, 2003). In pigs, infection manifested primarily as a respiratory disease with some encephalitis but with a relatively low mortality rate whereas in humans, it manifested as a severe febrile encephalitis with high case fatality (Chua, 2003). Additionally, up to 25% of cases exhibited respiratory signs and a significant number of patients experienced delayed relapsed encephalitis with a significant fatality rate (~18%). Dogs in close proximity to infected pigs demonstrated a high seroprevalence to NiV and

\* Corresponding author. CSIRO Livestock Industries, Australian Animal Health Laboratory, 5 Portarlington Rd., Geelong, VIC 3220, Australia. Fax: +61 3 5227 5555.

E-mail addresses: [Katharine.Bossart@csiro.au](mailto:Katharine.Bossart@csiro.au) (K.N. Bossart), [Mary.Tachedjian@csiro.au](mailto:Mary.Tachedjian@csiro.au) (M. Tachedjian), [Jennifer.McEachern@csiro.au](mailto:Jennifer.McEachern@csiro.au) (J.A. McEachern), [Gary.Crameri@csiro.au](mailto:Gary.Crameri@csiro.au) (G. Crameri), [zhongyuzhu@ncifcrf.gov](mailto:zhongyuzhu@ncifcrf.gov) (Z. Zhu), [dimitrov@ncifcrf.gov](mailto:dimitrov@ncifcrf.gov) (D.S. Dimitrov), [cbroder@usuhs.mil](mailto:cbroder@usuhs.mil) (C.C. Broder), [Linfa.Wang@csiro.au](mailto:Linfa.Wang@csiro.au) (L.-F. Wang).

<sup>1</sup> These authors contributed equally to this work.

although only two dogs with active disease were examined, both demonstrated respiratory disease and abnormalities in the brain (Hooper et al., 2001). Clinical features of NiV infected cats resembled those seen in humans and pigs, particularly with respect to tropism for endothelial cells and the respiratory epithelium (Hooper et al., 2001; Middleton et al., 2002). Cats have been used in experimental infection models with both HeV and NiV (Mungall et al., 2006; Williamson et al., 1998) and like HeV-infected horses, HeV- and NiV-infected cats exhibit severe respiratory disease and a high mortality rate but encephalitis has not been identified although virus does enter the central nervous system (CNS).

Paramyxoviruses are large, enveloped, negative-sense single stranded RNA viruses that generally have a narrow host range (Yin et al., 2006). HeV and NiV have been classified into the new *Henipavirus* genus of the family *Paramyxoviridae*, and are unique in that they exhibit a broad species tropism and cause fatal disease in both animals and humans (Eaton et al., 2006, 2005; Hooper et al., 2001). Paramyxoviruses contain two major membrane-anchored envelope glycoproteins that are required for infection of a receptive host cell. All members contain a fusion (F) glycoprotein which mediates pH-independent membrane fusion between the virus and its host cell, while the second is the attachment glycoprotein which binds the host cell receptor (reviewed in Yin et al., 2006). HeV and NiV possess attachment glycoproteins that lack hemagglutinin and neuraminidase activities and are designated G for glycoprotein (reviewed in (Bossart and Broder, in press)). Other than morbilliviruses, henipaviruses are the only other paramyxoviruses characterized to date known to utilize host cell proteins as receptors. Recently, ephrin-B2 ligand (EFNB2) was identified as a receptor employed by HeV and NiV for infection (Bonaparte et al., 2005; Negrete et al., 2005) and shortly thereafter, ephrin-B3 ligand (EFNB3) was identified as a receptor for NiV (Negrete et al., 2006). Ephrin molecules are highly conserved across vertebrate species and are members of a family of receptor tyrosine kinase ligands (Drescher, 2002; Poliakov et al., 2004). The ephrin-B ligands contain transmembrane and cytoplasmic domains and participate in bi-directional signaling events upon receptor binding; forward signaling into an ephrin receptor (Eph) bearing cell and reverse signaling where they signal into the cell upon which they are expressed (Palmer and Klein, 2003). Human EFNB2 and EFNB3 share 39% amino acid identity. The high level of conservation of these receptors across many species has helped explain why HeV and NiV, unlike other paramyxoviruses, are capable of such a broad species tropism. EFNB2 is highly expressed on neurons, smooth muscle, arterial endothelial cells and capillaries; closely paralleling the known tissue tropism of HeV and NiV *in vivo*. EFNB3 is expressed in specific regions of the central nervous system (CNS) and may facilitate pathogenesis in certain neural subsets.

The underlying host and viral factors influencing the pathogenicity of HeV and NiV in different species is largely unknown. To begin to understand receptor molecule function across various susceptible animal species, we cloned and characterized EFNB2 and EFNB3 from human, horse, pig, cat, dog, mouse and two flying foxes, *Pteropus alecto*, and *Pteropus vampyrus*. HeV and

NiV were capable of infecting cells expressing any and all of the various species of EFNB2 and EFNB3. Importantly, there did not appear to be differences in receptor function from different species or receptor usage by HeV and NiV. Soluble EFNB2 and G-specific human monoclonal antibodies (hmAbs) blocked henipavirus infection mediated by either ephrin from all species, suggesting all EFNB2 and EFNB3 molecules bind an overlapping domain of the henipavirus attachment glycoprotein (G). Ephrin receptors EphB3 and EphB4 inhibited HeV and NiV infections significantly suggesting that the Eph binding domain of the ephrin ligands may be important for binding the henipavirus G glycoprotein. Our results demonstrate that both EFNB2 and EFNB3 molecules from a variety of animals can function as highly efficient receptors for both NiV and HeV. These findings may have important implications for our understanding of the epidemiology and pathogenesis of these viruses as well as for the development of novel therapeutics.

## Results

### *Selection and cloning of ephrin ligands*

In order to assess whether ephrin ligands played a role in the observed differential henipavirus disease manifestations, we chose to clone and characterize the EFNB2 and EFNB3 molecules from a variety of alternative species known to be naturally or experimentally susceptible to infection. Specifically we included human, horse, pig, cat, dog, mouse and the flying foxes *P. alecto* and *P. vampyrus*. Human, horse, pig, cat and dog all exhibited different clinical symptoms and mortality rates during the HeV and NiV outbreaks. *P. alecto* and *P. vampyrus* were chosen as they are the natural reservoir for HeV and NiV but only develop subclinical disease upon infection (Middleton et al., 2007). NiV and HeV do not cause disease in mice after subcutaneous administration (Crameri, G and Eaton, B.T., unpublished observations) or with either an intranasal or intraperitoneal challenge of NiV (Wong et al., 2003), although they are lethal if administered intracranially. The absence of systemic henipavirus disease in mice is unique and differences in EFN molecules could account for such differences as EFNB2 is widely expressed, whereas EFNB3 expression is restricted to the CNS. To ensure no cell culture adapted mutations existed; fresh kidney and liver tissues were obtained for all species with the exception of human, where cDNA clones were already available (Bonaparte et al., 2005). We found that liver consistently gave good yields of high quality RNA and was thus used for most EFN cloning except for the cloning of EFNB3 from *P. alecto* where brain tissue was used. For horse, cat, *P. alecto* and *P. vampyrus* no sequence data were available for EFNB2 and/or EFNB3 molecules. By using a combination of RT-PCR and 5' and 3' RACE strategies as detailed in Materials and methods, we were able to generate full-length cDNA clones from each species. For the pig, dog and mouse, specific primers were designed based on published sequence data and the full-length EFNB2 and EFNB3 open reading frames were amplified from cDNA. For human, specific primers were used to amplify both ephrin ligands from pre-existing DNA constructs. For each

species an individual ephrin ligand clone was chosen for sequencing and characterization (Table 1).

#### Analysis of EFNB2 and EFNB3 DNA and protein sequences

The EFNB2 DNA sequences were aligned to one another and percent identities are shown in Table 2. There was a high level of conservation among all species, ranging from 93 to 95%, with the exception of mouse whose identity was slightly lower, 91%. Similar results were obtained when all EFNB3 DNA sequences were aligned to one another. Here the percent identity ranged from 91 to 95% and again mouse demonstrated the lowest level of conservation (Table 2). Because we were interested in the function of EFNB2 and EFNB3 molecules as viral receptors we next aligned the putative amino acids (aa) sequences of EFNB2 and EFNB3 molecules and results are shown in Figs. 1 and 2, respectively. As was seen with the DNA alignments, the EFNB2 proteins had significant percent identities ranging from 95 to 96% (Table 2). Although most aa changes were single substitutions, we identified a small region (residues 182–187) in both bat sequences that appeared to be significantly different. These aa changes were not present in any other species and could have represented changes in EFNB2 that decreased henipavirus receptor function. The EFNB3 molecules also had high sequence identities, ranging from 95 to 98% (Table 2). Like EFNB2, most substitutions were single aa changes; however, aa 21 and 22 varied in different species and the region between residues 200 and 208 had several changes. Bat and mouse were different from all other species in these regions and like EFNB2, these changes could affect henipavirus receptor function.

