Functional Characterization of the Attachment Glycoprotein of *Nipah virus*: Role in Fusion, Inhibition of *Henipavirus* Infection, Generation of Chimeric Proteins, and Assembly of Chimeric Viruses

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DEDICATION

This thesis is dedicated to my parents, Ron and Sue Sawatsky. Without their support and encouragement, it simply would not have happened.

I would also like to acknowledge the rest of my family (especially the aunts, uncles, and cousins who live in Winnipeg) for helping me maintain my sanity (?) during my tenure in graduate school. I couldn't have done it without you.

Quotes to live by:

"Life is too important to be taken seriously." —Oscar Wilde

"The scientific method is nothing more than a system of rules to keep us from lying to each other."
—Christopher Moore, *Fluke: or, I Know Why the Winged Whale Sings*

"Ninety-five percent of all the species that have ever existed are now extinct, so don't look so goddamn smug."
—Christopher Moore, *Fluke: Or, I Know Why the Winged Whale Sings*

ABSTRACT

Nipah virus (NiV) and *Hendra virus* (HeV) have been identified as the causes of outbreaks of fatal meningitis, encephalitis, and respiratory disease in Australia, Malaysia, Bangladesh, and India from 1994 until 2004. In order to accommodate the unique genomic characteristics of NiV and HeV, a new genus within the family *Paramyxoviridae* was created, named *Henipavirus*. NiV encodes two surface glycoproteins: the attachment glycoprotein (G) binds to the cellular receptor for the virus, while the fusion glycoprotein (F) mediates membrane fusion between the virus and cell membranes. Expression of F and G in the same cell results in cell-cell fusion in transfected cell monolayers, while expression of F and G on their own in cell monolayers does not result in fusion. Co-culture of singly-transfected F and G cells also does not result in fusion. Expression of NiV G in transgenic CRFK cells results in resistance to NiV- and HeV-induced cytopathic effect. Additionally, neither NiV nor HeV nucleic acid could be detected in CRFK-NiV G that had been exposed to NiV or HeV. NiV G expression also prevents NiV F+NiV G-mediated cell-cell fusion, but does not affect cell surface expression of either virus receptor, ephrin-B2 and ephrin-B3. Chimeric glycoproteins derived from NiV G and CDV H were constructed and characterized. None of the chimeric glycoproteins were able to fuse when co-expressed with either NiV F or CDV F. Only one of the chimeric glycoproteins

(H_{145}/G_{458}) was detected on the cell surface by immunofluorescence assay (IFA). None of the chimeric glycoproteins altered cell surface expression levels of ephrin-B2 and ephrin-B3. Finally, recombinant NiV genomes (rNiV and rNiV eGFPG) were constructed, as well as chimeric CDV genomes with NiV ORF substitutions (rCDV eGFPH NiVFG and rCDV eGFPH NiVMFG). The only chimeric virus that was generated, rCDV eGFPH NiVFG, was assessed for its release from infected cells. rCDV eGFPH NiVFG was poorly released from infected cells without a freeze-thaw cycle, but was also found to induce the cell-surface down-regulation of the viral receptors ephrin-B2 and ephrin-B3.

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LIST OF ABBREVIATIONS

aa	Amino acid
APMV-4	*Avian paramyxovirus type 4*
ATP	Adenosine triphosphate
ATU	Additional transcriptional unit
BDV	*Borna disease virus*
BHK-21	Baby hamster kidney cell line
BME	β-mercaptoethanol
BPIV-3	*Bovine parainfluenza virus type 3*
BRSV	*Bovine respiratory syncytial virus*
BSL4	Biosafety Level 4
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary DNA
CDV	*Canine distemper virus*
CHO-K1	Chinese hamster ovary cell line
CMV_{IE}	*Cytomegalovirus* immediate early promoter
CNS	Central nervous system
CPE	Cytopathic effect
CRFK	Crandell feline kidney cells
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
eGFP	Enhanced green fluorescent protein
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
F	Paramyxovirus fusion glycoprotein

FBS	Fetal bovine serum
FACS	Fluorescence activated cell sorting
fGAPDH	Feline glyceraldehyde 3-phosphate dehydrogenase
FITC	Fluorescein isothiocyanate
FMDV	*Foot-and-mouth-disease virus*
G	*Henipavirus* attachment glycoprotein
H	*Morbillivirus* hemagglutinin glycoprotein
HA	*Influenza virus* hemagglutinin
HeV	*Hendra virus*
HIV	*Human immunodeficiency virus*
HMPV	*Human metapneumovirus*
HN	Paramyxovirus hemagglutinin-neuraminidase glycoprotein
HPIV-1	*Human parainfluenza virus type 1*
HPIV-2	*Human parainfluenza virus type 2*
HPIV-3	*Human parainfluenza virus type 3*
HPIV-4a	*Human parainfluenza virus type 4a*
HPIV-4b	*Human parainfluenza virus type 4b*
HR	Heptad repeat region
HRP	Horseradish peroxidase
HRSV	*Human respiratory syncytial virus*
IFA	Immunofluorescence assay
IL-12	Interleukin-12
IRES	Internal ribosomal entry site
JEV	*Japanese encephalitis virus*
kDa	Kilodalton
LTR	Long terminal repeat
M	Paramyxovirus matrix protein
MAb	Monoclonal antibody

MDBK	Madin-Darby bovine kidney cells
MDCK	Madin-Darby canine kidney cells
MEM	Modified Eagle's medium
MeV	*Measles virus*
mRNA	Messenger RNA
MuV	*Mumps virus*
MVA	Modified vaccinia virus strain Ankara
NA	*Influenza virus* neuraminidase
NDV	*Newcastle disease virus*
NiV	*Nipah virus*
NP	Nucleoprotein
nt	Nucleotide
NTR	Non-translated region
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFU	Plaque-forming unit
PNGase F	Peptide-N-glycosidase F
PPRV	*Peste-des-petits-ruminants virus*
PVDF	Polyvinylidene difluoride
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNP	Ribonucleoprotein
R-PE	R-phycoerythrin
rpm	Revolutions per minute
RPV	*Rinderpest virus*

RTase	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
RV	*Rabies virus*
SARS-CoV	*Severe acute respiratory syndrome-associated coronavirus*
SDS	Sodium docecylsulfate
SeV	*Sendai virus*
SLAM	Signalling lymphocyte activation molecule
ssRNA	Single stranded RNA
SV41	*Simian virus 41*
SV5	*Simian paramyxovirus 5*
$TCID_{50}$	Tissue culture infectious dose 50%
TM	Transmembrane domain
TPMV	*Tupaia paramyxovirus*
VLP	Virus-like particle
vRNA	Viral genomic RNA
VSV	*Vesicular stomatitis virus*

INTRODUCTION

Discovery of *Hendra virus*

Hendra virus (HeV) was identified as the cause of an outbreak of respiratory disease among horses in a stable in the Hendra suburb of Brisbane in the state of Queensland, Australia (Fig. 1). The trainer of the horses was hospitalized for respiratory distress and symptoms which were consistent with *Legionella* infection, but no link between horse and human disease was suspected at the time. The hospitalized trainer eventually died of meningitis (inflammation of the brain lining, or meninges), while another stable hand became ill with respiratory symptoms, but recovered (Murray *et al.*, 1995a; Murray *et al.*, 1995b). In total, the new agent infected 20 horses, 13 of which died, and also caused two human infections, one of which was fatal. A syncytium-forming virus was eventually isolated by inoculation of Vero cells with diseased horse tissue. This virus could also grow in the Madin-Darby bovine kidney (MDBK), rabbit kidney (RK13), and baby hamster kidney (BHK) cell lines. A syncytium-forming virus was also isolated by inoculation of LLC-MK2 (rhesus monkey kidney) cells with material from the deceased trainer's kidney. Ultrastructural analysis of the virus

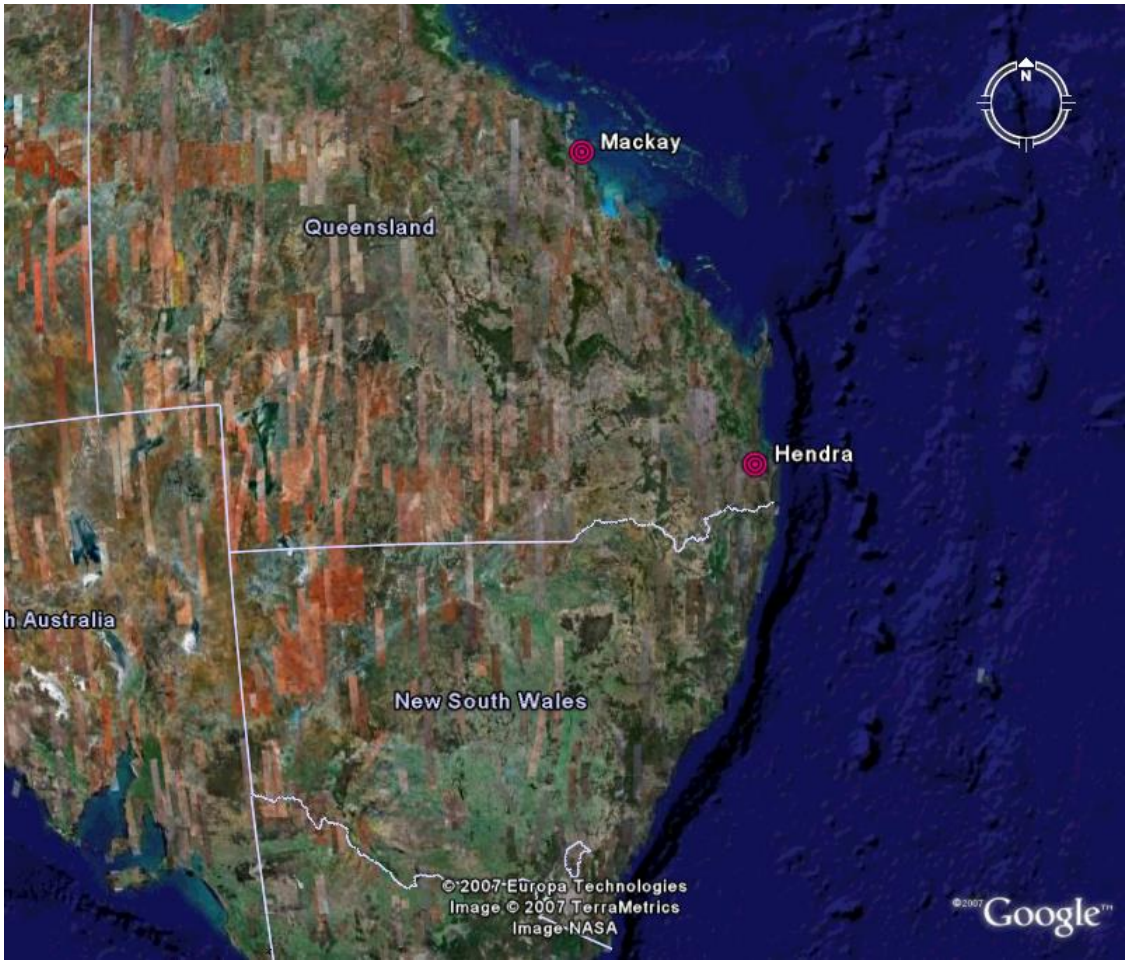

Figure 1: Map indicating known outbreaks of HeV in Australia (shown by ⊙). In 1994, the original outbreak of HeV occurred in the Hendra suburb of Brisbane in Queensland. Another human infection occurred 13 months later in Mackay on the north coast of Queensland. Map was created using Google Earth version 4.0.2737.

revealed typical paramyxovirus features (i.e. pleomorphic shape, herringbone nucleocapsid, and surface spike projections). The virus also cross-reacted with convalescent sera from horses and humans, and antisera against measles, canine distemper, and rinderpest viruses all failed to neutralize the virus. Diseased tissue from the lungs and spleens of infected horses was injected into recipient horses, which became sick with high fever and severe respiratory distress. A second farmer from Mackay in north Queensland, Australia (Fig. 1), assisted in the treatment subsequent necropsies of horses that had retrospectively diagnosed with HeV. The farmer experienced aseptic meningitis at the time, but recovered (O'Sullivan *et al.*, 1997). Some 13 months later, the farmer contracted acute progressive encephalitis and died (Allworth *et al.*, 1995; Rogers *et al.*, 1996). There is still some controversy over whether this represents a persistent infection or exposure to some other unknown factor (Hooper *et al.*, 1996). In mid-January of 1999, a third focus of HeV infection occurred, causing a nine-year-old thoroughbred mare to contract pneumonia (Field *et al.*, 2000).

Discovery of *Nipah virus* and subsequent outbreaks

From September 29, 1998, until April 4, 1999, 229 cases of acute febrile encephalitis among pig farmers and abattoir workers were reported to the Malaysian Ministry of Health. A further nine cases of febrile encephalitis,

including one fatality, among abattoir workers were reported in Singapore (Fig. 2). Three clusters were identified in Malaysia (Anonymous, 1999b; Anonymous, 1999a). The first cluster occurred near the city of Ipoh (Fig. 2) in the state of Perak from September, 1998, until February, 1999. The second cluster occurred near the city of Sikamat in the state of Negri Sembilan (Fig. 2) from December, 1998, until January, 1999. The third and largest cluster occurred in near the city of Bukit Pelandok in the state of Negri Sembilan (Fig. 2) in December, 1998. Two cases also occurred in the state Selangor. Encephalitis cases occurred primarily among men who had close contact to swine either at farms or in abattoirs. Japanese encephalitis virus (JEV) was initially suspected, and vaccination programs were started. However, a lack of efficacy in the vaccination campaign coupled with the close association with swine suggested that another infectious agent was responsible for the outbreak. Subsequent studies have confirmed the strong association of pigs with this outbreak (Amal *et al.*, 2000; Parashar *et al.*, 2000; Chew *et al.*, 2000; Sahani *et al.*, 2001). Person-to-person transmission was not documented during the outbreak. Tissue culture isolation was performed by inoculation of cells with central nervous system (CNS) material. Electron microscopy, immunofluorescent, and sequence analysis indicated that the virus was a paramyxovirus, and was closely related, but not identical, to HeV. In an effort to control the spread of this Hendra-like virus, Malaysian authorities

Figure 2: Map indicating known outbreaks of NiV in pensinsular Malaysia (shown by ◎). In 1998, the original outbreak of NiV occurred in Ipah, Malaysia and spread south to Singapore. Other key outbreak locations are shown for Bukit Pelandok, Kampung Sungei Nipah, Selangor, and Sikamat. Map was created using Google Earth version 4.0.2737.

banned the transport of pigs within the country and quarantined pigs within a 5 km perimeter of recognized outbreak areas were culled. Outbreak control in the states of Perak, Selangor, and Negri Sembilan focussed on the culling of pigs, and over 1 million pigs were culled to try and bring the outbreak under control. The Hendra-like virus was named Nipah virus (NiV) (Uppal, 2000), since it was first isolated from a patient from the village of Sungei Nipah ("Nipah river village") in the state of Negri Sembilan (Fig. 2) (Tan *et al.*, 1999; Chua *et al.*, 2000). In total, 265 confirmed cases were recorded with 105 associated fatalities (Chua *et al.*, 2000).

Between April 26 and May 26, 2001, 9 people died in the village of Chandpur in the Meherpur district of Bangladesh (Fig. 3). These people experienced a febrile illness with neurological symptoms. Another 18 in the same village were said to have had the same kind of disease but recovered. Subsequent investigation found that at least two of the individuals had been infected with a Nipah/Hendra-like virus (Anonymous, 2003). Between January 11 and 28, 2003, another outbreak of severe encephalitis was reported in at least 17 people in the villages of Chalksita and Biljoania in the district of Naogaon (Fig. 3), with eight fatalities (Anonymous, 2004a). Four of these patients had antibodies against a NiV antigen (Hsu *et al.*, 2004). From January 12 until 17, 2004, 12 people in Goalanda, district of Rajbari (Fig. 3), experienced febrile ill

Figure 3: Map indicating known outbreaks of NiV in Bangladesh and India (shown by ◎). Outbreak locations in Faridpur, Chandpur, Meherpur, Rajbari, and Naogaon, Bangladesh, and Siliguri in West Bengal, India are shown. Map was created using Google Earth version 4.0.2737.

progressing to coma; 10 of these people died. Through April 5, 2004, 29 cases were identified, including 14 laboratory confirmed cases, of which 22 died. Confirmed cases were identified from regions up to 150 km away, in Joypurhat, Naogaon, Natore, Faridpur, Gopalganj, Manikganj, and Dhaka districts (Anonymous, 2004a). Notably in this outbreak, person-to-person transmission was documented in the NiV outbreak in Faridpur district (Fig. 3) (Anonymous, 2004b). Cases tended to cluster within household and family groups. Prior to onset of illness, 33 of 36 cases had close contact with at least one person with confirmed or probable NiV infection. Patients in this outbreak also developed acute respiratory distress syndrome, which was not seen in prior outbreaks (Anonymous, 2004b).

Another outbreak of NiV-associated encephalitis was identified in January and February of 2001 in Siliguri, West Bengal, India (Fig. 3) (Chadha *et al.*, 2006). This area borders China, Bangladesh, and Nepal and has approximately 500,000 inhabitants. At the time, laboratory investigation did not identify an infectious agent. Based on the case definition, 66 human cases were identified with a fatality rate of approximately 74%. Patients showed signs of fever, headache and myalgia, vomiting, altered consciousness ranging from confusion to coma, respiratory symptoms ranging from rapid breathing to acute respiratory distress, and involuntary movements or convulsions. Since a likely infectious agent had

not been identified, patient samples were tested for IgM antibodies to JEV, West Nile virus, dengue virus, and measles virus (MeV), as well as *Leptospira*. Serum samples were also tested for IgG to hantavirus. All specimens tested negative for these agents. Testing of these patient specimens for antibody against NiV, and in some cases urine by NiV RT-PCR, revealed that IgM and IgG against NiV could be detected in serum and RNA from the M gene of NiV could be detected in urine. Furthermore, NiV N and M gene sequences were found to be more closely related to the Bangladesh NiV isolates than the Malaysian NiV isolates. Unfortunately, no virus isolates from this outbreak have been cultured, so further sequence analysis has been limited to archival patient material.

Henipavirus ecology and the reservoir species

The natural reservoir for both NiV and HeV appears to be fruit bats and flying foxes in the *Pteropus* genus of the order *Megachiroptera* (Halpin *et al.*, 1996; Young *et al.*, 1996; Halpin *et al.*, 2000; Olival & Daszak, 2005). Available evidence from experimental infections indicates that these bats are able to be persistently infected by both NiV and HeV, and shed infectious virus. In the wild, however, it has proven quite difficult to isolate infectious NiV and HeV from trapped fruit bats, although it is possible (Halpin *et al.*, 2000; Chua, 2003). Serological evidence has been obtained which indicates that bats from Malaysia (Chua *et al.*, 2002b),

and as far away as Cambodia (Olson *et al.*, 2002) and Madagascar (Iehlé *et al.*, 2007), have been exposed to a NiV-like virus as determined by the presence of anti-NiV antibodies in trapped bats. The circumstances leading to NiV outbreaks seems to be erratic, although there is some speculation that outbreaks have followed seasonal patterns and deforestation in Malaysia has also been implicated (Chua *et al.*, 2002a).

Henipavirus taxonomy and genetic analysis

Genetically, NiV and HeV are single-stranded RNA viruses with genomes of negative polarity totalling approximately 18.2 kb in length (Harcourt *et al.*, 2001; Harcourt *et al.*, 2000; Wang *et al.*, 2001). Partly on the basis of their relatively large genome sizes, NiV and HeV have been classified in the order *Mononegavirales*, within the family *Paramyxoviridae*, in a new genus named *Henipavirus* (Fig. 4) (Murray *et al.*, 1995b; Chua *et al.*, 2000; Mayo, 2002; Wang *et al.*, 2000). As paramyxoviruses, henipaviruses have a typical genome organization, shown in Fig. 5A. N is the nucleocapsid protein, which encapsulates viral genomic RNA in the virion (Fig. 6). P is a phosphoprotein and a polymerase cofactor which interacts with the viral ribonucleoprotein complex (Fig. 6). M is the matrix protein, and lines the inner leaflet of the viral membrane (Fig. 6). F and G are the surface glycoproteins (Fig. 6); G is responsible for

Family *Paramyxoviridae*

Subfamily *Paramyxovirinae*

Genus *Rubulavirus*

Human parainfluenza virus 2 (HPIV-2)
Human parainfluenza virus 4 (HPIV-4)
Mapuera virus (MPRV)
Mumps virus (MuV)
Porcine rubulavirus (PoRV)
Simian virus 5 (SV5)
Simian virus 41 (SV41)
Tioman virus (TiV, tentative)
Menangle virus (MenV, tentative)

Avulavirus

Avian paramyxovirus 2 (APMV-2)
Avian paramyxovirus 3 (APMV-3)
Avian paramyxovirus 4 (APMV-4)
Avian paramyxovirus 5 (APMV-5)
Avian paramyxovirus 6 (APMV-6)
Avian paramyxovirus 7 (APMV-7)
Avian paramyxovirus 8 (APMV-8)
Avian paramyxovirus 9 (APMV-9)
Newcastle disease virus (NDV)

Respirovirus

Bovine parainfluenza virus 3 (BPIV-3)
Human parainfluenza virus 1 (HPIV-1)
Human parainfluenza virus 3 (HPIV-3)
Sendai virus (SeV)
Simian virus 10 (SV10)

Henipavirus

***Hendra virus* (HeV)**
***Nipah virus* (NiV)**

Morbillivirus

Canine distemper virus (CDV)
Cetacean morbillivirus virus (CeMV)
Measles virus (MeV)
Peste-des-petits-ruminants virus (PPRV)
Phocine distemper virus (PDV)
Rinderpest virus (RPV)

Figure 4: Taxonomic structure of the subfamily *Paramyxovirinae* within the family *Paramyxoviridae*. The genus ***Henipavirus*** is highlighted.

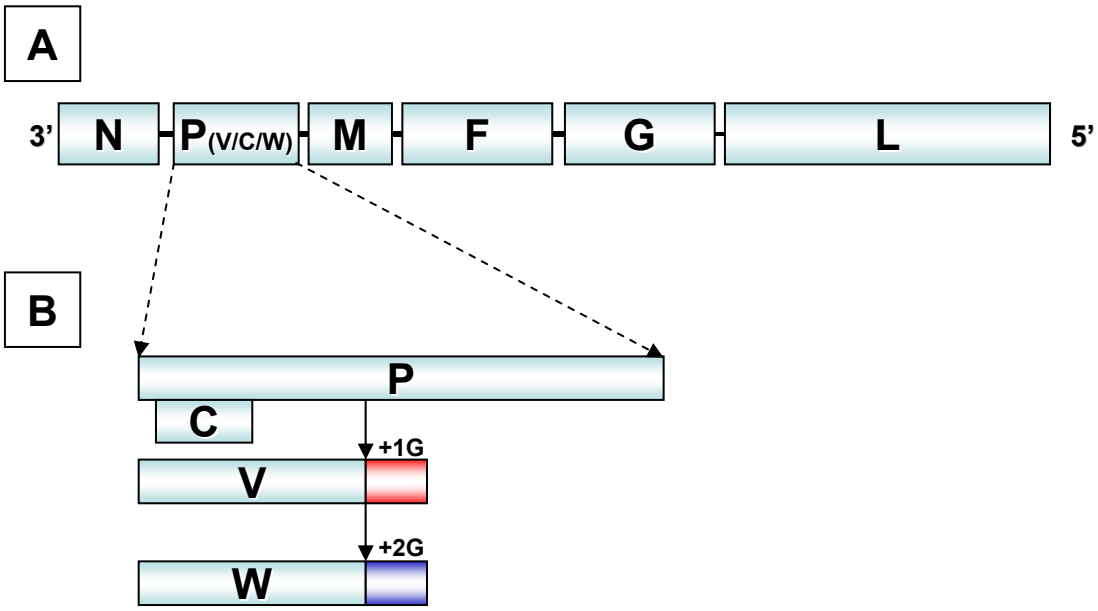

Figure 5: Schematic representation of the henipavirus genome. By convention, the genomes of (-)-ssRNA viruses are written 3'→5'. A) The full-length NiV genome. B) The co-transcriptional editing strategy of the P gene for the generation of the V and W transcripts (not to scale).

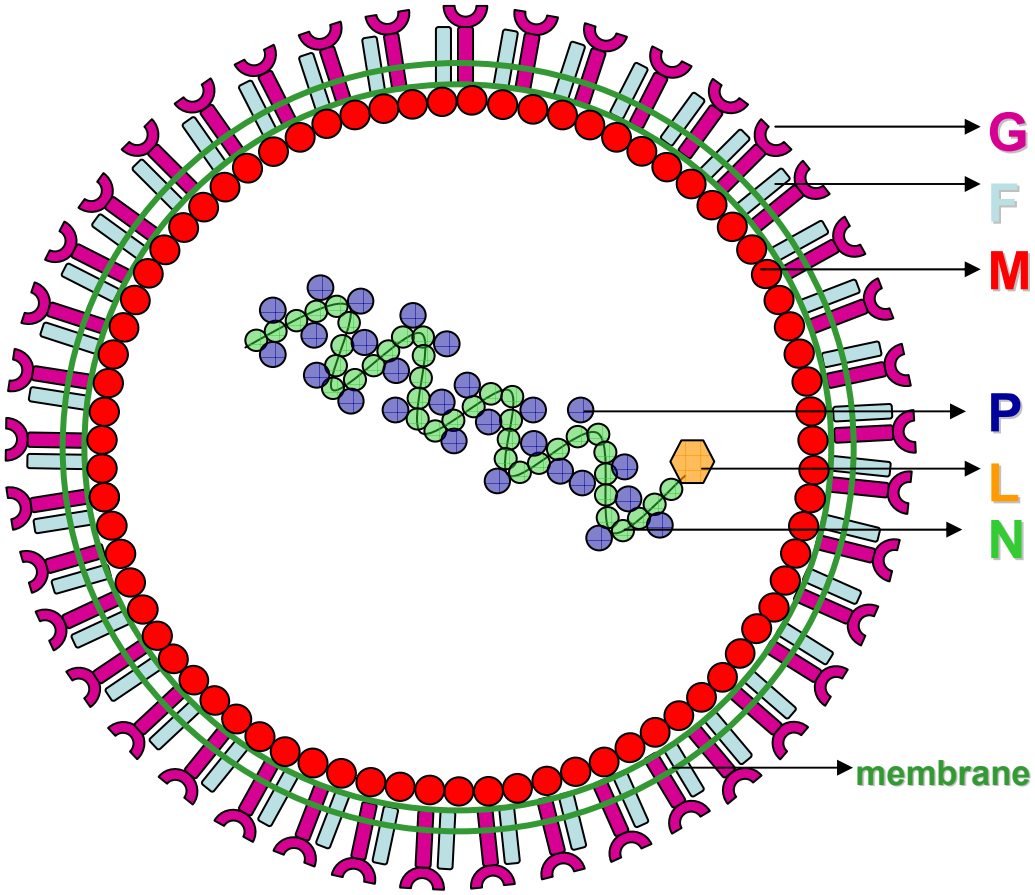

Figure 6: Schematic representation of the NiV virion. The genome is encapsidated by the N protein (green). The P (blue) and L (orange) proteins are also associated with the genome. The M protein (red) underlies the inner leaflet of the viral membrane. The F (light blue) and G (purple) surface glycoproteins are embedded in the viral membrane.

attaching to the host cell receptor and F is responsible for the process of viral-to-host cell membrane fusion. L is the RNA-dependent RNA polymerase, and is responsible for making viral mRNA as well as full-length viral genomic and anti-genomic RNA. The P gene is also capable of producing several other proteins via the process of co-transcriptional editing by addition of non-template G residues to P mRNA by the viral polymerase protein (Fig. 5B). The C protein could be produced from a distinct start codon upstream of the P start codon, while two additional proteins, V and W, could be produced via the process of co-transcriptional editing of the nascent P mRNA by insertion of one G residue (for V) or two G residues (for W) (Eaton *et al.*, 2006). Thus, V and W share the same N-terminus with P, but all differ in their C-terminal regions. Although V, C, and W have not been shown to be produced in infected cells, they have all been shown to block interferon activity when expressed in recombinant systems (Rodriguez *et al.*, 2002; Park *et al.*, 2003; Rodriguez & Horvath, 2004; Rodriguez *et al.*, 2003).