#### Expression of ephrin ligands

To discern whether the aa sequence differences had an effect on receptor function we established a transient expression

Table 2  
DNA and amino acid sequence identities of ephrin ligands

	EFNB2		EFNB3	
	DNA	Amino acid	DNA	Amino acid
Human	100	100	100	100
Horse	95	96	95	98
Pig	95	96	95	97
Cat	94	96	93	97
Dog	93	96	95	97
P. alecto	94	95	94	97
P. vampyrus	94	95	94	97
Mouse	91	96	91	95

All sequences were aligned to human reference sequence using Clone Manager Professional Suite version 8 software. For DNA sequences global-reference alignments were done using a standard linear scoring matrix. For amino acid sequences global alignments were done using a standard linear scoring matrix.

system in HeLa-USU cells, a cell line previously shown to be non-permissive for HeV and NiV (Bonaparte et al., 2005). All ephrin expression constructs were transfected overnight using equal amounts of DNA and a liposomal transfection reagent as described in Materials and methods. As the functional domains of EFNB2 and EFNB3 molecules were likely to be highly conserved in all species we speculated that the ephrin receptor (Eph) molecules, EphB3 and EphB4 could be used for EFN cell surface staining in all species. Live cells were used for immunofluorescence, and results for EFNB2 and EFNB3 expression are shown in Figs. 3 and 4, respectively. EphB3 bound all EFNB2 molecules similarly and verified cell surface expression of these proteins from all species. EphB3 also bound all EFNB3 molecules similarly and provided evidence of cell surface expression from all species. EphB4 bound all EFNB2 molecules similarly but was unable to bind EFNB3 (data not shown). To further examine differential Eph-EFN binding, recombinant EFN and Eph molecules were used for binding and competition assays. To increase assay sensitivity, multiplexed microsphere

Table 1  
Primers used for PCR amplification of EFN molecules

	EFNB2 primers	EFNB3 primers
<i>Internal</i>		
Forward	CTACCTGGACAAGGACTGG	CAGCCTGGAGCCTGTCTACTG
Reverse	GGATGATAATGTCACTGGG	GCCCATCCTGCACGATRTACAC
<i>RACE</i>		
5' RACE outer	CTTTATAATATTCATACTGGCCAACAG	GCTGGCGCTGCTCTTGCTGG
5' RACE inner	GAGTCCACTTTGGGGCAAATAATATCC	GTGACCCACCAGCAATGCAA
3' RACE outer	AGCACACGGCCACGCTGTCAC	GCTGGCGCTGCTCTTGCTGG
3' RACE inner	AGCACGCTGGCCACGCCAAGC	CACCCTGGTCTGGCTCCTT
<i>Cloning</i>		
Consensus Forward	GTCGACCACCATGGCYGYGAGRAGGGACTCC	GTCGACCACCATGGGGCCCCCATTCTGG
Consensus Reverse	GTCGACTCTCAGACCTTRTAGTAAATGTTT	GTCGACGAGRAGCCCTCATACCTTGATG
Mouse Forward	GTCGACCACCATGGCCATGGCCCGTCCAGGAG	GTCGACCACCATGGGGCCCCCATTTTGG
Mouse Reverse	GTCGACGGCCTCAGACCTTGTAATAATGTTG	GTCGACGAGGAGCCCTCATACCTTGTAATAG
Dog Forward <sup>a</sup>	GTCGACCACCATGGCCGCGAGGAG	GTCGACCACCATGGGGCCCTGCGTTC
Dog Reverse <sup>a</sup>	GTCGACTCAGACCTTGTAATAATG	GTCGACTCACCAGGACGGCCG
Cat-pig Forward		GTCGACCACCATGGGGCCCCSCGTTCTG
Cat-pig Reverse		GTCGACGGAGRAGCCCTCATACCTTGATG

<sup>a</sup> The same primers were used for dog and pig EFNB2.

Human	MAV-RRDSVWKYCWGVLMVL <b>C</b> RTAISKSIVLEPIYWNSSNSKFLPGQGLVLYPQIGDKLD	59
Horse	..A-...L.....R.....	59
Pig	..A-.....R.....	59
Cat	..-.....R.....	59
Dog	..A-.....R.....	59
<i>P. alecto</i>	..A-.....R.....	59
<i>P. vampyrus</i>	..A-.....R.....	59
Mouse	..RS.....L.....R.....	60
Human	II <b>C</b> PKVDSKTVGQYEYKVMVDKDQADRC <b>T</b> IKKENTPLLN <b>C</b> AKPDQDIKFTIKFQEFSP	119
Horse	.....R...V.....	119
Pig	.....R...V.....	119
Cat	.....E.....R...V.....	119
Dog	.....R...V.....	119
<i>P. alecto</i>	.....S.T.....R...V.....	119
<i>P. vampyrus</i>	.....S.T.....R...V.....	119
Mouse	.....R...V.....	120
Human	NLWGLEFQKNKDYIIISTSN <b>S</b> LEGLDNQEGGV <b>C</b> QTRAMKILMKVGVQDASSAGSTRNKDP	179
Horse	.....R.....P.HN..	179
Pig	.....R.....HNE..	179
Cat	.....R.....HN..	179
Dog	.....R.....A.HN..	179
<i>P. alecto</i>	.....HN..	179
<i>P. vampyrus</i>	.....HN..	179
Mouse	.....A..HG..	180
Human	<b>T</b> RRPELEAGTNGRSSTTSPFVKPNPGSSTDGNSAGHS <b>G</b> NNILGSE <b>V</b> ALFAGIAS <b>G</b> <b>C</b> II <b>F</b> I	239
Horse	.....	239
Pig	.....	239
Cat	.....S.....	239
Dog	.....S.....	239
<i>P. alecto</i>	..H..QAP.....S.....	239
<i>P. vampyrus</i>	..H..QAP.....S.....	239
Mouse	.....L.....	240
Human	<b>V</b> IIITLVVLL <b>L</b> KYRRRRHKHSPQHTTTLSLSTLATPKRSGNN <b>S</b> EPSDIIIP <b>L</b> RTADSV	299
Horse	.....A.....	299
Pig	.....A..I.....G.....	299
Cat	.....A.....S.....T.....	299
Dog	.....A.....G.....	299
<i>P. alecto</i>	.....A.....	299
<i>P. vampyrus</i>	.....A.....	299
Mouse	.....G.....V.....	300
Human	F <b>C</b> PHYEKVSGDYGHPVYIVQEMPPQSPANIYK <b>V</b>	333
Horse	.....	333
Pig	.....	333
Cat	.....	333
Dog	.....	333
<i>P. alecto</i>	.....	333
<i>P. vampyrus</i>	.....	333
Mouse	.....	334

Fig. 1. Amino acid sequence alignment of EFNB2 from different species. Species and amino acid number are listed on the left and right, respectively. Dots represent identical amino acids; hyphens represent gaps introduced to maximize sequence alignments. The transmembrane domain is indicated by boxed borders, cysteine residues are shaded in gray, and putative glycosylation sites are underlined. GenBank accession numbers: horse EFNB2:EF682140; pig EFNB2:EF682141; cat EFNB2:EF682138; dog EFNB2:EF682139; *P. alecto* EFNB2:EF682142; *P. vampyrus* EFNB2:EF682143; mouse EFNB2:NM\_010111.

assays were conducted using the Bio-Plex Protein Array System as described in Materials and methods. Briefly, EFN molecules were coupled to spectrally distinct fluorescently labeled microsphere subsets and Eph molecules were biotinylated. Binding of biotinylated Eph molecules to each EFN-coupled microsphere subset was quantified in the absence and presence of non-biotinylated Eph molecules and median fluorescent intensities (M.F.I) are shown in Fig. 5. Consistent with the immunofluorescence data, EphB3 bound EFNB2 and EFNB3 (Fig. 5A). Non-biotinylated EphB4 did not block EphB3 binding to either EFN molecule, non-biotinylated EphB3 was included as a control and completely blocked binding to both EFN molecules. EphB4 bound EFNB2 as expected (Fig. 5B); however, M.F.I. values were significantly lower as compared to EphB3-EFNB2 binding values. EphB4 bound EFNB3 poorly as indicated by

the low M.F.I. values. Non-biotinylated EphB3 significantly blocked binding of EphB4 to EFNB2. Although EphB4 binding to EFNB3 was low, non-biotinylated EphB3 appeared to further reduce EphB4 binding. Together these data suggest that both Eph receptors bind to an overlapping domain of EFN and that EphB3 has a stronger binding affinity than EphB4. Additionally, subtle changes in the Eph binding domain of EFNB3 are suggested as the binding of EphB3 was reduced as compared to EphB3-EFNB2 binding and the binding of EphB4 was almost undetectable.

#### Function of ephrin ligands as henipavirus receptors

To assess and compare each ephrin ligand and its ability to support infection by HeV and NiV, all ephrin ligand transfections

Human	MGPPHSGPGGVRVGLALLLGLVLSGLSLEPVYWSANKRFQAEGGYVLYPQIGDRDL	60
Horse	...R.....FF.....	60
Pig	..A.R.....LG.....	60
Cat	..A.R.....Y.....	60
Dog	..A.R.....LG.....	60
<i>P. alecto</i>	..A..R....E...F.FW.....	60
<i>P. vampyrus</i>	...R.....E....FW.....	60
Mouse	..A..F....Q.....FA.....	60
Human	LCPRARPPGPHSSPNYEFYKLYLVGGAQGRRC <sup>■</sup> EAPPAPNLLLT <sup>□</sup> DRPDLDLRF <sup>□</sup> TIKFQ <sup>□</sup> EY	120
Horse	.....	120
Pig	.....	120
Cat	.....	120
Dog	.....	120
<i>P. alecto</i>	.....	120
<i>P. vampyrus</i>	.....	120
Mouse	.....S.....E.....	120
Human	SPNLWGHEFRSHHDYI <sup>□</sup> IATSDGTREGLES <sup>□</sup> LQGGV <sup>□</sup> CLTRGMKVL <sup>□</sup> LRVQ <sup>□</sup> SPRGGAV <sup>□</sup> PRKP	180
Horse	.....A.....	180
Pig	.....A.....	180
Cat	.....A.....	180
Dog	.....A.....	180
<i>P. alecto</i>	.....A.....	180
<i>P. vampyrus</i>	.....A.....	180
Mouse	.....	180
Human	VSEPMERDRGAAHSLEPGKENLPGDPTSNATSRGAEGLPPPSM <sup>□</sup> PAVAGAAGGLALLLL	240
Horse	.....M.....	240
Pig	...V...T...TA...S.....	240
Cat	.....M.....	240
Dog	...V...T...TA...S.....	240
<i>P. alecto</i>	.....N..V.....	240
<i>P. vampyrus</i>	.....N..V.....	240
Mouse	.....A...RDTI...S.....M.....	240
Human	<sup>□</sup> GVAGAGGAMC <sup>□</sup> WRRRRRAKPS <sup>□</sup> ESRHPG <sup>□</sup> SGFGRG <sup>□</sup> SLGLGGGGMGP <sup>□</sup> PREAEPGELGIAL <sup>□</sup> RGG	300
Horse	.....	300
Pig	.....	300
Cat	.....W.....AV.....	300
Dog	.....	300
<i>P. alecto</i>	.....	300
<i>P. vampyrus</i>	.....S.....	300
Mouse	.....	300
Human	GAADPPFC <sup>□</sup> PHYEKVSGDYGHPVYIVQDGGPPQSPNNIYK <sup>□</sup> V	340
Horse	.....	340
Pig	.....	340
Cat	.....	340
Dog	.....	340
<i>P. alecto</i>	...E.....	340
<i>P. vampyrus</i>	...E.....	340
Mouse	..T.....	340

Fig. 2. Amino acid sequence alignment of EFNB3 from different species. Species and amino acid number are listed on the left and right, respectively. Dots represent identical amino acids. The transmembrane domain is indicated by boxed borders, cysteine residues are shaded in gray, and putative glycosylation sites are underlined. GenBank accession numbers: horse EFNB3:EF682146; pig EFNB3:EF682147; cat EFNB3:EF682144; dog EFNB3:EF682145; *P. alecto* EFNB3:EF682148; *P. vampyrus* EFNB3:EF682149; mouse EFNB3:NM\_007911.

and live virus infections were conducted simultaneously as described in Materials and methods. All cells were fixed 20 h post-infection and infected cells were visualized using immunofluorescence assays as described in Materials and methods. The ability of EFNB2 molecules from different species to support infection by HeV or NiV are shown in Fig. 6. There did not appear to be differences of species-specific EFNB2 molecules in their ability to support HeV infection. All EFNB2 molecules also functioned similarly as viral receptors for NiV. Interestingly, when comparing HeV and NiV infection in EFNB2 transfected cells, the progression of the cytopathic effects (CPE) in NiV-infected cells was further advanced as evidenced by the increased number of rounded cells suggesting that HeV had a slightly slower kinetics of infection. Previous studies have suggested that HeV G appears to have a lower affinity for EFNB2 than NiV G (Bossart et al., 2007) and the slower kinetics

of HeV infection was consistent with a lower receptor affinity. However, in the context of live virus infection, other viral glycoproteins may have influenced the kinetics of virus replication. Shown in Fig. 7 are the expression of EFNB3 molecules from different species and their ability to support HeV or NiV infection. EFNB3 molecules from all species functioned similarly for both HeV and NiV as demonstrated by the almost equivalent amounts of HeV- and NiV-infected cells in all panels. Interestingly, when comparing all infections, (Figs. 6 and 7), it appears that only HeV in EFNB2 transfected cells had a slower progression of CPE, all other infections seemed to progress similarly. Analogous experiments using 100-fold less virus were conducted and although the number of infected cells was significantly decreased, infection kinetics and receptor function closely paralleled results shown in Figs. 6 and 7 (data not shown).

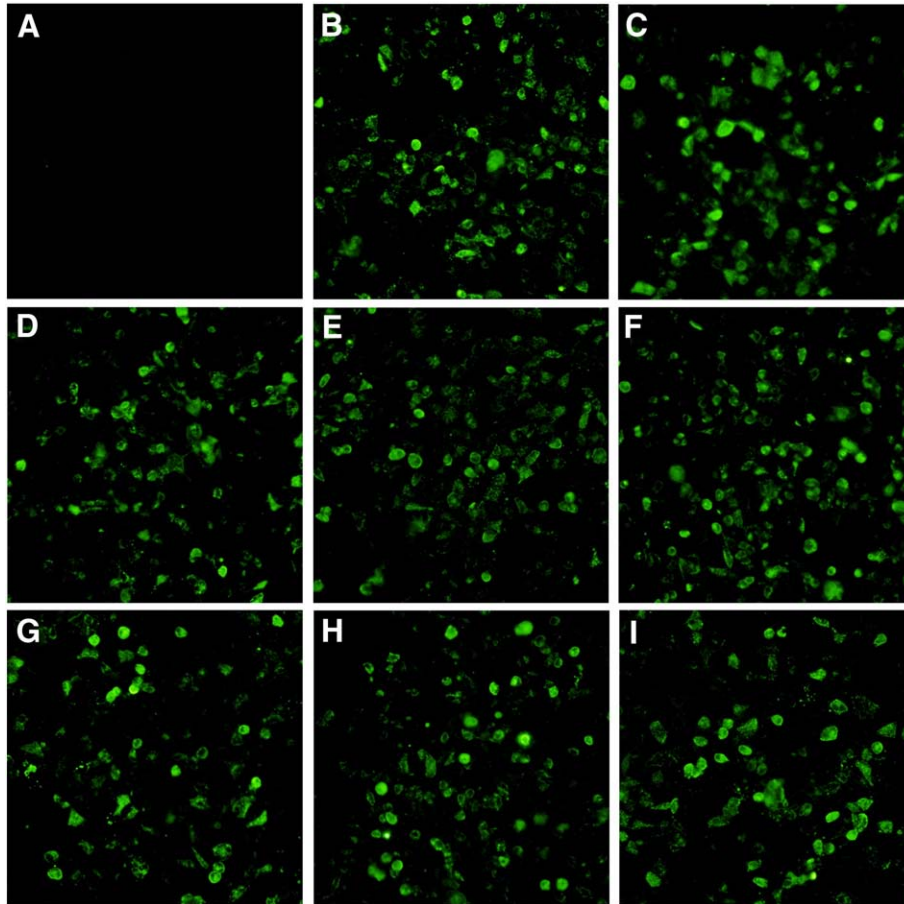


Fig. 3. Expression of species-specific EFNB2 molecules. HeLa-USU cells were grown to 80% confluence in 24 well plates and transfected with various EFNB2 constructs. Cells were probed with ephrin receptor EphB3 (His-epitope tagged) followed by a mouse anti-His FITC conjugate. A: vector control, B: human, C: horse, D: pig, E: cat, F: dog, G: *P. alecto*, H: *P. vampyrus*, I: mouse.