NiV and HeV share a high degree of sequence relationship. The sequence relationships are summarized in Table 1 (Harcourt *et al.*, 2000; Harcourt *et al.*, 2001). At the amino acid level, the open reading frames (ORFs) of the NiV genes have at least 80% sequence identity with their corresponding HeV ORFs, with the exception of the NiV P ORF, which has 67.6% amino acid identity with the HeV P

Table 1: Summary of the sequence comparisons of the NiV and HeV genes and ORFs

		ORF			3' NTR		5' NTR	
Gene	**Virus**	**Length**	**aa Identity**	**Nucleotide homology**	**Length**	**Homology**	**Length**	**Homology**
N	HeV	532 aa	92.1%	78.4%	57 nt	66.7%	568 nt	41.1%
	NiV	532 aa			57 nt		586 nt	
P	HeV	707 aa	67.6%	70.5%	105 nt	41.9%	469 nt	40.0%
	NiV	709 aa			105 nt		469 nt	
V	HeV	55 aa	81.1%	88.5%				
	NiV	52 aa						
C	HeV	166 aa	83.2%	85.0%				
	NiV	166 aa						
M	HeV	352 aa	89.0%	77.1%	100 nt	40.0%	200 nt	40.0%
	NiV	352 aa			100 nt		200 nt	
F	HeV	546 aa	88.1%	74.2%	272 nt	44.1%	418 nt	41.4%
	NiV	546 aa			284 nt		412 nt	
G	HeV	604 aa	83.3%	70.8%	233 nt	43.8%	516 nt	45.6%
	NiV	602 aa			233 nt		504 nt	
L	HeV	2,244 aa	87%	73%	153 nt	54%	67 nt	58%
	NiV	2,244 aa			153 nt		67 nt	

Data for HeV and NiV sequence comparisons for the N, P, V, C, M, F, and G ORFs and 3' and 5' NTRs were obtained from Harcourt *et al.* (2000) *Virology* **271**:334-349, with permission from Elsevier Ltd.. Data for HeV and NiV sequence comparisons for the L ORF and 3' and 5' NTRs were adapted from Harcourt *et al.* (2001) *Virology* **287**:192-201, with permission from Elsevier Ltd. The C proteins of HeV and NiV are produced from a start codon upstream of the P start codon. The V proteins of HeV and NiV share the N-terminus of the P protein, but differ at the C-terminus where a co-transcriptional G insertion in the mRNA by the L protein creates a truncated ORF. Hence, neither protein has a 3' or 5' NTR.

ORF. At the nucleotide level, NiV ORFs have sequence homology to the corresponding HeV ORFs varying from 70% to 90%. The NiV P and NiV G ORFs have the lowest levels of homology to their HeV counterparts at 70.5% and 70.8%, respectively. In general, the 3' and 5' non-translated regions (NTRs) have very low nucleotide sequence homology at approximately 40%-45% compared to the HeV 3' and 5' NTRs. The exceptions are the 3' and 5' NTRs of the L gene (54% and 58%, respectively) and the 3' NTR of the N gene (66.7%). The sequences of the Malaysian strain of NiV and the more recently isolated Bangladesh strain have also been compared (Harcourt *et al.*, 2005). The comparisons are summarized in Table 2. At the amino acid level, the ORFs share at least 90% identity. The W protein (100%), M protein (98.9%), F protein (98.4%), N protein (98.3%), and the L protein (98.2%) all have very high identity between the Malaysian and Bangladesh NiV strains. The P protein (92.0%) and the V protein (92.5%) have the lowest levels of similarity. The genome of the Bangladesh strain of NiV is 18,252 nt in length, which is 6 nt longer than the Malaysian strain, which is 18,246 nt. This difference is accounted for by the longer 3' NTR of the F gene (Harcourt *et al.*, 2005).

While NiV and HeV have significantly larger genomes (18.2 kb) than their counterparts of the subfamily *Paramyxovirinae* (approximately 15.5 kb), the ORFs of the N, P, M, F, G, and L genes all fall within the size ranges found in other

Table 2: Comparison of the ORFs, genes, and NTRs of the Malaysia and Bangladesh strains of NiV

		ORF			3' NTR		5' NTR	
Gene	**Virus**	**Length**	**aa Identity**	**Nucleotide homology**	**Length**	**Homology**	**Length**	**Homology**
N	Malaysia	532 aa	98.3%	94.3%	57 nt	100%	586 nt	90.8%
	Bangladesh	532 aa			57 nt		586 nt	
P	Malaysia	709 aa	92.0%	92.0%	105 nt	91.4%	469 nt	88.1%
	Bangladesh	709 aa			105 nt		469 nt	
V	Malaysia	52 aa	92.5%	95.7%				
	Bangladesh	55 aa						
C	Malaysia	166 aa	95.2%	97.6%				
	Bangladesh	166 aa						
W	Malaysia	47 aa	100%	98.5%				
	Bangladesh	47 aa						
M	Malaysia	352 aa	98.9%	93.4%	100 nt	86.0%	200 nt	83.5%
	Bangladesh	352 aa			100 nt		200 nt	
F	Malaysia	546 aa	98.4%	93.4%	284 nt	83.1%	412 nt	79.4%
	Bangladesh	546 aa			290 nt		412 nt	
G	Malaysia	602 aa	95.5%	93.0%	233 nt	75.5%	504 nt	80.8%
	Bangladesh	602 aa			233 nt		504 nt	
L	Malaysia	2244 aa	98.2%	93.4%	153 nt	82.4%	67 nt	80.6%
	Bangladesh	2244 aa			153 nt		67 nt	

Data for the sequence comparisons Malaysia and Bangladesh strains of NiV for the N, P, V, C, W, M, F, G, and L ORFs and 3' and 5' NTRs were adapted from public domain material in Harcourt *et al.* (2005) *Emerg. Infect. Dis.* **11**:1594-1597. The C protein of NiV is produced from a start codon upstream of the P start codon. The V and W proteins of NiV share the N-terminus of the P protein, but differ at the C-terminus where a co-transcriptional G insertion in the mRNA by the L protein creates a truncated ORF. Hence, neither the V, C, or W proteins have a 3' or 5' NTR.

members of the subfamily (Harcourt *et al.*, 2001). The size discrepancies in genome lengths of NiV and HeV when compared to other members of the subfamily can be accounted for by the sizes of the 3' and 5' NTRs the genes, shown in Table 3. The NiV N gene 5' NTR (586 nt), P gene 5' NTR (469 nt), F gene 5' NTR (412 nt), and G gene 3' and 5' NTRs (233 and 504 nt, respectively) are all significantly larger than those of other members of the subfamily *Paramyxovirinae*. The NiV P gene 3' NTR (105 nt), M gene 3' NTR (100 nt), and L gene 3' NTR (153 nt) are also larger than comparable *Paramyxovirinae* sequences, but the difference are not as large. The 3' and 5' genomic termini (defined as the number of nucleotides before the N gene or after the L gene) of NiV and HeV are 55 nt and 33 nt long, respectively (Harcourt *et al.*, 2001). The length of the 3' genomic terminus of many members of the order *Mononegavirales* is highly conserved, and is summarized in Table 4. Other paramyxoviruses, such as MeV, canine distemper virus (CDV), rinderpest virus (RPV), human parainfluenza virus type 3 (HPIV-3), Sendai virus (SeV), mumps virus (MuV), simian virus 5 (SV5), and Newcastle disease virus (NDV) and Ebola virus, in the family *Filoviridae*, all have 3' genomic termini with lengths of 55 nt. Vesicular stomatitis virus (VSV) (50 nt, family *Rhabdoviridae*) and respiratory syncytial virus (RSV) (44 nt, family *Paramyxoviridae*, subfamily *Pneumovirinae*) have different 3' genomic terminus lengths. The length of the 5' genomic terminus seems to be more

Table 3: Summary of the sizes of the 3' and 5' NTRs and the ORFs of the NiV and HeV genes and their comparison to ranges found within the subfamily *Paramyxovirinae*

Gene	Virus	3' NTR	5' NTR	ORF
N	NiV	57 nt	586 nt	1,599 nt
	HeV	57 nt	568 nt	1,599 nt
	Paramyxovirinae	52-96 nt	43-111 nt	1,530-1,650 nt
P	NiV	105 nt	469 nt	2,130 nt
	HeV	105 nt	469 nt	2,124 nt
	Paramyxovirinae	33-79 nt	66-123 nt	1,176-1,812 nt
M	NiV	100 nt	200 nt	1,059 nt
	HeV	100 nt	200 nt	1,059 nt
	Paramyxovirinae	32-36 nt	61-426 nt	1,008-1,134 nt
F	NiV	284 nt	412 nt	1,641 nt
	HeV	272 nt	418 nt	1,641 nt
	Paramyxovirinae	28-583 nt	38-137 nt	1,590-1,989 nt
G	NiV	233 nt	504 nt	1,809 nt
	HeV	233 nt	516 nt	1,815 nt
	Paramyxovirinae	20-78 nt	66-111 nt	1,698-1,854 nt
L	NiV	153 nt	67 nt	6,735 nt
	HeV	153 nt	67 nt	6,735 nt
	Paramyxovirinae	8-47 nt	34-137 nt	6,552-6,786 nt

Data are adapted from Harcourt *et al.* (2001) *Virology* **287**:192-201, with permission from Elsevier Ltd.. Rows labelled "Paramyxovirinae" show the range of 3' and 5' NTR and ORF sizes for other members of the subfamily *Paramyxovirinae* in the genera *Morbillivirus* (MeV and CDV), *Rubulavirus* (MuV and SV5), and *Respirovirus* (SeV and HPIV3). The large range of 5' NTR sizes for *Paramyxovirinae* M genes is due to the large 5' NTRs of morbilliviruses, while the large range of 3' NTR sizes for *Paramyxovirinae* F genes is due to the large 3' NTR of MeV.

Table 4: Comparison of the lengths of the 3' and 5' genomic termini of NiV and HeV with other members of the family *Paramyxoviridae* and other selected viruses

Virus	Family	Subfamily	3' terminus	5' terminus
NiV	*Paramyxoviridae*	*Paramyxovirinae*	55 nt	33 nt
HeV			55 nt	33 nt
MeV			55 nt	40 nt
CDV			55 nt	41 nt
RPV			55 nt	40 nt
HPIV3			55 nt	44 nt
SeV			55 nt	57 nt
MuV			55 nt	24 nt
SV5			55 nt	31 nt
NDV			55 nt	114 nt
RSV		*Pneumovirinae*	44 nt	154 nt
Ebola virus	*Filoviridae*		55 nt	678 nt
VSV	*Rhabdoviridae*		50 nt	59 nt

Abbreviations: NiV, Nipah virus; HeV, Hendra virus; MeV, measles virus; CDV, canine distemper virus; RPV, rinderpest virus; HPIV3, human parainfluenza virus type 3; SeV, Sendai virus; MuV, mumps virus; SV5, simian virus 5; NDV, Newcastle disease virus; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus. Adapted from Harcourt *et al.* (2001) *Virology* **287**:192-201, with permission from Elsevier Ltd.

variable, with only MuV (24 nt) and SV5 (31 nt) having shorter 5' terminal genomic sequences. The 5' genomic terminus of other paramyxoviruses ranges from 40-57 nt, with only NDV having a significantly longer 5' terminal genomic sequence (114 nt). By contrast, *Tupaia paramyxovirus* (TPMV), a recently-isolated unclassified member of the family *Paramyxoviridae* (Springfeld *et al.*, 2005; Tidona *et al.*, 1999), has a 3' genomic terminus of 55 nt, but its 5' genomic terminus is 590 nt in length, which is significantly larger than any other member of the family.