#### Functional mapping of the G receptor binding domain using soluble receptor molecules

Previously we have demonstrated that soluble EFNB2 could block HeV and NiV infection in Vero cells and EFNB2 transfected cells at concentrations as low as 10  $\mu\text{g/ml}$  (Bonaparte et al., 2005). In the current studies we tested soluble receptors as inhibitors of HeV and NiV infection in EFNB2 and EFNB3 transfected cells. Percent inhibitions were calculated as described in Materials and methods and results are shown in Table 3. EFNB3-mediated infections were completely blocked by soluble EFNB2 for both viruses, strongly suggesting HeV and NiV bind EFNB2 and EFNB3 molecules through an overlapping receptor binding domain. Although effective, soluble EFNB2 was less efficient at inhibiting NiV infection in EFNB2 transfected cells even though there was two fold less virus as compared to HeV. These data provide further evidence that NiV G may have increased affinity for EFNB2 as compared to HeV. When soluble EFNB3 was used as a competitive inhibitor, it was less effective against both viruses regardless of the species (Table 3). When used at 10  $\mu\text{g/ml}$ , no effect was seen (data not shown). When increased to 25  $\mu\text{g/ml}$ , EFNB3-mediated infections were significantly inhibited and EFNB2-mediated infections were only partially blocked. As both receptors appear

to bind to the same domain of G it would seem likely that soluble EFNB3 had a reduced affinity for HeV and NiV as compared to EFNB2 and could not adequately compete for G binding. A difference in affinity would be consistent with soluble EFNB2 completely blocking EFNB3-mediated infections (Table 3). Soluble EFNB3 was more effective blocking EFNB2-mediated HeV infection as compared to EFNB2-mediated NiV infection, further suggesting that NiV has an increased affinity for EFNB2 as compared to HeV.

#### Functional mapping of the G receptor binding domain using antibodies

Recently, m101 (Zhu et al., 2006) and m102.4 (Zhu et al., in press), two recombinant human mAb, were identified whose epitopes overlap with the EFNB2 binding domain of the henipavirus G glycoproteins. Both antibodies can neutralize HeV and NiV, although m101 is more effective against HeV (Zhu et al., 2006) whereas m102.4 can potently neutralize both viruses with somewhat higher potency for NiV (Zhu et al., in press). To further evaluate the overlapping EFNB2 and EFNB3 binding domain of G and to determine if the differential neutralization profiles of m101 and m102.4 correlated with differential inhibition of EFNB2 and/or EFNB3-mediated infection, we evaluated

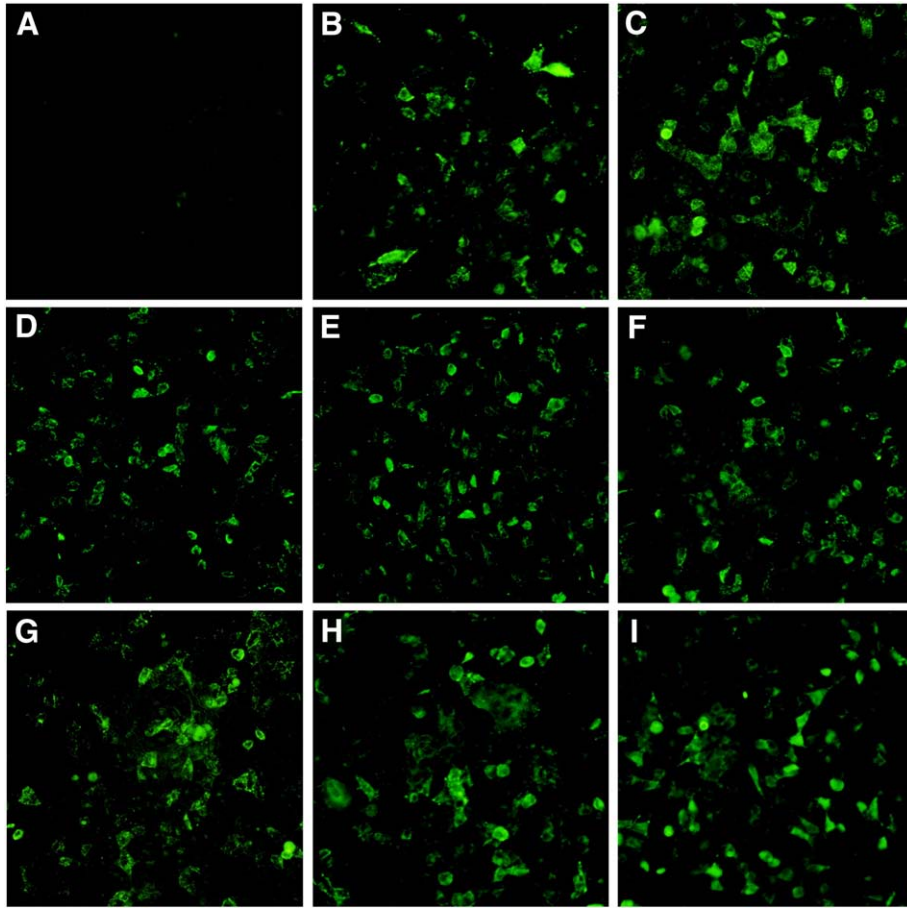


Fig. 4. Expression of species-specific EFNB3 molecules. HeLa-USU cells were grown to 80% confluence in 24 well plates and transfected with various EFNB3 constructs. Cells were probed with ephrin receptor EphB3 (His-epitope tagged) followed by a mouse anti-His FITC conjugate. A: vector control, B: human, C: horse, D: pig, E: cat, F: dog, G: *P. alecto*, H: *P. vampyrus*, I: mouse.

m101 and m102.4 for their ability to block HeV and NiV infections. Percent inhibitions were calculated as described in Materials and methods and results are shown in Table 3. Human mAb m101 inhibited both EFNB2- and EFNB3-mediated HeV infections in all species. NiV was significantly less susceptible to inhibition by m101 and although low, inhibition levels were

similar in all species for both receptors. Together these data demonstrate that the levels of m101 inhibition were dependent on the virus used but not the specific EFN and suggest that m101 bound an overlapping EFNB2 and EFNB3 binding domain of the attachment glycoprotein. Human mAb m102.4 was able to potentially block EFNB2-mediated infections in all species for both

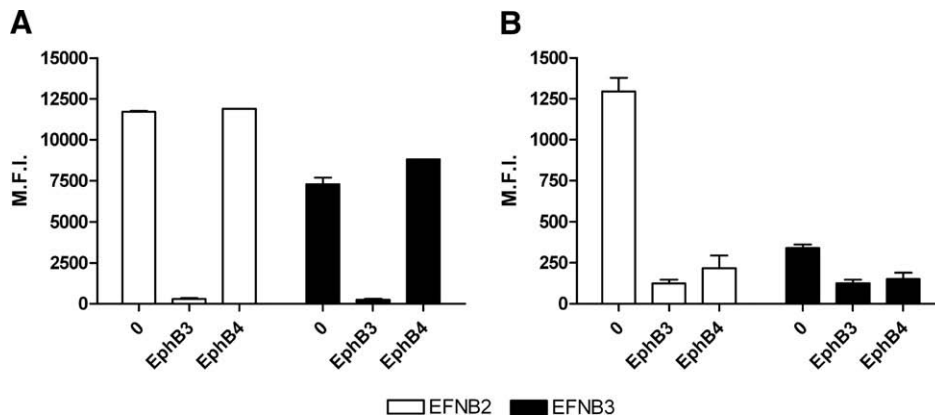


Fig. 5. Binding of EphB3 and EphB4 to recombinant EFN. Biotinylated EphB3/Fc (panel A) or biotinylated EphB4 (panel B) were assayed for EFN binding using a multiplexed microsphere assay as detailed in Materials and methods. Median fluorescence intensities (M.F.I.) are shown on the y-axis for each microsphere population; EFNB2 (white bars) and EFNB3 (black bars). Binding was measured in the absence (0) or presence of non-biotinylated Eph receptors as indicated on the x-axis. All assays were done in duplicate and the mean M.F.I. is shown. Error bars represent the range of M.F.I.

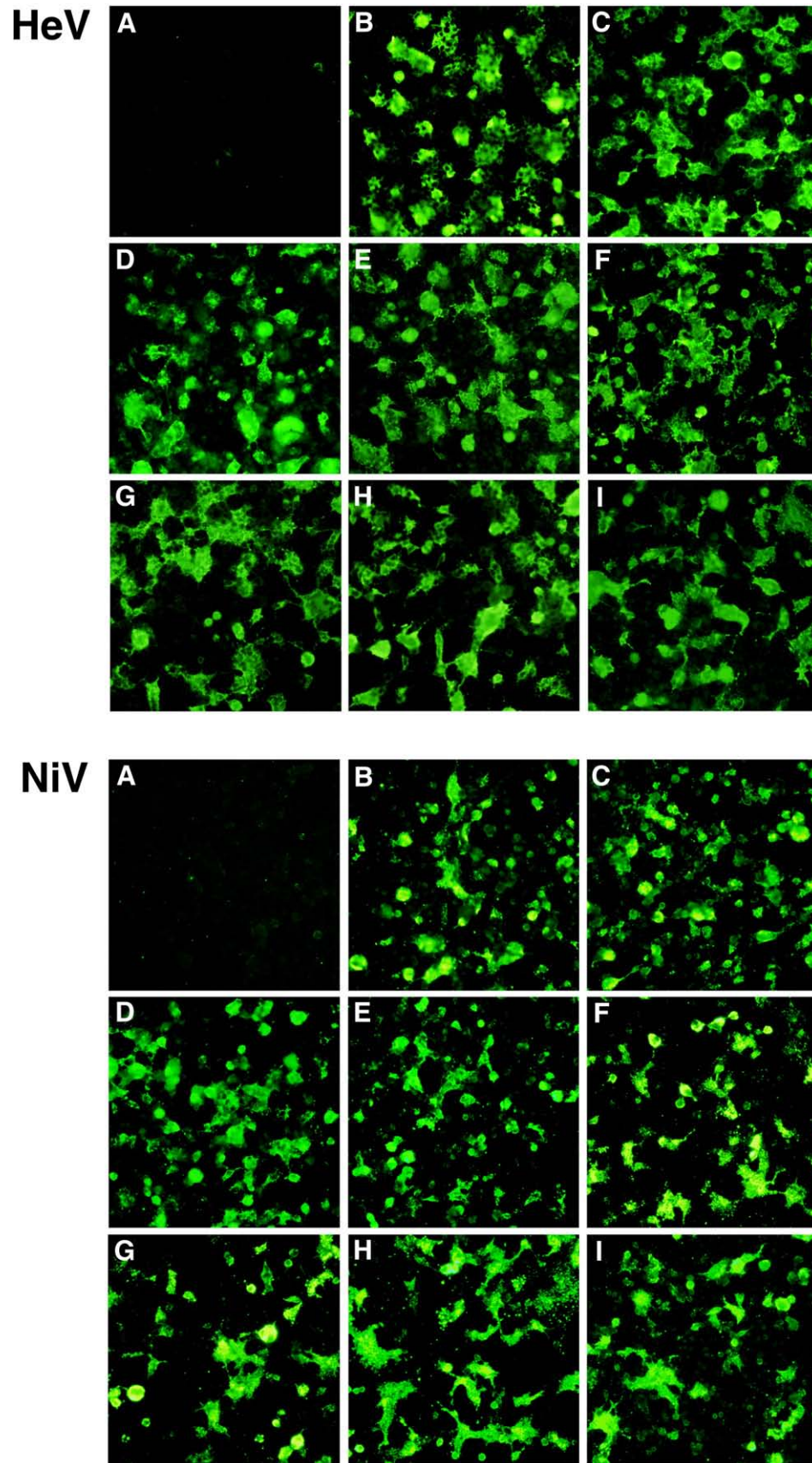


Fig. 6. HeV and NiV infection of EFNB2-transfected HeLa-USU cells. HeLa-USU cells were grown to 80% confluence in 24 well plates and transfected with various EFNB2 constructs. Transfected cells were infected with either HeV or NiV as described in Materials and methods. All infections were incubated for 20 h fixed in methanol and immunofluorescently stained for P protein prior to digital microscopy. A: vector control, B: human, C: horse, D: pig, E: cat, F: dog, G: *P. alecto*, H: *P. vampyrus*, I: mouse.

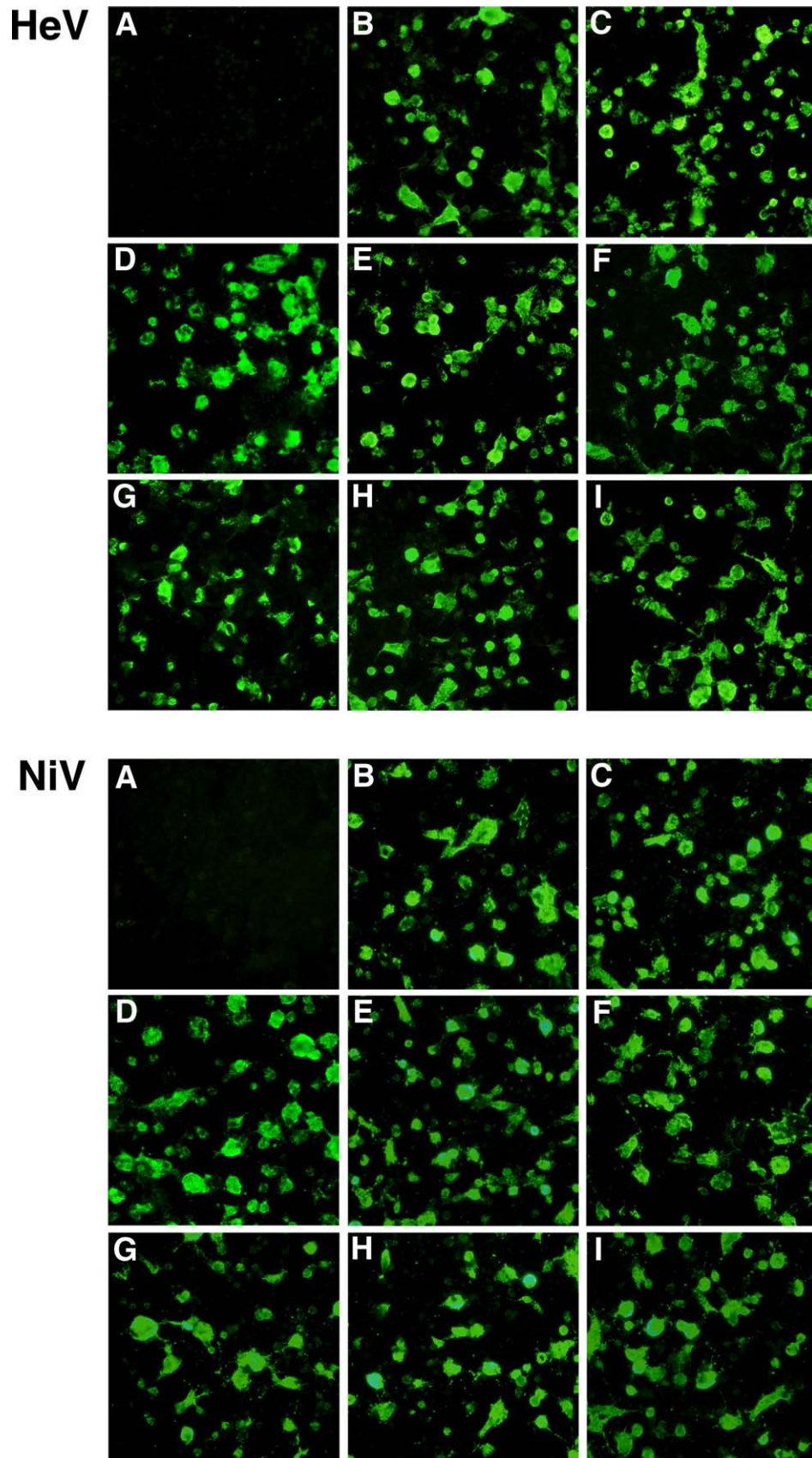


Fig. 7. HeV and NiV infection of EFNB3-transfected HeLa-USU cells. HeLa-USU cells were grown to 80% confluence in 24 well plates and transfected with various EFNB3 constructs. Transfected cells were infected with either HeV or NiV as described in Materials and methods. All infections were incubated for 20 h fixed in methanol and immunofluorescently stained for P protein prior to digital microscopy. A: vector control, B: human, C: horse, D: pig, E: cat, F: dog, G: *P. alecto*, H: *P. vampyrus*, I: mouse.

Table 3  
Percent inhibition<sup>a</sup> of HeV and NiV infection by molecules that target the henipavirus attachment glycoprotein