Though they have high sequence similarity to each other, NiV and HeV have relatively low similarity to viruses in other genera within the family *Paramyxoviridae* (Chan *et al.*, 2001), summarized in Table 5. The highest similarity between NiV and morbilliviruses such as MeV and CDV is seen in the M and L proteins, which is approximately 40-45%. NiV has even more limited similarity to MeV and CDV when a comparison is made between the N and F proteins, where similarity is approximately 30%. This trend also holds when NiV is compared with members of the *Respirovirus* genus (human parainfluenza virus type 1 [HPIV-1], HPIV-3, and SeV). The M proteins have approximately 30-35% similarity to NiV M, while the similarity between the *Respirovirus* L proteins and NiV L is almost 40%. It is interesting to note that the NiV G and HeV proteins have higher sequence similarity to *Respirovirus* HN proteins than to *Morbillivirus* H proteins, despite having overall lower similarity to the respiroviruses. NiV

Table 5: Comparison of the NiV and HeV ORF sequences to other members of the family *Paramyxoviridae*

Virus	Accession #	Sequence similarity					
NiV	**AY029767**	**N**	**P**	**M**	**F**	**G/H/HN**	**L**
HeV	AF017149	92%	64%	88%	85%	78%	85%
MeV	AF016162	32%	19%	44%	32%	16%	46%
CDV	AF014953	30%	21%	43%	27%	15%	44%
HPIV1	X66908	21%	17%	34%	27%	24%	–
HPIV3	Z11575	25%	17%	31%	27%	25%	39%
SeV	M19661	22%	17%	35%	26%	24%	38%
MuV	AB000388	25%	16%	21%	27%	18%	29%
HPIV4a	P17240	26%	15%	21%	–	19%	–
HPIV4b	P17241	25%	14%	22%	–	–	–
SV5	AF052755	27%	14%	20%	27%	20%	29%
NDV	AF077761	28%	15%	22%	25%	21%	26%
HRSV	AF013254	17%	11%	14%	20%	14%	19%
BRSV	AF092942	15%	10%	13%	19%	12%	19%

Abbreviations: NiV, Nipah virus; HeV, Hendra virus; MeV, measles virus; CDV, canine distemper virus; HPIV1, human parainfluenza virus type 1; HPIV3, human parainfluenza virus type 3; SeV, Sendai virus; MuV, mumps virus; HPIV4a, human parainfluenza virus type 4a; HPIV4b, human parainfluenza virus type 4b; SV5, simian virus 5; NDV, Newcastle disease virus; HRSV, human respiratory syncytial virus; BRSV, bovine respiratory syncytial virus. The paramyxovirus sequence similarities listed are shown relative to the corresponding NiV protein. The genera *Henipavirus* (HeV), *Morbillivirus* (MeV and CDV), *Respirovirus* (HPIV1, HPIV3, and SeV), *Rubulavirus* (MuV, HPIV4a, HPIV4b, and SV5), and *Avulavirus* (NDV) from the subfamily *Paramyxovirinae*, and the genus *Pneumovirus* from the subfamily *Pneumovirinae* (HRSV and BRSV) are represented. Adapted from Chan *et al.* (2001) *J. Gen. Virol.* **82**:2151-2155, with permission from the Society for General Microbiology.

and HeV have very limited sequence similarity to members of the *Rubulavirus* and *Pneumovirus* genera, with sequence similarities of approximately 15-25% for any given protein comparison.

The paramyxovirus attachment glycoprotein and virus entry

Attachment of virions to susceptible cells occurs via the binding of the attachment protein to a cellular receptor. Vaccine and laboratory-adapted strains of MeV are able to use the quasi-ubiquitous surface molecule CD46 as their entry receptor (Manchester *et al.*, 2000; Schneider *et al.*, 2002), which is expressed on many cell types. Wild-type strains of morbilliviruses, such as MeV, CDV, and RPV, also use what has come to be known as the universal morbillivirus receptor, signalling lymphocyte activation molecule (SLAM or CD150), for entry to susceptible cells (Hsu *et al.*, 1998; Schneider *et al.*, 2002; von Messling *et al.*, 2005; Baron, 2005). These appear to be the only receptors used by wild-type morbilliviruses for cell entry, although the existence of an additional morbillivirus receptor has been postulated (Nielsen *et al.*, 2003). This is based largely on the observation that MeV can replicate in cells that do not express either CD46 or SLAM, as well as evidence obtained from recombinant MeVs that have been engineered to selectively not recognize CD46 or SLAM, or both, but which are able to enter and replicate in CD46- and SLAM-deficient cell lines (also

known as "SLAM- or CD46-blind" viruses) (Hashimoto *et al.*, 2002; Massé *et al.*, 2002; Andres *et al.*, 2003). A recombinant CDV has also been reported which is able to enter and replicate in endothelial cells using a SLAM-blind wild-type CDV H protein (von Messling *et al.*, 2005). Many groups are currently attempting to identify any additional receptor(s). Rubulaviruses and respiroviruses use sialic acids as their entry receptors, which are cleaved by the neuraminidase activity encoded by the protein. The NiV and HeV attachment proteins have neither hemagglutinating nor neuraminidase activities, and are the first identified members of the subfamily *Paramyxovirinae* to not have either activity (Yu *et al.*, 1998).

Until recently, the receptor or receptors for NiV and/or HeV had remained unidentified. However, two entry receptors have been recently characterized for NiV and HeV. Two groups employing different methods, identified ephrin-B2 (previously known as Htk-L, ELF-2, LERK-5, or NLERK-1) (Eph Nomenclature Committee, 1997) as a receptor for NiV and HeV (Aguilar *et al.*, 2006; Bonaparte *et al.*, 2005). This has been verified by transfection of an ephrin-B2 expression plasmid into a fusion-resistant cell line followed by infection with NiV or HeV. Ephrin-B2 is sufficient to allow infection of a fusion-resistant cell line, thus indicating its role as receptor for virus entry (Bonaparte *et al.*, 2005). The closely related ligand ephrin-B3 (previously known as NLERK-2,

Elk-L3, EFL-6, ELF-3, or LERK-8) (Eph Nomenclature Committee, 1997) was identified as a receptor for NiV based on its ability to bind soluble NiV G and the ability of NiV F+G-pseudotyped VSV particles to enter cells expressing ephrin-B3 (Negrete *et al.*, 2006). It has been demonstrated that the closely-related ligand ephrin-B1 is not able to bind to the NiV attachment glycoprotein, and thus is likely not a receptor for henipaviruses (Negrete *et al.*, 2006). NiV and HeV are the first known viruses to use these surface proteins as entry receptors.

Viral entry and replication

Once virus-cell membrane fusion has proceeded to an extent that fusion pores of sufficient size have formed, the viral ribonucleoprotein (RNP) complex is released into the cell. The RNP complex is comprised of the viral (-)-ssRNA genomic RNA, the nucleocapsid protein (N), the phosphoprotein (P), and the large RNA-dependent RNA polymerase (L), which must enter with the genome in order for proper transcription and replication to occur (Lamb & Kolakofsky, 1996).

The N protein directly binds to the viral genomic RNA at hexamer intervals, such that viruses of the subfamily *Paramyxovirinae* must have hexameric genome lengths in order for efficient replication to take place. This phenomenon is known as "The Rule of Six", and applies only to members of the

Morbillivirus, *Respirovirus*, *Rubulavirus*, *Avulavirus*, and *Henipavirus* genera (Calain & Roux, 1993; Durbin *et al.*, 1997b; Kolakofsky *et al.*, 1998; Peeters *et al.*, 2000; Halpin *et al.*, 2004). This hexameric genome length requirement is not seen in any other virus family, and does not hold for members of the related subfamily *Pneumovirinae* in the family *Paramyxoviridae* such as RSV or HMPV. The (-)-sense genome is replicated via a (+)-sense antigenomic intermediate and is eventually packaged into progeny virions (Lamb & Kolakofsky, 1996).

Membrane fusion and the attachment glycoprotein

After binding to the host cell receptor by NiV G, a type-specific interaction occurs between NiV F and G to initiate fusion between the viral and host cell membranes. As is the case for other paramyxoviruses, this process occurs at neutral pH, rather than acid-induced fusion in post-endocytic compartments typical of other viruses such as influenza and VSV (Hernandez *et al.*, 1996). It has been demonstrated for numerous paramyxoviruses that co-expression of the fusion and attachment glycoproteins is required for fusion. Examples include HPIV-1 (Yao *et al.*, 1997), human parainfluenza virus type 2 (HPIV-2) (Hu *et al.*, 1992; Heminway *et al.*, 1994; Tsurudome *et al.*, 1995), simian virus 41 (SV41) (Tsurudome *et al.*, 1995), HPIV-3 (Ebata *et al.*, 1991; Ebata *et al.*, 1992; Heminway *et al.*, 1994; Tanaka & Galinski, 1995; Bagai & Lamb, 1995), human parainfluenza

virus type 4a (HPIV-4a) (Nishio *et al.*, 1994), SeV (Yao *et al.*, 1997), MuV (Tanabayashi *et al.*, 1992), MeV (Wild *et al.*, 1993; Cattaneo & Rose, 1993), CDV, RPV, NDV (Morrison *et al.*, 1991; Zeng *et al.*, 2004), and NiV and HeV (Bossart *et al.*, 2001; Bossart *et al.*, 2002; Tamin *et al.*, 2002). Additionally, provided that the complementing heterologous glycoproteins are of sufficiently close relation, they may also substitute for the homologous protein in the fusion process. This is possible for MeV and CDV (Bar-Lev Stern *et al.*, 1995), HPIV-1, HPIV-3, and SeV (Bousse *et al.*, 1994; Yao *et al.*, 1997), HPIV-2, HPIV-4a, MuV, and SV41 (Tsurudome *et al.*, 1998), and NiV and HeV (Tamin *et al.*, 2002; Bossart *et al.*, 2002). It must be noted that this function complementarity seems to segregate with genetic relatedness, since only viruses within the same genus seem to able to perform this function.

Based on the lack of fusion activity by singly-expressed fusion proteins, the paramyxovirus attachment protein has a vital role in fusion (Cattaneo & Rose, 1993; Nishio *et al.*, 1994; Dutch *et al.*, 1998; Zeng *et al.*, 2004; Bagai *et al.*, 1993; Hu *et al.*, 1992; Wild *et al.*, 1991; Morrison *et al.*, 1991; Gitman & Loyter, 1984; Merz & Wolinsky, 1983). It has been proposed that binding of the attachment of paramyxovirus attachment glycoprotein to the host cell receptor causes a change in the conformation of the protein which is transmitted to the fusion glycoprotein via the interacting amino acid residues between the fusion

and attachment glycoproteins (Dallocchio *et al.*, 1995; Stone-Hulslander & Morrison, 1997; Deng *et al.*, 1999; Crennell *et al.*, 2000; Takimoto *et al.*, 2002). This is borne out by experimental evidence which indicates that the interaction and fusion promotion domains of paramyxovirus attachment proteins can be separated by mutation or domain swapping (Sergel *et al.*, 1993a; Sergel *et al.*, 1993b; Mirza *et al.*, 1994; Deng *et al.*, 1995; Hummel & Bellini, 1995; Tanabayashi & Compans, 1996; Deng *et al.*, 1997; Porotto *et al.*, 2003); this allows the generation of attachment proteins which have receptor binding function, but lack fusion promotion function when co-expressed with the homologous fusion protein. The induced conformational change causes a radical change in the structure of the fusion protein as it converts from its metastable pre-fusion conformation to its post-fusion stable conformation (Hsu *et al.*, 1981; Lamb, 1993; Russell *et al.*, 2001). The initial triggering of the fusion protein exposes the fusion peptide, a small hydrophobic sequence, which is buried within the structure of the pre-fusion conformation of the protein (Hernandez *et al.*, 1996). The initial stages of paramyxovirus fusion are shown schematically in Fig. 7. Exposure of the fusion peptide then results in further conformational changes in the fusion protein which eventually lead to the insertion of the fusion peptide into the opposing membrane of the host cell. At this point, it is thought that interactions between the adjacent heptad repeat (HR) regions cause the fusion protein alpha-