Inhibitor	Ligand	Virus	Human	Horse	Pig	Cat	Dog	P. alecto	P. vampyrus	Mouse
Soluble EFNB2 (10 µg/ml)	EFNB2	HeV	99.8	98.5	99.3	98.3	99.3	99.3	99.5	99.3
		NiV	87.3	84.6	90.9	88.6	91.8	90.5	90.9	89.1
	EFNB3	HeV	100	100	100	100	100	100	100	100
		NiV	100	100	100	100	100	100	100	100
Soluble EFNB3 (25 µg/ml)	EFNB2	HeV	61.9	62.0	69.3	64.0	67.7	66.1	63.0	62.8
		NiV	22.7	24.5	31.8	35.6	32.7	32.8	27.8	29.6
	EFNB3	HeV	89.5	88.5	88.4	88.8	88.6	89.2	88.2	89.9
		NiV	88.2	81.2	80.7	81.5	85.4	89.4	84.4	87.1
m101 (10 µg/ml)	EFNB2	HeV	74.3	79.9	86.0	87.0	76.9	82.4	84.0	88.0
		NiV	8.1	3.9	17.4	13.9	17.8	14.0	22.1	17.0
	EFNB3	HeV	99.6	99.1	99.1	98.6	99.4	99.6	99.1	99.1
		NiV	23.2	19.0	16.6	16.1	19.1	23.1	23.2	23.0
m101 (40 µg/ml)	EFNB2	HeV	99.5	99.6	84.0	82.0	79.7	87.3	85.2	88.5
		NiV	9.4	25.0	24.1	16.1	23.0	26.3	23.2	20.4
	EFNB3	HeV	99.4	99.1	99.3	99.6	99.1	99.6	99.3	99.6
		NiV	29.7	30.2	25.3	32.2	25.4	27.2	28.1	26.7
m102.4 (10 µg/ml)	EFNB2	HeV	90.3	89.4	90.8	92.9	91.0	86.9	92.0	88.9
		NiV	73.6	75.0	74.3	73.6	75.7	71.4	73.6	74.3
	EFNB3	HeV	98.7	98.4	94.7	94.2	94.7	98.2	94.9	93.8
		NiV	99.7	99.4	99.7	99.7	99.7	99.4	99.7	99.7
m102.4 (50 µg/ml)	EFNB2	HeV	92.5	92.4	93.1	92.9	94.1	94.0	94.0	93.3
		NiV	80.0	81.4	78.6	79.3	83.6	81.4	82.9	81.4
	EFNB3	HeV	99.7	99.6	99.1	99.3	99.5	99.7	99.6	99.5
		NiV	99.4	99.4	99.4	99.4	99.4	99.7	99.7	99.7

<sup>a</sup> Percent inhibitions were calculated as detailed in Materials and methods.

viruses with slightly higher activity against HeV compared to NiV. Human mAb m102.4 was also able to inhibit EFNB3-mediated infections for both viruses in all species and inhibition was increased (>99%) as compared to inhibition of EFNB2-mediated infection. Together these data suggest that like m101, m102.4 blocked infection of HeV and NiV by binding to an overlapping EFNB2 and EFNB3 binding domain of the attachment glycoprotein. Importantly, for m102.4, HeV and NiV neutralization was achieved in the context of all EFN receptors from all tested species demonstrating its potential as a potent cross-reactive therapeutic for NiV and HeV infections in animals and humans.

#### *EFN receptor binding domains overlap with the henipavirus G binding domain*

Finally, we sought to determine if the Eph binding domain of EFNB2 and EFNB3 were involved in G glycoprotein binding. Using the multiplexed EFN-Eph microsphere assay as described in Materials and methods, we assessed whether soluble versions of the henipavirus attachment glycoproteins (sG) could inhibit Eph-EFN binding and results are shown in Fig. 8. Binding of EphB3 to EFNB2 was significantly inhibited by sG-HeV and sG-NiV (Fig. 8A). EphB3 binding to EFNB3 was also significantly reduced, although inhibition by sG-HeV

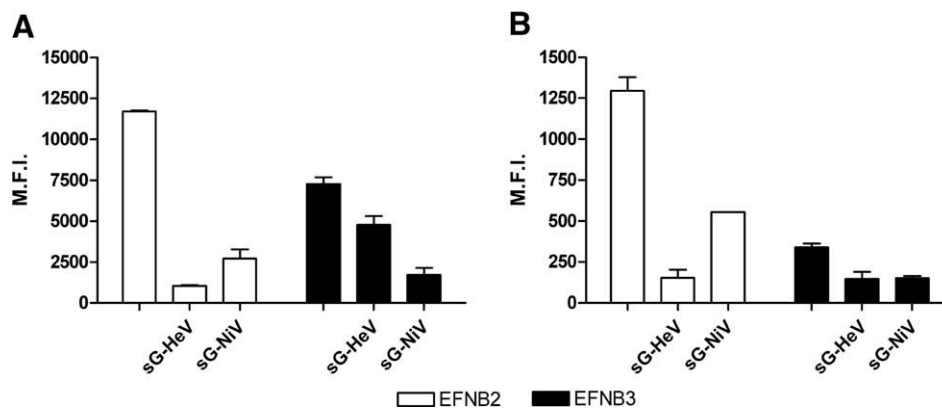


Fig. 8. Competition of Eph binding by sG. Biotinylated EphB3/Fc (panel A) or biotinylated EphB4 (panel B) were assayed for EFN binding using a multiplexed microsphere assay as detailed in Materials and methods. Median fluorescence intensities (M.F.I.) are shown on the y-axis for each microsphere population; EFNB2 (white bars) and EFNB3 (black bars). Binding was measured in the absence (0) or presence of sG as indicated on the x-axis. All assays were done in duplicate and the mean M.F.I. is shown. Error bars represent the range of M.F.I.

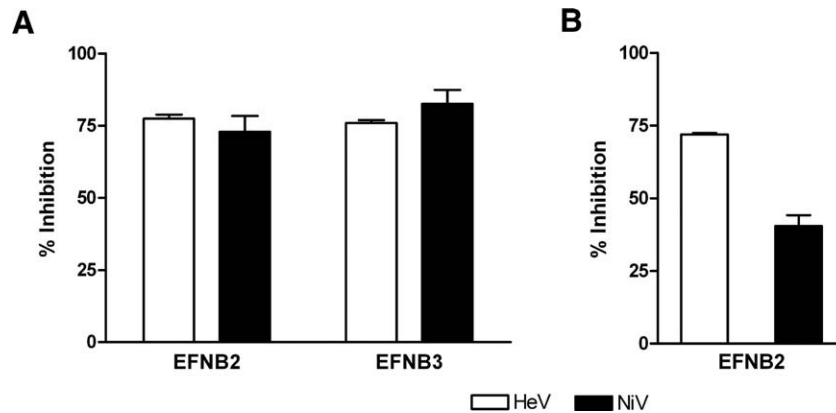


Fig. 9. Percent Inhibition of HeV and NiV infection by Eph receptors. HeLa-USU cells were grown to 80% confluence in 24 well plates and transfected with human EFNB2 and EFNB3 constructs. Transfected cells were incubated in the presence or absence of EphB3 or EphB4 for 30 min and subsequently infected with either HeV or NiV as described in Materials and methods. HeV and NiV infected cells were immunofluorescently labeled for P protein and counted in all groups. Percent inhibitions were calculated as detailed in Materials and methods. Eph inhibition assays were done in duplicate and the average percent inhibition of HeV (white bars) or NiV (black bars) is shown. Error bars represent the range of percent inhibition. Inhibition of EFNB2 and EFNB3-mediated infections by EphB3 is shown in Panel A; inhibition of EFNB2-mediated infections by EphB4 is shown in Panel B.

was less efficient. Binding of EphB4 to EFNB2 was significantly reduced by sG (Fig. 8B) and here, inhibition by sG-NiV was less efficient. As demonstrated previously, EphB4 bound EFNB3 poorly, nonetheless both sG molecules further reduced EphB4 binding. Together these data suggest that the Eph binding domains of EFNB2 and EFNB3 overlap with the henipavirus attachment protein binding domain. Furthermore, both EphB3 and EphB4 binding were inhibited by sG, which provides further evidence that EphB3 and EphB4 bind through an overlapping binding domain of EFN. Next we assessed Eph receptors for their ability to block HeV and NiV infections in EFN transfected cells. Because binding of EphB4 to EFNB3 was not detected by immunofluorescence and was very low using recombinant EFNB3, EphB4 was only assayed for its ability to block EFNB2-mediated henipavirus infections. Due to the Eph concentrations employed (50  $\mu\text{g/ml}$ ) and reagent limitations, we were only able to use human EFN transfected cells in henipavirus Eph inhibition assays and results are shown in Fig. 9. Percent inhibitions were calculated as described in Materials and methods. EphB3 blocked approximately 75% of EFNB2- and EFNB3-mediated infections for both HeV and NiV (Fig. 9A). EphB4 blocked approximately 70% of EFNB2-mediated infections for HeV and approximately 40% of EFNB2-mediated infections for NiV (Fig. 9B). These data provide further evidence that the overlapping EphB3/EphB4 binding domain of EFNB2 and EFNB3 overlaps with the domain that binds the henipavirus G glycoproteins. Importantly, EphB3 blocked HeV and NiV infections regardless of the EFN used suggesting it may potentially represent a new avenue for therapeutic antiviral drug development.