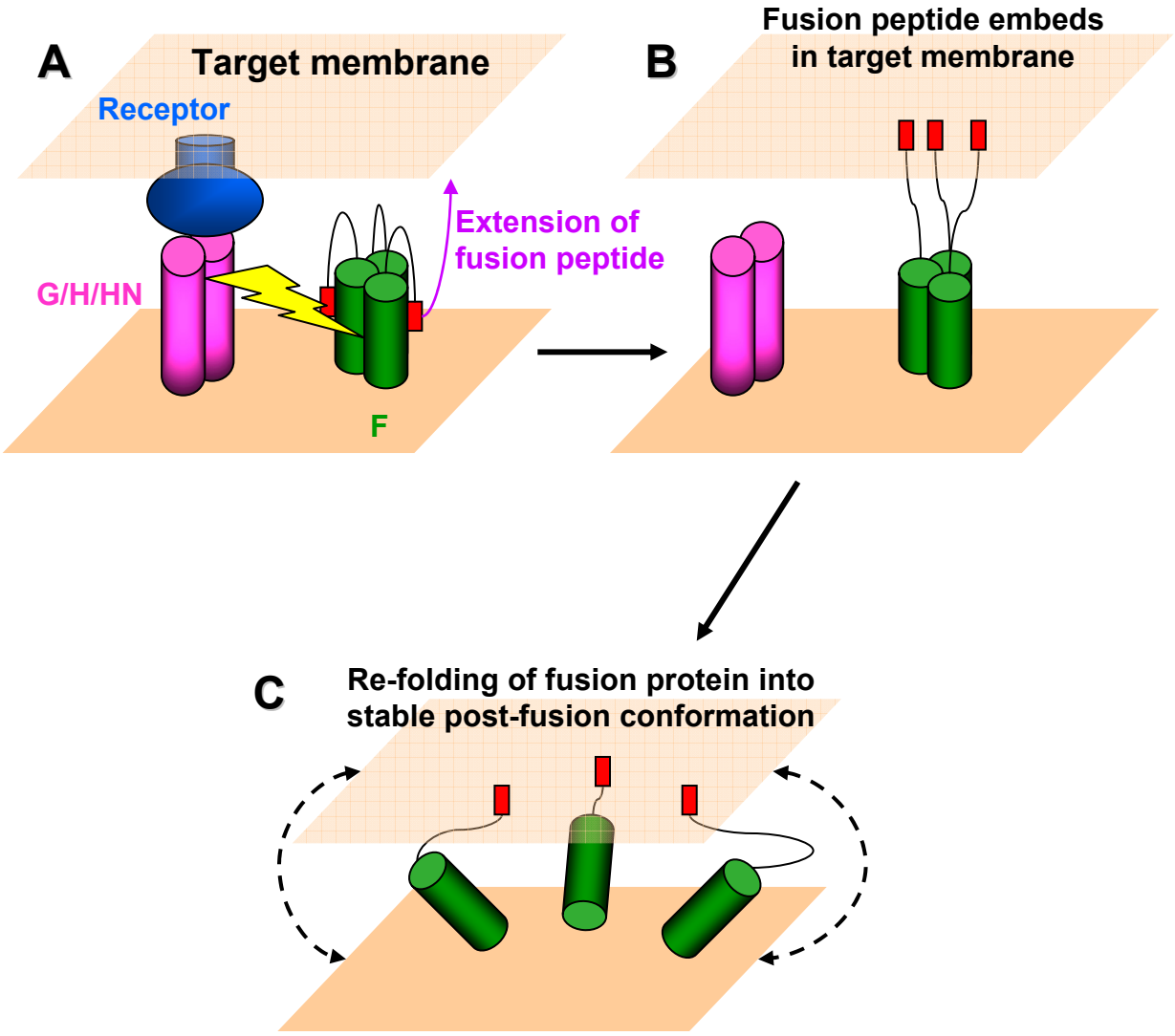

Figure 7: Schematic representation of membrane fusion mediated by the paramyxovirus glycoproteins. A) Binding of the receptor on the target membrane by the attachment glycoprotein (G/H/HN) transduces a signal (yellow lightning bolt) that activates the fusion glycoprotein (F). B) Activation of the F protein causes extension of the fusion peptide into the target membrane. C) The F protein folds into its stable post-fusion conformation, in the process bringing the target and viral membranes in close proximity to each other (indicated by the dashed lines). This results in mixing of the lipids in the outer leaflets of each membrane and hemi-fusion pore which ultimately grow into full fusion pores.

helical domains to fold back upon themselves and draw the viral and host cell membranes closer together, beginning the process of fusion. The initial interaction between the membranes results in exchange of the outer membrane leaflets leading to a structure known as hemifusion. The hemifusion pore can flicker back and forth between hemifusion and fusion before stable exchange of the inner leaflets results in the opening of a full fusion pore. Increased viral glycoprotein density around fusion pores results in further enlargement of the pore (Aroeti & Henis, 1991).

The attachment glycoprotein and viral interference

The function of binding to the cellular receptor has some intriguing consequences for expression studies. First, the possibility exists that if recombinant attachment protein is expressed in susceptible cells, it might interact with the receptor and prevent exogenously-applied virus from attaching and entering the cell. When the glycoproteins of some retroviruses (Delwart & Panganiban, 1989; Czub *et al.*, 1995; Hunt *et al.*, 1999; Ponferrada *et al.*, 2003), influenza virus (Marschall *et al.*, 1997), HPIV-3 (Horga *et al.*, 2000), HBV (Breiner *et al.*, 2001), and gammaherpesviruses (Geraghty *et al.*, 2000) are expressed in susceptible cell lines, their interaction and blockage of the receptor renders these cells refractive to infection with a homologous virus that uses the same receptor.

The mechanisms by which these proteins confer resistance differ somewhat. In most cases, expression of the attachment protein of some viruses, such as MeV H (Lecouturier *et al.*, 1996; Tanaka *et al.*, 2002; Welstead *et al.*, 2004), induces a down-regulation of the receptor so that it is either no longer present on the cell surface, or is present in very small amounts. The HPIV-3 HN protein, which possesses both hemagglutination and neuraminidase functions, is somewhat unique in that it induces the destruction of its cellular receptor, terminal sialic acids, via cleavage of these sugar moieties by neuraminidase activity of the attachment glycoprotein (Horga *et al.*, 2000); the net effect, however, is the same.

Structure-function relationships in the paramyxovirus attachment glycoprotein

The general structure of paramyxovirus attachment proteins was initially determined when the crystal structure for NDV HN was solved (Crennell *et al.*, 2000). Paramyxovirus attachment proteins are type II transmembrane glycoproteins, with a cytoplasmic N-terminus and an extracellular C-terminus. Paramyxovirus attachment glycoproteins must attach to the host cell via a receptor (Li & Qi, 2002; Fournier *et al.*, 2004; Hu *et al.*, 2004) and must interact with the fusion glycoprotein after attachment to initiate the process of membrane fusion between the viral envelope and the host cell plasma membrane

(Dallocchio *et al.*, 1995; Stone-Hulslander & Morrison, 1997; Deng *et al.*, 1999; Crennell *et al.*, 2000; Takimoto *et al.*, 2002). In addition to functions involved in virus entry, paramyxovirus attachment glycoproteins also have domains which are important for protein structure. Most paramyxovirus attachment glycoproteins form disulfide-linked dimers, and possibly higher-order trimers or tetramers as well (Herrler & Compans, 1983; Malvoisin & Wild, 1993; Mirza *et al.*, 1993; Parks & Pohlmann, 1995; Plemper *et al.*, 2000). The attachment and fusion glycoproteins also have presumed functions in virus assembly and budding through interaction with the matrix protein inside the viral particle (Sanderson *et al.*, 1993; Sanderson *et al.*, 1994; Ali & Nayak, 2000) and an interaction between either or both of the glycoproteins with the matrix protein during intracellular transport to ensure proper sorting to the correct cellular compartment (Pantua *et al.*, 2006). As a major surface glycoprotein, the paramyxovirus attachment protein is a target for the neutralizing immune response. For instance, the H glycoprotein of MeV is the major target for neutralizing antibodies during an immune response to infection (de Swart *et al.*, 2005).

The HN proteins of NDV, HPIV-3, and SV5 have all been crystallized and have similar features in their 3D structures (Crennell *et al.*, 2000; Lawrence *et al.*, 2004; Yuan *et al.*, 2005). The globular ectodomain of the paramyxovirus HN protein consists of six β-sheets each composed of 4 anti-parallel β-strands

arranged radially around the central axis of the protein, referred to as a β-propeller. Sequence comparisons and homology modelling of MeV H and CDV H has indicated that the morbillivirus H proteins share this same structure (Langedijk *et al.*, 1997; Massé *et al.*, 2004). The sites for CD46 and SLAM binding by MeV H and sialic acid binding by NDV HN have been mapped to a number of different residues in the C-terminal 35% of these proteins. A "minimal dimerization domain" has been determined for MeV H (Plemper *et al.*, 2000), and cysteines at positions 139 and 154 of MeV H are also involved in homodimerization through the formation of intermolecular disulfide bonds (Sato *et al.*, 1995).

Regions responsible for the interaction of the attachment glycoprotein with the fusion glycoprotein have also been identified in some paramyxoviruses. NDV HN has two separate regions which interact with F in the stalk region of the protein (Tsurudome *et al.*, 1995; Gravel & Morrison, 2003), while membrane-proximal residues in the ectodomain of SeV HN are important for interaction with SeV F (Tanabayashi & Compans, 1996). The morbilliviruses (e.g. MeV and CDV) have remained largely uncharacterized in this respect.

The regions of the NiV G attachment glycoprotein responsible for interaction with NiV F or HeV F and protein dimerization and multimerization have not been fully identified. Neutralizing epitopes of both HeV G and NiV G

have been characterized in some detail (White *et al.*, 2005; Zhu *et al.*, 2006). Further work has focussed on elucidation of residues responsible for binding of the attachment glycoprotein to the receptors ephrin-B2 and ephrin-B3. Guillaume *et al.* first identified the residues Trp504, Glu505, Gln530, Thr531, Ala532, Glu533, and Asn557 as being important for binding of NiV G to ephrin-B2 (Guillaume *et al.*, 2006). More recent work has shown that while NiV G and HeV G use ephrin-B2 with equal efficiency, NiV G likely uses ephrin-B3 more efficiently, and that binding of ephrin-B3 can be localized to the residue Val507 of NiV G (Negrete *et al.*, 2007). The authors of this study claim that this might explain the pathology seen in NiV and HeV infection since ephrin-B3 is expressed more predominantly in the brain stem than ephrin-B2, and infection of the brain stem during NiV infection has been strongly associated with death. This hypothesis, however, needs to be demonstrated in an animal model to be fully convincing. Bishop *et al.* have demonstrated that several other clusters of residues of HeV G (Asp257, Asp260, Gly439, Lys443, Gly449, Lys465, and Asp468) are also involved in receptor binding (Bishop *et al.*, 2007), but it is unclear how these distinct residues relate to those identified in previous studies of receptor binding function.

Rationale: Expression and fusogenic behaviour of NiV G

Paramyxoviruses have at least two glycoproteins that are expressed on the surface of virions. These glycoproteins are responsible for attachment and entry into cells during the initial stages of infection. After the attachment protein binds the cellular receptor, the fusion protein is activated and the fusion peptide is exposed. During this process, the fusion protein undergoes a series of radical structural changes in which the fusion peptide is inserted into the opposing host cell membrane, followed by folding of the heptad repeat domains such that the opposing membranes are brought together in close proximity. This process ultimately leads to the formation of fusion pores through which the viral RNP complex is released into the cytoplasm. Most paramyxoviruses require both the fusion and attachment glycoprotein for cell-cell fusion to occur (Ebata *et al.*, 1991; Wild *et al.*, 1991; Ebata *et al.*, 1992; Tanabayashi *et al.*, 1992; Hu *et al.*, 1992; Cattaneo & Rose, 1993; Heminway *et al.*, 1994; Nishio *et al.*, 1994; Tsurudome *et al.*, 1995; Bagai & Lamb, 1995; Tanaka & Galinski, 1995; Yao *et al.*, 1997; Bossart *et al.*, 2001; Bossart *et al.*, 2002; Tamin *et al.*, 2002; Zeng *et al.*, 2004; Morrison *et al.*, 1991). In limited instances, if two viruses are closely related, the glycoproteins are able to functionally substitute for each other. This has been shown experimentally with the MeV and CDV glycoproteins, where the combinations of MV F+CDV H and CDV F+MV H are able induce cell-cell fusion (Bar-Lev Stern

et al., 1995) as is the combination of RPV F+MeV H (Tamin *et al.*, 2002). This has also been shown with the combinations HPIV-2 F+MuV HN and HPIV-4a F+HPIV-2 HN (Tsurudome *et al.*, 1998). In all cases, the glycoproteins come from virus species with the same genus. Some paramyxoviruses, however, have fusion proteins that are capable of mediating cell-cell fusion in the absence of a homologous attachment. These include the F proteins of SV5 (Horvath *et al.*, 1992; Dutch *et al.*, 1998), peste-des-petits-ruminants virus (PPRV) (Seth & Shaila, 2001), human RSV (Branigan *et al.*, 2005), and human metapneumovirus (HMPV) (Schowalter *et al.*, 2006). The F protein of SeV, when expressed on its own, can also bind to asialoglycoprotein receptor on the surface of opposing cells and mediate cell-cell fusion in the absence its corresponding attachment protein (Markwell *et al.*, 1985; Bitzer *et al.*, 1997; Leyrer *et al.*, 1998a). The mechanism by which fusion is triggered in paramyxovirus F protein-only fusion is unclear.

Rationale: Receptor interference and protection of cells from NiV and HeV infection

Viral attachment proteins are responsible for binding to receptors on susceptible host cells. In many other viral families, the attachment protein also mediates virus-cell membrane fusion via an internal fusion peptide.

Paramyxovirus attachment glycoproteins lack this function, and it is instead performed by the fusion protein.

The function of binding to the cellular receptor has some interesting consequences for expression studies. First, the possibility exists that if recombinant attachment protein is expressed in susceptible cells, it might interact intracellularly with the receptor and prevent exogenously-applied virus from attaching and entering the cell. When the glycoproteins of some retroviruses (Delwart & Panganiban, 1989; Czub *et al.*, 1995; Hunt *et al.*, 1999; Ponferrada *et al.*, 2003), influenza virus (Marschall *et al.*, 1997), HPIV-3 (Horga *et al.*, 2000), HBV (Breiner *et al.*, 2001), and gammaherpesviruses (Geraghty *et al.*, 2000) are expressed in susceptible cell lines, their interaction and blockage of the receptor renders these cells refractive to infection with a homologous virus that uses the same receptor (Fig. 8). The mechanisms by which these proteins confer resistance differ somewhat. In most cases, expression of the attachment protein of some viruses, such as MeV H (Lecouturier *et al.*, 1996; Tanaka *et al.*, 2002; Welstead *et al.*, 2004), induces a down-regulation of the receptor so that it is either no longer present on the cell surface, or is present in very small amounts. The HPIV-3 HN protein, which possesses both hemagglutination and neuraminidase functions, is somewhat unique in that it induces the destruction

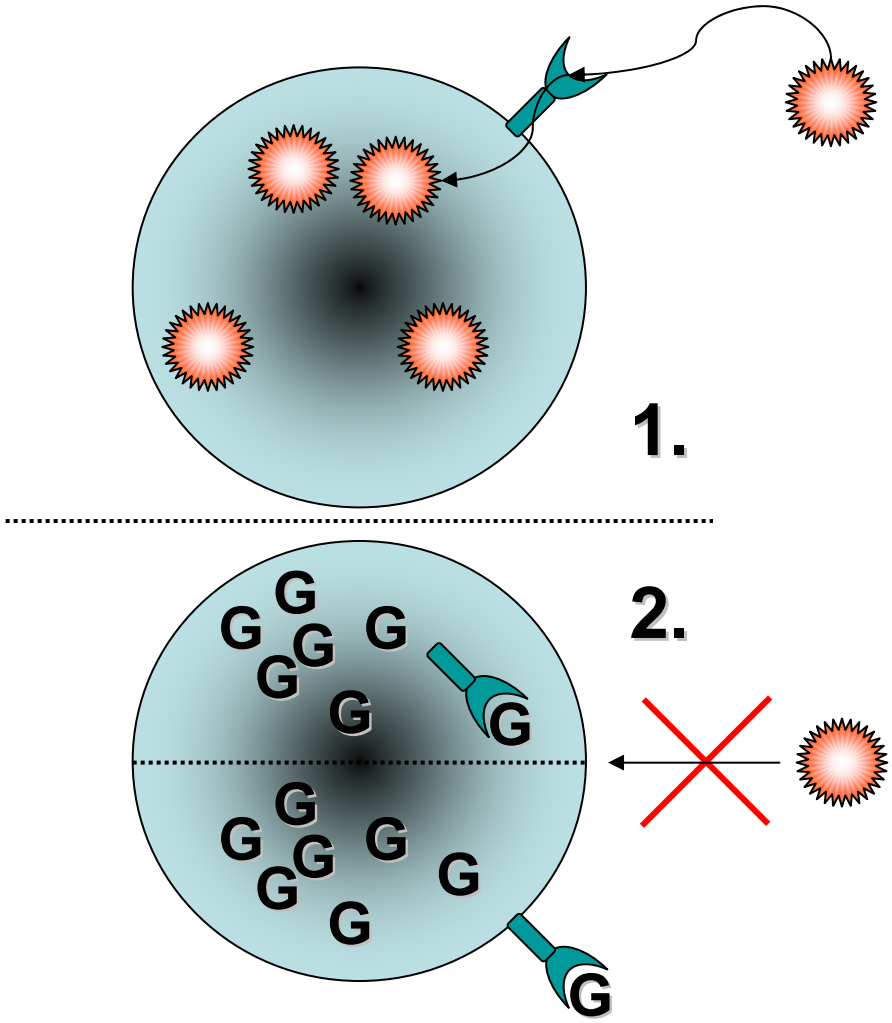

Figure 8: Scenarios for viral interference.

1.) NiV is able to bind to the cellular receptor and enter, thereby causing infection.

2.) In cells expressing NiV G, a hypothesized interaction occurs between NiV G and the receptor either in an intracellular compartment (top half of cell) or on the cell surface (bottom half of cell) such that there is no functional receptor on the cell surface and NiV cannot bind and enter the cell.

of its cellular receptor, terminal sialic acids, via its neuraminidase activity (Horga *et al.*, 2000); the net effect, however, is the same.

Rationale: Functional characterization of NiV G

Thus far, the domain of NiV G responsible for binding to the cellular receptor has not been identified. However, the identification of specific domains in other paramyxovirus attachment glycoproteins, such as those of MeV H, CDV H, and NDV HN, responsible for binding of their respective cellular receptors, gives fairly good clues as to where this domain might be located in NiV G.

The general structure of paramyxovirus attachment proteins was initially determined when the crystal structure for NDV HN was solved (Crennell *et al.*, 2000). Paramyxovirus attachment proteins are type II transmembrane glycoproteins, with a cytoplasmic C-terminus and an extracellular N-terminus. The attachment glycoproteins have several presumed functional domains. First, they must attach to the host cell via a receptor (Li & Qi, 2002; Fournier *et al.*, 2004; Hu *et al.*, 2004). Second, most paramyxovirus attachment glycoproteins form disulfide-linked dimers, and possibly higher-order trimers or tetramers as well (Herrler & Compans, 1983; Malvoisin & Wild, 1993; Mirza *et al.*, 1993; Parks & Pohlmann, 1995; Plemper *et al.*, 2000). Third, the attachment glycoprotein must interact with the fusion glycoprotein after attachment to initiate the process of

membrane fusion between the viral envelope and the host cell plasma membrane (Dallocchio *et al.*, 1995; Stone-Hulslander & Morrison, 1997; Deng *et al.*, 1999; Crennell *et al.*, 2000; Takimoto *et al.*, 2002). Fourth, it is thought that one or both of the glycoproteins (fusion and/or attachment) interacts with the matrix protein inside the viral particle (Sanderson *et al.*, 1993; Sanderson *et al.*, 1994; Ali & Nayak, 2000). Fifth, any interaction between either or both of the glycoproteins and the matrix protein is also likely to ensure proper sorting to the correct cellular compartment during the process of viral budding and egress (Pantua *et al.*, 2006). Sixth, the H glycoprotein of MeV is the major target for neutralizing antibodies during an immune response to infection (de Swart *et al.*, 2005), so NiV G is likely to have a number of specific epitopes.

The HN proteins of NDV, HPIV-3, and SV5 have all been crystallized and have similar features in their 3D structures (Crennell *et al.*, 2000; Lawrence *et al.*, 2004; Yuan *et al.*, 2005). The globular ectodomain of the paramyxovirus HN protein is of six β-sheets each composed of 4 anti-parallel β-strands arranged radially around the central axis of the protein, referred to as a β-propeller. Sequence comparisons and homology modelling of MeV H and CDV H has indicated that the morbillivirus H proteins share this same structure (Langedijk *et al.*, 1997; Massé *et al.*, 2004). CD46 binding by MeV H has been mapped to residues a number of different residues in several studies: Ser546 and Asn481 (Li

& Qi, 2002); Val451 (Lecouturier *et al.*, 1996); Ser548 and Phe549 (Massé *et al.*, 2002); Ala428, Leu464, Tyr481, Ile487, Ala527, Tyr452, and Pro486 (Vongpunsawad *et al.*, 2004); and aa's 473-477 (Patterson *et al.*, 1999). Gly211 and Arg243 have also been implicated in CD46 binding (Bartz *et al.*, 1996). SLAM binding by MeV H has been mapped to the following residues: residues 429-438 (Hu *et al.*, 2004); TyrY529, Asp530, Arg533, Tyr553, Thr531, Phe552, and Pro554 (Vongpunsawad *et al.*, 2004); Asp505, Asp507, and Arg533 (Massé *et al.*, 2004). There is also evidence that the CD46 and SLAM binding sites overlap on the MeV H globular head (Santiago *et al.*, 2002; Massé *et al.*, 2004) and substitutions at residues 481 and 533 have been shown to abrogate binding to both CD46 and SLAM (Hadac *et al.*, 2004). Residues Glu401, Arg416, and Tyr526 of NDV HN are key for receptor binding (Connaris *et al.*, 2002), and Phe220, Ser222, and Leu224 have been implicated in NDV HN receptor binding efficiency, although mutation of these residues also affects HN dimerization (Corey *et al.*, 2003). Residue Ile175 of NDV HN is also important for binding, but is located in the neuraminidase active site and can be rescued by compensatory mutations (Li *et al.*, 2004). As can be seen from the list, the majority of residues involved in receptor binding by MeV H (CD46 and/or SLAM) and NDV HN are located in the C-terminal ~35% of the respective proteins. A "minimal dimerization domain" has been determined for MeV H (Plemper *et al.*, 2000), and cysteines at positions 139 and

154 of MeV H are also involved in homodimerization through the formation of intermolecular disulfide bonds (Sato *et al.*, 1995).

Regions responsible for the interaction of the attachment glycoprotein with the fusion glycoprotein have also been identified in some paramyxoviruses. NDV HN has two separate regions which interact with F. The NDV F HR interacts with residues 124-152 of NDV HN, with mutations Ile133 and Leu140 having the greatest effect on interaction (Gravel & Morrison, 2003), although the involvement of this region of NDV HN has been disputed in a recent study (Melanson & Iorio, 2006). For HPIV-2, residues 148-209 of HN interact with F, which also maps to residues 154-215 of NDV HN (Tsurudome *et al.*, 1995). The 82 aa's directly adjacent to the TM domain of SeV HN are important for interaction with SeV F (Tanabayashi & Compans, 1996).

Chimeric glycoproteins were created by fusion of portions of the NiV G ORF with complementary domains from CDV H. Since the CDV and NiV glycoproteins are not able to functionally complement each other in the induction of cell-cell fusion and use different receptors (canine SLAM vs. ephrin-B2 and ephrin-B3), it was hoped that some of the chimeras would retain some of the functions of the full-length parental glycoproteins, but lose others.

Rationale: Reverse genetics and chimeric viruses

For many years, the study of the molecular determinants of viral pathogenesis and virulence for non-segmented (-)-ssRNA viruses was hampered by the lack of a system whereby viruses could be genetically manipulated. Very few tools exist that allow for the genetic manipulation of RNA, and even if such tools could be used, any attempt to generate infectious virus from this genomic RNA would fail. Eukaryotic cells do not possess any means by which they can transcribe RNA from an RNA template. This problem was solved over a decade ago when the first reverse genetics system for non-segmented (-)-ssRNA viruses were developed (Conzelmann, 1996; Conzelmann, 1998; Conzelmann & Meyers, 1996). The solution lies in cloning (-)-sense RNA genome as an antigenomic cDNA upstream of an RNA polymerase promoter that is able to drive transcription of the full-length cDNA genomic copy. By supplying the requisite RNA polymerase and plasmids expressing the viral proteins necessary to generate the viral RNP complex, viral replication can be initiated *in vitro*.

Rabies virus (RV) (Schnell *et al.*, 1994), which is a member of the *Lyssavirus* genus (family *Rhabdoviridae*), and VSV (Whelan *et al.*, 1995), which is a member of the *Vesiculovirus* genus (family *Rhabdoviridae*), were the first (-)-ssRNA viral genomes to be rescued. Both viruses are relatively small (12 kb for RV and 11 kb for VSV) and have relatively uncomplicated genomes consisting of 5

transcriptional units: 3'-N-P-M-G-L-5'. The success of these two reverse genetics systems has quickly led to the cloning of many more viral genomes and the development of accompanying rescue systems for these genomes.

The first paramyxovirus to be successfully rescued by a reverse genetic approach was MeV (Radecke *et al.*, 1995). The complete genome of the laboratory-adapted Edmonston strain of MeV was assembled in a plasmid upstream of a bacteriophage T7 RNA polymerase (RNAP) promoter. This plasmid, along with a T7 RNAP-driven expression plasmid for MeV L, was then transfected into a helper cell line which expressed the MeV N and P proteins. Syncytia developed in these cells and virus could easily be isolated, indicating that successful virus transcription and replication had occurred. Several years later, another approach was used to rescue MeV in which the T7 RNAP-expressing helper cell line was replaced with modified vaccinia virus strain Ankara expressing the phage T7 RNAP (MVA-T7) (Schneider *et al.*, 1997). MVA-T7 is useful because it expresses T7 RNAP to a high level, but MVA is assembly-deficient in most cell lines and has been reported to not contaminate the culture medium and any resulting recombinant virus (Fuerst *et al.*, 1986; Meyer *et al.*, 1991; Wyatt *et al.*, 1995; Sutter *et al.*, 1995). In this rescue system, MVA-T7 co-infection provides the T7 RNAP needed to drive transcription of the genome, while the plasmids necessary for forming the RNP complex (N, P, and L) were

provided in *trans* by transfection. However, it is not just the laboratory-adapted strains of MeV that are of interest to researchers. Virulent strains of MeV still circulate in different parts of the world, so a reverse genetics system for the Edmonston strain would only prove useful to a point. To that end, a rescue system was also developed for a highly pathogenic MeV strain (IC-B) that had originally been isolated after passage in B95a cells (Takeda *et al.*, 2000). As before, the genomic RNA cloned as antigenomic cDNA into a plasmid where transcription was driven by the T7 RNAP promoter and expression plasmids for the RNP proteins N, P, and L were supplied. This rescue system, however, used a different vaccinia virus strain, vTF7-3, but which also expresses the T7 RNAP. Although the data are unpublished, anecdotal evidence indicates that recovery of infectious virus from cells that have been transfected with the MeV antigenome plasmid, the RNP protein expression plasmids, and an expression plasmid for T7 RNAP has been unsuccessful.