## Discussion

HeV and NiV can use at least two different receptors for infection, and it was speculated that differences could exist between the receptors from one animal species to another influencing the pathogenic process. For these reasons we cloned

and characterized both of these receptors from a host of naturally or experimentally susceptible species and examined their function as henipavirus receptors. We found that there was almost no variation in receptor function, regardless of species, suggesting that host receptor molecules do not play a major role in differential infection. We specifically included mouse EFN molecules in the current study as these animals are highly resistant to systemic infection with HeV and NiV. Recently, it was reported that two rodent cell lines known to express EFNB2 were resistant to NiV infection (Yoneda et al., 2006). Our results demonstrate that mouse EFN molecules are highly functional henipavirus receptors when expressed in HeLa-USU cells, suggesting that resistance to HeV and NiV infection in mice is most likely due to a post-entry block in virus replication. It is possible that receptor expression and distribution vary in different species, although given their high level of conservation and essential functions, we believe this to be unlikely. Nonetheless, we are currently investigating this possibility. We also demonstrated that HeV and NiV used EFNB2 and EFNB3 similarly indicating that the differences in disease were unlikely to be due to selective use of receptors by either virus. HeV appeared to have a slightly slower kinetics of infection in EFNB2 transfected cells as compared to NiV regardless of the source species. These observed differences are consistent with previous data which demonstrated that NiV G had a slightly increased association constant ( $k_a$ ) for EFNB2 (Bossart et al., 2007). However, HeV- and NiV-specific proteins other than the attachment glycoprotein, including the fusion glycoprotein or those involved with the replication machinery, could also lead to the observed replication differences as cell type-specific differences in HeV and NiV replication have been observed previously (Cramer, G., unpublished observation). EFNB3-mediated infections were similar for both HeV and NiV in all species with CPE similar to EFNB2-mediated NiV infections. Previous work has demonstrated that NiV G has an increased affinity for EFNB2 as compared to EFNB3 (Negrete et al., 2006). The difference in affinity was not attributed to differences in the

association constants ( $k_a$ ) but to differences in disassociation constants ( $k_d$ ). Importantly, this “faster off rate” of EFNB3 does not appear to influence infectivity *in vitro* as there was no difference in the kinetics of EFNB2- and EFNB3-mediated NiV infections. Analysis of all infectivity data suggests that the previously described association constants of HeV and NiV G for their receptors do correlate with live virus infection.

Recent data using biochemical and pseudovirus assays have suggested that HeV and NiV G bind EFNB2 and EFNB3 molecules through an overlapping receptor binding domain (Bishop et al., 2007; Negrete et al., 2006). To further examine this possibility using live HeV and NiV we tested two classes of entry inhibitors, soluble EFN molecules and G-specific human mAb known to interfere with EFNB2 binding. Soluble EFNB2 blocked EFNB2- and EFNB3-mediated HeV and NiV infections in all species indeed establishing that both receptor molecules bind to the same or overlapping domain of G. Soluble EFNB3 could neutralize EFNB3-mediated infections but only partially neutralized EFNB2-mediated infections. We hypothesize that this is not due to where EFNB3 binds on G but due to a lower affinity not sufficient for EFNB2 competition. The previously described decreased disassociation constant would be consistent with these data. The hmAb m101 and m102.4 blocked infection of HeV and NiV regardless of the receptor used, indicating independently that the receptor binding domain for both EFNB2 and EFNB3 molecules overlapped with the epitopes of G that bind m101 and m102.4. The differential neutralization of HeV and NiV by m101 most likely represents minor amino acid differences between the attachment proteins that influence m101 binding. We also found that m102.4 was slightly more effective against HeV at the high antibody concentrations used in these studies. Interestingly, we have previously observed that at lower concentrations this antibody was more active against NiV compared to HeV resulting correspondingly to lower IC50 although at the highest concentration it neutralized HeV better than NiV (Zhu et al., *in press*). The mechanisms of such concentration-dependent differential neutralization are complex and are currently being investigated. Regardless, the neutralization by m102.4 was very potent and cross-reactive, suggesting potential use of this antibody as a therapeutic agent for henipavirus infection (Zhu et al., *in press*).

Finally, we evaluated EFN receptor binding domains to determine if they played a role in henipavirus binding and infection. Due to sensitivity problems and limited reagents, we established a new microsphere assay capable of detecting EFN-Eph interactions which was particularly useful for EFN-EphB4 binding studies and Eph competition studies. We were able to demonstrate that EphB3 and EphB4 most likely bound an overlapping binding domain of EFN and that sG could block Eph binding. Consequently, we evaluated Eph receptors for their ability to inhibit henipavirus infection and found that EphB3 significantly inhibited henipavirus infections regardless of receptor use whereas EphB4 bound and inhibited EFNB2-dependent infections. EphB4 inhibited HeV better than NiV in EFNB2-expressing cells and we speculate that this may be due to relative differences in EFNB2 affinity as compared to EphB4. Together the Eph binding and inhibition data suggested an

overlapping Eph binding domain within EFNB2 and EFNB3 which overlapped with the henipavirus G glycoprotein binding domain. It will be interesting to determine if binding of the henipavirus G glycoprotein mimics EphB3 and EphB4 binding and leads to signaling through the ephrin ligands. Ephrin ligands and their receptors participate in bi-directional signaling events and research suggests that this bi-directional signaling is crucial in controlling vascular homeostasis (Augustin and Reiss, 2003). If the henipavirus G glycoproteins stimulate one-way signaling via the ephrin ligands, it may promote improper endothelial cell migration leading to imbalance in the vascular system, possibly explaining the general vasculitis seen in HeV and NiV infected individuals.

## Materials and methods

### *Tissue samples and total RNA isolation*

Fresh kidney and liver tissue samples were obtained from horse, pig, cat, dog, *P. alecto*, *P. vampyrus* and mouse and were stored in RNAlater (Ambion Inc. Austin, TX, USA) at  $-80\text{ }^{\circ}\text{C}$ . RNA was extracted using RNeasy Mini kit (Qiagen Pty. Ltd., Clifton Hill, VIC, Australia) following the manufacturer's protocol. Tissue homogenization and disruption was as described previously (Tachedjian et al., 2006). Briefly, 80 mg of tissue stored in RNAlater was added to tubes containing 1.8 ml RLT lysis buffer (RNeasy) containing  $18\text{ }\mu\text{l}$   $\beta$ -mercaptoethanol and  $15\times 2.3\text{ mm}$  diethyl pyrocarbonate (Sigma-Aldrich, St. Louis, MO, USA) treated stainless steel balls (BioSpec Products Inc., Bartlesville, OK, USA). Samples were then homogenized for 3 cycles of 30 s using a Mini-Bead Beater 8 (Biospec Products Inc.), centrifuged to pellet cell debris and the supernatant transferred to a new tube. All samples were then processed as per the manufacturer's instructions supplied with the RNeasy kit. RNA was either used immediately for cDNA synthesis or stored at  $-80\text{ }^{\circ}\text{C}$  for future use.

### *Synthesis of cDNA*

Procedures previously described (Tachedjian et al., 2006) were used with the following modifications. Between 1.5 and  $2.0\text{ }\mu\text{g}$  of total RNA was denatured for 10 min at  $65\text{ }^{\circ}\text{C}$  then added to a  $20\text{ }\mu\text{l}$  reaction mixture containing 1X reverse transcription (RT) buffer (Qiagen Pty. Ltd.), 10 units RNasin (Promega, Madison, WI, USA), 0.5 mM dNTPs (Qiagen Pty. Ltd.), 4  $\mu\text{M}$  random hexamers (Promega), 1  $\mu\text{M}$  oligo dT<sub>12–18</sub> (Qiagen Pty. Ltd.) and 4 units Omniscript-RT (Qiagen Pty. Ltd.). First strand synthesis was performed for 5 min at room temperature then 2 h at  $37\text{ }^{\circ}\text{C}$ . Reactions were immediately stored at  $-20\text{ }^{\circ}\text{C}$ .

### *5' and 3' RACE*

Alignments of known EFNB2 and EFNB3 DNA sequences were conducted using Clone Manager Professional Suite version 8 (Scientific and Educational Software, Cary, NC, USA) and highly conserved regions were identified for both EFNB2 and

EFNB3 molecules. Internal primers were designed for both molecules (Table 1) and used in PCR to generate a 747 base pair (bp) internal fragment of EFNB2 (horse, cat, *P. alecto* and *P. vampyrus*) and a 900 bp internal fragment for EFNB3 (horse, *P. alecto* and *P. vampyrus*). PCR products were sequenced and primers were designed for 5' and 3' RACE (Table 1). 5' and 3' RACE was performed using the Ambion FirstChoice™ RLM-RACE kit (Applied Biosystems, Scoresby, VIC, Australia) with modifications for cDNA synthesis and PCR amplifications as previously described (Tachedjian et al., 2006).