In subsequent years, reverse genetics rescue systems have been developed for several other paramyxoviruses. Two other morbilliviruses, CDV (Gassen *et al.*, 2000) and RPV (Baron & Barrett, 1997), were recovered using an MVA-T7-based method. A rescue system for SeV, the type species for the genus *Respirovirus*, has also been established using vaccinia virus vTF7-3 to drive antigenome transcription (Garcin *et al.*, 1995; Kato *et al.*, 1996). Another group

has subsequently shown that MVA-T7 in fact seems to be better for SeV rescue than vTF7-3 (Leyrer *et al.*, 1998b). Rescue systems were developed for HPIV-3 by two separate groups, although one group used MVA-T7 (Durbin *et al.*, 1997a) and the other used vTF7-3 (Hoffman & Banerjeee, 1997) to drive antigenome transcription. An infectious clone was developed more recently for MuV, the type species for the genus *Rubulavirus*, using MVA-T7 (Clarke *et al.*, 2000). Finally, rescue systems for two strains of NDV have been developed. The first was developed for the generation of the lentogenic (low virulence) NDV strain Clone-30 using the cell line BSR T7/5, which stably expresses the T7 RNAP (Römer-Oberdörfer *et al.*, 1999). The second was developed for another lentogenic NDV strain, LaSota, but this group used a recombinant fowlpox virus (fpEFLT7pol) to drive antigenome transcription (Peeters *et al.*, 1999). Thus, all paramyxovirus rescue systems employ similar strategies, but the most profound differences occur in the choice of the method by which to provide the T7 RNAP needed to transcribe the antigenome. Recently, one group has developed a rescue system that has generated infectious MeV and Borna disease virus (BDV) from cDNA using an RNAP II promoter instead of a T7 RNAP promoter (Martin *et al.*, 2006). This represents a fundamentally new development in the reverse genetics field, largely because non-segmented (-)-sense ssRNA viruses can now be rescued without the need for an additional polymerase such as T7 RNAP. The

only plasmids that must be transfected in such a system are the RNP protein expression plasmids and the cDNA antigenome plasmid. The elimination of helper virus infection (i.e. MVA-T7 or vTF7-3) should facilitate virus rescues in future studies.

Of course, the great promise of reverse genetics lies in the ability to genetically manipulate the cDNA antigenome and modify parts of the viral genome, or add or subtract viral components. One of the more popular modifications thus far has been the inclusion of an eGFP reporter as an additional transcriptional unit (ATU). One group has developed a MeV clone that includes an eGFP gene as an ATU immediately before the N gene, which results in high amounts of fluorescence in infected cells and allowed the visualization of MeV spread in the neural cells of infected animals (Duprex *et al.*, 2000). The same type of approach has been used with the 5804P strain of CDV by insertion of eGFP ATUs either before the N gene, between the P and M genes, or between the H and L genes (von Messling *et al.*, 2004). These eGFP-expressing viruses were easily seen in infected lymphoid tissue in the recently developed lethal ferret model of CDV infection (von Messling *et al.*, 2003). Another group added two different variants of eGFP as an ATU between the P and M genes of RPV. One variant expressed GFP as a cytoplasmic protein, while the other variant of eGFP had an N-terminal signal sequence to direct its secretion into the

extracellular space (Walsh *et al.*, 2000a). The authors of this study hoped that the RPV with the secretory eGFP would be useful as a marker vaccine, since animals vaccinated with this virus would also generate antibodies against eGFP. SV5 has been engineered with eGFP as an ATU between the HN and L genes (He *et al.*, 1997). A recombinant NDV based on the Clone-30 vaccine strain was also developed to include an eGFP ATU between the F and HN genes (Engel-Herbert *et al.*, 2003). In most cases, the extra eGFP gene is maintained in the recombinant viruses over 10-20 passages regardless of genomic location, indicating that paramyxovirus replication is fairly plastic and that the genome is able to tolerate a fairly large amount of extra genetic material. While a number of viruses have been modified to include eGFP as an extra gene, it is important to note that other reporter proteins with similar utility to eGFP can also be used in recombinant virus systems. In similar applications of this approach, one group developed a variant of NDV that expressed another reporter protein, chloramphenical acetyltransferase (CAT), as an ATU between the HN and L genes (Krishnamurthy *et al.*, 2000), while several other groups have developed luciferase-expressing viruses. One used SeV and included firefly luciferase as an ATU before the N gene (Hasan *et al.*, 1997), and the other used CDV and included luciferase as an ATU between the P and M genes (Parks *et al.*, 2002).

While the inclusion of eGFP and other reporter genes in viral genomes is useful for tracking virus infections and spread in a variety of tissues, the real utility of reverse genetics lies in the ability to knock out certain viral genes or introduce foreign or chimeric genes into fully infectious viral particles. NDV has been used to express NDV-APMV-4 HN chimeric glycoproteins for the development of marker vaccines for poultry (Peeters *et al.*, 2001). NDV has also been used to express an additional HPIV-3 HN protein for vaccination in humans (Bukreyev *et al.*, 2005). With the emergence of avian influenza and the on-going presence of other avian diseases, NDV has gained recognition as a vaccine vector by carrying some of the more immunogenic proteins from avian influenza viruses. Two groups have independently developed chimeric NDVs, one that used the backbone of the NDV lentogenic Clone-30 strain and expressed the hemagglutinin (HA) from the H5N2 strain of avian influenza (Veits *et al.*, 2006), while the other used the NDV backbone and expressed the HA from the H7N7 strain of avian influenza (Park *et al.*, 2006). The H7 in one of the chimeric viruses was also modified by removal of its cytoplasmic tail and transmembrane domain, and replacement with those of NDV F (Park *et al.*, 2006). Both of these approaches were able to induce immunity in birds against either H5N2 or H7N7 avian influenza virus as well as NDV.

MeV has been modified to delete the F and H glycoprotein genes, which were replaced with the G glycoprotein from VSV (Spielhofer *et al.*, 1998). The VSV G protein was also modified in another recombinant chimeric virus with its cytoplasmic tail substituted with that of the MeV F protein to aid in incorporation into virus particles (Spielhofer *et al.*, 1998). MeV have also been generated which lack the M gene, which has proven useful in the study of MeV persistence in the brain and in addressing questions of MeV virion assembly (Cathomen *et al.*, 1998a). In order to study the differences in receptor usage between wild-type and vaccine strains of MeV, recombinant chimeric MeV have been generated with vaccine viruses expressing wild-type glycoproteins or wild-type viruses expression vaccine strain glycoproteins, or a combination of both (Johnston *et al.*, 1999; Takeda *et al.*, 1999). These viruses have proven useful in determining that the MeV H glycoprotein is one of the major determinants of disease in MeV infection. Other studies, performed with the wild-type strain 5804Han89 and the vaccine strain Onderstepoort of CDV, have also shown similar behaviour for the H protein of CDV (von Messling *et al.*, 2001). Recombinant bovine parainfluenza virus type 3 (BPIV-3) has also been used to carry various severe acute respiratory syndrome-associated coronavirus (SARS-CoV) proteins in several vaccination studies (Buchholz *et al.*, 2004; Bukreyev *et al.*, 2004). Recombinant HPIV-3 has also been used in studies to test the

immunogenicity of combinations of the Ebola virus nucleoprotein (NP) and glycoprotein (GP) (Bukreyev *et al.*, 2006). In a somewhat different approach, MeV has also been engineered to express potential immunotherapeutic proteins such as the p35 and p40 subunits (separated by an internal ribosome entry site [IRES] element) of human interleukin (IL)-12 from a single transcript between the H and L genes of MeV (Singh, 1999). Morbillivirus genomes have also been explored as epitope delivery systems for vaccine platforms. The $3D^{pol}$ protein of foot-and-mouth-disease virus (FMDV) was inserted as an ATU between the P and M genes of RPV (Baron *et al.*, 1999). The resulting virus was slightly attenuated, but conferred no protection against FMDV challenge in vaccinated animals. However, a chimeric RPV with the F and H glycoproteins of PPRV substituted for those of RPV was able to elicit protection in vaccinated animals upon challenge with PPRV, so the generation of chimeric viruses has some merit in this regard (Das *et al.*, 2000). Unfortunately, foreign genes are not always incorporated into viral particles, as was observed when the HA of an H3N2 strain of influenza virus was inserted into RPV between the P and M genes (Walsh *et al.*, 2000b). This virus, though, was still able to elicit protective immunity in vaccinated animals.

Infectious paramyxovirus virions are assembled at the plasma membrane, and require the participation of a number of different proteins during this

process. For paramyxoviruses, the M protein is thought to largely coordinate the steps of viral assembly and direct viral proteins to assembly sites (Pantua *et al.*, 2006). During the maturation processes of the fusion and attachment glycoproteins, the matrix proteins of several paramyxoviruses are known to bind to the glycoproteins via their cytoplasmic tails (Sanderson *et al.*, 1993; Sanderson *et al.*, 1994; Ali & Nayak, 2000). In some virus species, matrix protein expression can also drive the formation of viral particles, as demonstrated by experiments where VLPs have been generated by expression of the matrix protein. It must be noted that in order for proper particle formation to occur, other viral proteins, such as surface glycoproteins, must often be co-expressed for particle formation to occur. Recent studies in which NDV VLPs were generated have sought to elucidate which proteins are required for budding (Pantua *et al.*, 2006). VLPs composed of the NDV NP, M protein, F protein, and HN protein were released with efficiencies equal to that of authentic virus and had comparable particle buoyant densities. Expression of M alone also resulted in VLP formation, but not when NP, F, or HN were expressed alone. Interestingly, this group also found that NP and HN were incorporated into nascent particles due to interactions with M, but that F was incorporated through indirect interactions with HN, and possibly NP. It has recently been found that NiV M drives the budding of NiV VLPs, both with and without the co-expression of NiV F and G (Ciancanelli &

Basler, 2006) and that NiV M has a central role in the release of VLPs (Patch *et al.*, 2007). In any case, the paramyxovirus matrix protein seems to direct the internal and surface components of virus particles to the appropriate cellular locations for assembly of progeny virions.

AIMS OF THE THESIS

The attachment glycoproteins of paramyxoviruses have a number of different functions in the viral life cycle. Among these proposed functions are attachment to the viral receptor, triggering of fusion or fusion promotion between the viral and host cell membranes, homooligomerization of attachment protein units, and interaction with the matrix protein in the course of virus assembly and budding. The attachment glycoprotein is also the location of important neutralizing epitopes during an immune response. With this in mind, the research projects were designed to attempt to answer the following questions:

1. What are the fusion requirements of the NiV glycoproteins?
2. Does cellular expression of NiV G render these cells resistant to infection by NiV (and possibly HeV)?
3. Does cellular expression of NiV G result in cell surface down-regulation of the *Henipavirus* receptors ephrin-B2 and ephrin-B3?
4. Are chimeric glycoproteins derived from NiV G and CDV H correctly expressed and transported to the cells surface?

5. Are chimeric glycoproteins able to induce fusion when co-expressed with NiV F or CDV F?
6. Are chimeric glycoproteins able to down-regulate the cell surface expression of ephrin-B2 and ephrin-B3?
7. Are chimeric viruses (rCDV eGFPH NiVFG and rCDV eGFPH NiVMFG) correctly assembled and released from infected cells?
8. Is rCDV eGFPH NiVFG able to induce cell surface down-regulation of ephrin-B2 and ephrin-B3?

MATERIALS & METHODS

Cloning of the NiV glycoproteins

The NiV F and G ORFs were initially cloned into the pBK-CMV eukaryotic expression vector (Stratagene). Since our previous attempts to express NiV F and NiV G had failed thus far, the NiV glycoprotein ORFs were excised from pBK-CMV using *Nhe* I and *Hin*d III (New England Biolabs) and cloned into one of two retroviral vectors, pczCFG5 IEGZ (Lindemann *et al.*, 2001) or pHITBE. For insertion into pczCFG5 IEGZ, pczCFG5 IEGZ was digested with *Swa* I (New England Biolabs) and dephosphorylated with calf intestinal alkaline phosphatase. pBK-CMV-NiV F and pBK-CMV-NiV G were digested with *Nhe* I and *Hin*d III (New England Biolabs), filled in with Klenow fragment (Roche), and the gel-purified fragments were ligated to *Swa* I-digested pczCFG5 IEGZ using T4 DNA ligase (New England Biolabs) to give the plasmids pczCFG5-NiV F and pczCFG5-NiV G. For insertion into pHITBE, pHITBE was digested with *Eco*R I (New England Biolabs), filled in with Klenow fragment (Roche), and then digested with *Hin*d III (New England Biolabs). NiV F and NiV G were excised from pBK-CMV first by digestion with *Nhe* I (New England Biolabs), followed by

Klenow fragment (Roche) fill-in, digestion with *Hin*d III (New England Biolabs), gel purification, and finally ligated to the *Nhe* I- and *Hin*d III-digested pHITBE using T4 DNA ligase (New England Biolabs). All restriction digestion reactions were incubated at 37˚C, except *Swa* I, which was incubated at 25˚C. T4 DNA ligase reactions were incubated at either 16˚C or 4˚C overnight.

The orientation of NiV F in pczCFG5-NiV F was verified by digestion with *Xho* I and *Eco*R V (New England Biolabs). NiV G orientation in pczCFG5-NiV G was verified by digestion with *Sal* I and *Eco*N I (New England Biolabs). The presence of NiV F and NiV G in pHITBE-NiV F and pHITBE-NiV G was verified by digestion with *Kpn* I and *Sac* I (New England Biolabs). Expected fragment sizes generated by restriction digestion of the NiV F and NiV G expression plasmids are shown in Table 6.

Expression of the NiV glycoproteins

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Oakville, Ontario, Canada) and supplemented with 10% fetal bovine serum (FBS) (Wisent, St. Bruno, Québec, Canada) that had been heat-inactivated for 1 hour at 56˚C. 293T cells were selected because of their high transfectibility. Cells were grown at 37˚C in a humidified atmosphere containing 5% CO_2. Plasmids were transfected into 293T cells in order to assess their

Table 6: Expected fragment sizes for verification of NiV glycoprotein ORF insertion

Vector	Enzyme	NiV F forward	NiV F reverse	Enzyme	NiV G forward	NiV G reverse
pczCFG5 IEGZ	*Xho* I	2962 bp 7402 bp	3508 bp 6856 bp	*Eco*R V	34 bp 2188 bp 2648 bp 5660 bp	34 bp 2188 bp 3652 bp 4656 bp
	Sal I	75 bp 2184 bp 8105 bp	75 bp 3302 bp 6987 bp	*Eco*N I	1510 bp 9020 bp	1121 bp 9409 bp
pHITBE	*Kpn* I	1459 bp 2905 bp 5290 bp		*Kpn* I	1422 bp 1459 bp 1632 bp 5290 bp	
	Sac I	4361 bp 5293 bp		*Sac* I	4527 bp 5293 bp	

functional expression (induction of cell-cell fusion), since we had no effective antibodies at the time. 4 μg each of pczCFG5-NiV F, pczCFG5-NiV G, pHITBE-NiV F, and pHITBE-NiV G were transfected into 293T cells alone or in combination in six-well dishes using 5 μL of Lipofectamine 2000 (Invitrogen) per well in a total volume of 1 mL of OptiMEM (Gibco, Brampton, Ontario, Canada). Cells were incubated overnight at 37˚C and were examined the next day for syncytia.