#### Cloning of full length EFNB2 and EFNB3

For horse, cat, *P. alecto* and *P. vampyrus* EFNB2 molecules and for horse, *P. alecto* and *P. vampyrus* EFNB3 molecules, consensus primers were designed based on RACE sequences. For pig, dog and mouse EFNB2 molecules and cat, pig, dog and mouse EFNB3 molecules, primers were designed based on previously published sequences (GenBank accession numbers: pig EFNB2:AJ667124 and DB784906; dog EFNB2:XM849457; mouse EFNB2:NM\_010111; cat EFNB3:6308350020, AANG01159385 and AANG01159384; pig EFNB3:AC127472; dog EFNB3:XM844752; mouse EFNB3:NM\_007911). Consensus primers were used for subcloning of human EFNB2 and EFNB3 molecules. All cloning primers are listed in Table 1. For each species except human, cDNA was synthesized as described above and 1.5 µl of cDNA was used as template for PCR in a 25 µl reaction. For human EFNB2 and EFNB3 molecules 1 µl of previously obtained expression plasmids (Bonaparte et al., 2005) were used as PCR templates. All PCR reactions were performed using Qiagen's Proofstart DNA polymerase with Q solution (Qiagen Pty. Ltd.) with the following settings: 94 °C for 5 min initially and then 94 °C for 1 min, 60 °C (EFNB2) or 61 °C (EFNB3) for 1 min, 72 °C for 1 min 15 s; 45 cycles, 72 °C for 10 min. All primers generated PCR products with a 5' Kozak sequence (CCACC), 3' stop codon and flanking 5' and 3' *Sa*II sites. PCR products were gel purified (Qiagen Pty. Ltd.) and cloned into the TOPO vector (Invitrogen Australia Pty. Ltd., Mount Waverley, VIC, Australia) and subsequently subcloned into the pcDNA3.1 myc-his expression vector (Invitrogen Australia Pty. Ltd.). All clones were verified by PCR, restriction digest and 2-fold DNA sequencing. For mouse EFNB2, a mutation was introduced in the first codon during subcloning (atg to ttg). This mutation gives rise to a truncated mouse EFNB2 that is missing the first two amino acids.

#### Transient expression of EFN molecules

HeLa-USU cells (Bonaparte et al., 2005; Bossart et al., 2005) were seeded in 24 well plates in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Australia Pty. Ltd.) containing 10% fetal calf serum (FCS) (Invitrogen Australia Pty. Ltd.) and 2 mM glutamax (Invitrogen Australia Pty. Ltd.) (DMEM-10) to achieve 215,000 cells per well. HeLa-USU cells were transfected with either a plasmid encoding EFNB2, EFNB3 or vector alone using Fugene transfection reagent (Roche, Indianapolis, IN, USA) per the manufacturer's instructions.

DNA-Fugene mixtures were added to cells and incubated overnight at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Medium was removed and cells were washed once with Ca<sup>++</sup> and Mg<sup>++</sup> free PBS (PBSA). For HeV and NiV infections, 200 µl of DMEM containing 10% FCS and 2 mM glutamax was added per well. For immunofluorescence, 200 µl of DMEM containing 1% bovine serum albumin (BSA) and 2 mM glutamax was added per well.

#### Immunofluorescence assays for EFN molecules

All assays were done using live transfected cells. Cells were incubated in DMEM containing 1% BSA and 2 mM glutamax for 30 min at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were washed three times with PBS containing 0.05% Tween-20 (PBST). 800 ng recombinant mouse EphB3/Fc (Bio-Scientific Pty. Ltd., Gympie, NSW, Australia) or 400 ng recombinant human EphB4 (Bio-Scientific Pty. Ltd.) were added to each well in 200 µl DMEM containing 1% BSA and 2 mM glutamax and incubated for 30 min at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were washed three times with PBST. 200 µl of fluorescein isothiocyanate (FITC) conjugated mouse anti-His antibody (Milenyi Biotec Australia Pty. Ltd, NSW, Australia) diluted 1:100 in DMEM containing 1% BSA and 2 mM glutamax was added per well and incubated in the dark for 30 min at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were washed three times with PBST and 0.5 ml PBSA was added per well. FITC immunofluorescence was visualized using an Olympus IX71 inverted microscope (Olympus Australia, Mt. Waverley, VIC, Australia) coupled to an Olympus DP70 high resolution color camera and all images were acquired at an original magnification of 20×.

#### Eph-EFN binding and competition assays

10 µg of recombinant mouse EFNB2/Fc or recombinant human EFNB3/Fc (Bio-Scientific Pty. Ltd.) were coupled to 5 × 10<sup>5</sup> carboxylated (COOH) microspheres (Fisher Biotec., Wembley, WA, Australia), bead sets #42 and #46, respectively, as described previously (Bossart et al., 2007). 10 µg recombinant mouse EphB3/Fc (Bio-Scientific Pty. Ltd.) or 5 µg recombinant human EphB4 (Bio-Scientific Pty. Ltd.) were biotinylated using a Lightning-Link™ Biotin conjugation kit (Innova Biosciences Ltd., Babraham, Cambridge, UK) following the manufacturer's protocol. For all multiplexed assays, multiScreen-BV 1.2 µm hydrophilic, low protein binding, 96-well filter plates (Millipore Australia Pty Ltd, North Ryde, NSW, Australia) were pre-wet with PBSA. EFN-coupled microspheres were vortexed and sonicated for 1 min. PBSA was removed from pre-wet 96-well filter plates using a vacuum manifold and 100 µl of PBSA containing 1500 microspheres of each bead set was added per well. PBSA was removed using a vacuum manifold and 100 µl of biotinylated EphB3/Fc (1:500) or biotinylated EphB4 (1:500) was added per well and incubated for 30 min at room temperature with shaking in the dark. Liquid was removed using the vacuum manifold and 100 µl of streptavidin-phycoerythrin (1:1000) (Qiagen Pty. Ltd.) was added per well and incubated for

30 min as described above. For competition assays, 50  $\mu$ l of non-biotinylated EphB3/Fc or EphB4 (50  $\mu$ g/ml) or 50  $\mu$ l sG-HeV or sG-NiV (Bossart et al., 2007) (10  $\mu$ g/ml) were added prior to the addition of biotinylated Eph receptors and incubated for 30 min as described above. Liquid was removed using the vacuum manifold and biotinylated Eph receptors and streptavidin–phycoerythrin were added as described above. All samples were assayed for median fluorescence intensities (M.F.I.) on the Bio-Plex Protein Array System as previously described (Bossart et al., 2007).

#### *HeV and NiV infection and immunofluorescence assay*

All live virus experiments were conducted under strict bio-containment procedures in a biological safety level-4 (BSL4) laboratory. To each well of transfected HeLa-USU monolayers,  $1.6 \times 10^6$  TCID<sub>50</sub> HeV or NiV was added. All infections were done in DMEM-10 in a total volume of 400  $\mu$ l per well and were incubated overnight at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. The culture medium was discarded the next day, and plates immersed in ice-cold absolute methanol for 20 min prior to air-drying outside the BSL4 laboratory. Infected cells were immunofluorescently labeled with anti-P antiserum and viewed as previously described (Bonaparte et al., 2005).

#### *HeV and NiV inhibition assays*

The amount of virus chosen for all inhibition assays was based on what has been used previously, 8000 TCID<sub>50</sub> (Bonaparte et al., 2005). As HeV infection progressed more slowly in transfected cells than NiV, we chose to use two fold more virus for HeV as compared to NiV. For inhibition by soluble receptors, 40  $\mu$ g recombinant mouse EFNB2/Fc (Bio-Scientific Pty. Ltd.) or 100  $\mu$ g recombinant human EFNB3/Fc (Bio-Scientific Pty. Ltd.) were mixed with  $1.6 \times 10^5$  TCID<sub>50</sub> HeV or  $0.8 \times 10^5$  TCID<sub>50</sub> NiV in 4 ml DMEM-10. For inhibition by antibodies, 40  $\mu$ g or 160  $\mu$ g m101 (Zhu et al., 2006) or 40  $\mu$ g or 200  $\mu$ g affinity purified m102.4 (Zhu et al., in press) were mixed with  $1.6 \times 10^5$  TCID<sub>50</sub> HeV or  $0.8 \times 10^5$  TCID<sub>50</sub> NiV in 4 ml DMEM-10. All virus–inhibitor mixtures were incubated for 30 min at 37 °C, media from transfected HeLa-USU cells was removed and 400  $\mu$ l of virus–inhibitor mixtures were added per well and cells were incubated overnight at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. For inhibition by Eph receptors, 20  $\mu$ g recombinant mouse EphB3/Fc (Bio-Scientific Pty. Ltd.) or 20  $\mu$ g recombinant human EphB4 (Bio-Scientific Pty. Ltd.) were added to transfected HeLa-USU monolayers in 200  $\mu$ l (100  $\mu$ g/ml) and incubated for 30 min at 37 °C. 200  $\mu$ l DMEM-10 containing  $3.2 \times 10^5$  TCID<sub>50</sub> HeV or  $1.6 \times 10^5$  TCID<sub>50</sub> NiV was added to each well. Eph–virus mixtures were incubated on cell monolayers for 30 min at 37 °C, washed once with PBSA and further incubated in 400  $\mu$ l DMEM-10 overnight at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were fixed, immunofluorescently labeled with anti-P antiserum and viewed as previously described. Percent inhibitions were calculated for each species using the following equation:  $100 \times (1 - \text{number of infected cells in species-specific inhibi-$

tor-treated well/number of infected cells in species-specific untreated well).

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