Production of polyclonal NiV immune serum in guinea pigs

In order to develop antibodies to detect NiV proteins, two female guinea pigs (Hartley, 500 g, Charles River, Québec) were inoculated intraperitoneally with 10^5 plaque-forming units (PFU) of NiV per guinea pig. The guinea pigs were boosted intraperitoneally with a further 10^5 PFU per guinea pig at 14 days post-inoculation, and were terminally bled at 28 days post-inoculation. Guinea pigs were anesthetized prior to inoculation, bleeding, or exsanguination by intramuscular administration of xylozyne (5 mg/kg) and ketamine (40 mg/kg). The generation of the swine antisera against NiV G has been described previously (Weingartl *et al.*, 2006). All animal work was performed under biosafety level 4 (BSL4) conditions and according to Canadian Council of Animal Care guidelines.

Western blots

For detection by Western blot, transfected cells were incubated for 24 or 48 hours in six-well dishes, washed in PBS, and resuspended by scraping or pipetting. The cells were centrifuged for 5 minutes at 500x*g* and the supernatant was discarded. Cell pellets were lysed in 1X SDS gel loading buffer and sheared successively through 18-, 20-, 22-, and 25-gauge needles to break up genomic DNA. For Western blots, cells were lysed in 1X SDS gel loading buffer (50 mM Tris-HCl pH 7.5, 1% SDS, 8.75% glycerol, and 0.125% bromophenol blue) with 4% β-mercaptoethanol (BME; Fisher). Lysates were boiled for 5 minutes before being run on 10% resolving SDS polyacrylamide gels. Protein gels were transferred to polyvinylidene difluoride (PVDF) (Amersham, Baie d'Urfe, Québec, Canada) membranes using the Mini-PROTEAN 3 Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Mississauga, Ontario, Canada) overnight at 30 volts in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3). Membranes were blocked for at least 1 hour at room temperature in blocking buffer (5% skim milk and 0.1% Tween-20 in PBS). Membranes were washed 3 times for 5 minutes in PBS+0.1% Tween-20 (PBS-T) and then probed with a guinea pig anti-NiV immune serum or swine anti-NiV G serum as the primary antibody; NiV antisera were diluted 1:1,000 in blocking buffer containing 1% normal rabbit serum (Sigma, Oakville, Ontario, Canada). Primary

antibody incubation was performed at room temperature for 2 hours on a rocker. Membranes were washed 3 times for 10 minutes each in PBS-T. A rabbit anti-guinea pig horseradish peroxidase (HRP) conjugated antibody (Sigma, Oakville, Ontario, Canada) was used as the secondary antibody at a dilution of 1:10,000 in blocking buffer. Secondary antibody incubation was performed at room temperature for 1 hour, followed by washing with PBS-T 3 times for 10 minutes. Blots were developed using the ECL$^+$ kit (Amersham, Baie d'Urfe, Québec, Canada) and exposed to Hyperfilm (Amersham, Baie d'Urfe, Québec, Canada) to visualize bands.

Production of retroviral particles and generation of transgenic cell lines expressing the NiV glycoproteins

Retroviral particles for transductions were produced by adaptation of previously described methods (Soneoka *et al.*, 1995). The pczCFG5 IEGZ vector contains a replication-deficient retroviral genome with a Ψ packaging sequence from Moloney murine leukemia virus. A schematic is shown of how the expression cassette in the retroviral vector is transcribed when supplied by transfection (Fig. 9A) and when transgenic cells are made by transduction (Fig. 9B). Plasmids (4 μg each of pczCFG5 IEGZ, pczCFG5-NiV F, or pczCFG5-NiV G) were co-transfected into 293T cells with separate plasmids encoding VSV G and

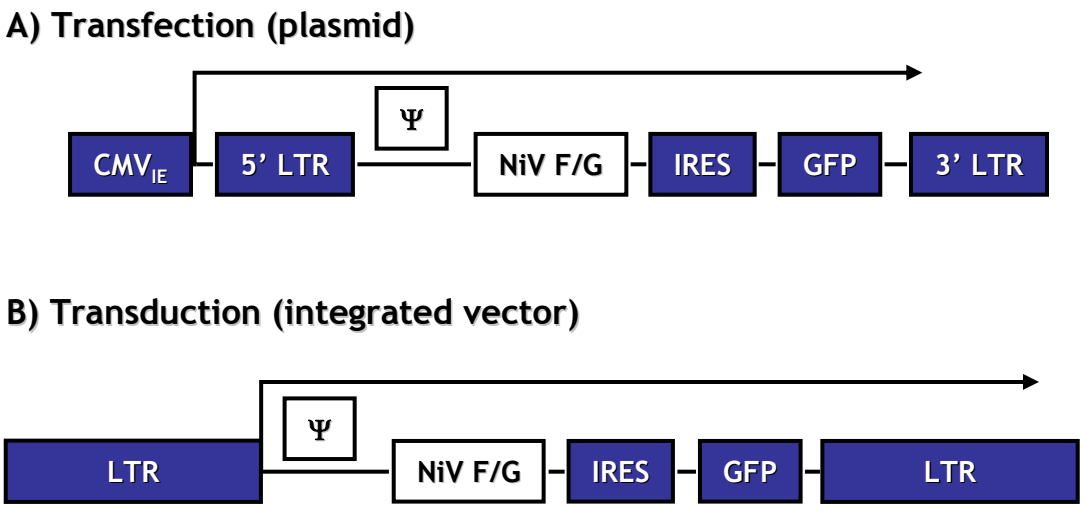

Figure 9: Schematic representation of transcription from the retroviral expression vectors. A) When transfected into cells, transcription is driven from a CMV_{IE} promoter, although some transcription is driven by the 5′ long terminal repeat (LTR) in the vector. B) When retroviral particles are produced and used to transduce target cells, the 5′ and 3′ LTRs of the retroviral vector are duplicated at each end of the genome. In this proviral form of the genome, transcription is driven exclusively by the LTR.

murine retroviral gag-pol ORFs. After transfection, the newly transcribed plasmid genomes are packaged by the gag and pol protein products and viral particles are pseudotyped with VSV G. VSV G mediates binding and entry via a phospholipid receptor that is present on many mammalian cells. Retroviral particles were either used in transductions immediately or else were harvested and stored at -70˚C until further use. Target CRFK cell monolayers were transduced with undiluted retroviral particles. CRFK cells were selected because they are easily infected by NiV and HeV with a very clear and fast cytopathic effect (CPE). Transgenic cells resulting from the transduction of CRFK cells with pczCFG5 IEGZ, pcz-CFG5-NiV F, or pczCFG5-NiV G particles were designated CRFK-pcz, CRFK-NiV F, and CRFK-NiV G, respectively. Untransduced CRFK cells were referred to as CRFK wt. Monolayers were transduced twice at 16-24 hour intervals with retroviral particles to ensure high levels of integrated provirus. The pczCFG5 IEGZ vector also contains a GFP-Zeocin resistance fusion gene. Transgenic cells were selected for this drug marker by treatment with Zeocin (Invitrogen, Burlington, Ontario, Canada) at 400 μg/mL for 2 weeks.

Exposure of transgenic cells to NiV and HeV

NiV and HeV virus stocks (approximately 10^6 tissue culture infectious dose 50% [$TCID_{50}$]/mL) were diluted in serum-free OptiMEM (Gibco,

Burlington, Ontario, Canada) and virus was added to transgenic CRFK cells in 24-well dishes in 10-fold serial dilutions corresponding to $5x10^5$ $TCID_{50}$/well to 5 $TCID_{50}$/well. Virus was adsorbed at 37˚C for 1 hour, then the inoculum was removed and replaced with fresh DMEM containing 2% FBS. Cells were monitored daily for CPE over the course of five days, after which the medium was removed and the cells were fixed in PBS-buffered 3.7% formaldehyde overnight at 4˚C. The following day, the fixative was removed and the plates were sealed in plastic bags with fresh PBS-buffered 3.7% formaldehyde and dunked out of BSL4. The plates were left at 4˚C overnight, after which time the bags were opened, and the plates were washed with water. 500 µL of water was added to each well and the cells were photographed with a Zeiss Axiovert inverted light microscope.

RT-PCR for viral nucleic acid in NiV- and HeV-exposed transgenic cells

CRFK wt, CRFK-pcz, CRFK-NiV F, and CRFK-NiV G cells were seeded into two 24-well dishes. The cells were exposed to HeV and NiV as described above in dilutions from $5x10^4$ $TCID_{50}$ to 5 $TCID_{50}$ per well. At 5 days post-exposure, surviving cells were trypsinized and half were taken for RNA extraction using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada). Extracted RNA from each sample was first subjected to first-strand cDNA

synthesis using the SensiScript Reverse Transcriptase kit (Qiagen) and a reverse transcription primer (designated RT). The resulting cDNA was amplified using the Master Mix PCR kit (Qiagen). Primer sets used for the detection of HeV nucleic acid are shown in Table 7, and those used for the detection of NiV nucleic acid are shown in Table 8. Primers were designed to target HeV and NiV (+)-sense mRNA from the N, M, and G genes, and (-)-sense viral genomic RNA (vRNA) at the N/P, M/F, and F/G gene junctions. RTase reaction conditions are shown in Table 9. PCR reaction conditions are shown in Table 10 and cycling parameters are shown in Table 11.

RNA extracts from all CRFK cells and CRFK-derived cells were verified with an internal mRNA control using primers for feline glyceraldehyde 3-phosphate dehydrogenase mRNA (fGAPDH; GenBank accession number AB038241), fGAPDH1 fwd (5'-TTCCACGGCACAGTCAAGGCTGAGA-3') and fGAPDH1 rev (5'-GGTGCAGGAGGCATTGCTGACAATC-3'). Amplification of mRNA in all samples gave the expected 294-bp RT-PCR product (see Fig. 19, bottom panel). Feline glyceraldehyde-3-phosphate dehydrogenase (fGAPDH) RT-PCR conditions are shown in Table 12 and cycling parameters are shown in Table 13. All PCR reactions were performed using either a Biometra T1 or a T-Gradient Thermoblock (Montreal Biotech Inc.)

Table 7: Primer sets used for two-step RT-PCR detection of HeV nucleic acid

Primer name	Sequence (5'→3')	Target region
HeV 623 RT rev	GGGCTTCGATGGTAATGACGGC	
HeV N 159 fwd	CGAAACTCGGTCGAGATGGGCGG	HeV N mRNA
HeV N 556 rev	GCTATCTACAAACGGGGTCTTTCCC	
HeV 5656 RT rev	CCTTGGGATCATGTAGATGCCGG	
HeV M 5263 fwd	CAACTACATGTACATGATTTGCTATGG	HeV M mRNA
HeV M 5579 rev	CCAGTTGAATCTGATCCACATTGCGGC	
HeV 9444 RT rev	CCCCTTGTGAGATTGGTCGGTATTCTCTG	
HeV G 8865 fwd	TCAAGAGCCTGTCTCAACTATCAAG	HeV G mRNA
HeV G 9265 rev	ATGGTGCTGGATGTGTCAATTAGTG	
HeV 1999 RT fwd	GTAGTAAGTCTCACTGGTGATGGG	
HeV N 2074 fwd	GCTGAATAGGCTATCTACTATGCACTGG	HeV N/P vRNA
HeV P 2497 rev	CTTGGTTGTTGGATGCTTGATCGTCCG	
HeV 6118 RT fwd	CACAGGGAAGATCCTAAAGGGGTG	
HeV M 6227 fwd	CAGTAAGTTACACAGGTACAATACA	HeV M/F vRNA
HeV F 6477 rev	CAGACCAGTTGATTGACTATATCTA	
HeV 8546 RT fwd	GTACCTATTTGAATCAATCGACATTGG	
HeV F 8559 fwd	TCAATCGACATTGGATTCTCTATTC	HeV F/G vRNA
HeV G 8856 rev	ATGACTTGCGAGTATTAGCTCTTAT	

Primer sets were designed for two-stage amplification of HeV N, M, and G mRNA, or sections of the intergenic regions between HeV N and P, M and F, and F and G. The numbering in the primer names denotes where the first nucleotide of the primer binds to the HeV genome. Primers named "RT" for "reverse transcription" (e.g. HeV 623 RT rev) were used for first-strand cDNA synthesis, while the other two primers in the set (e.g. HeV N 159 fwd and HeV N 556 rev) were used to amplify the specific HeV nucleic acid segment. RT primers with the designation "fwd" were designed to bind to the (-)-sense strand of the HeV genome, while RT primers designated "rev" were designed to bind to the (+)-sense strand of either the HeV antigenome or mRNA.

Table 8: Primer sets used for two-step RT-PCR detection of NiV nucleic acid

Primer name	Sequence (5'→3')	Target region
NiV 534 RT rev	CCCTTGCTGCTGTCTCGAGC	
NiV N 115 fwd	GAGTGATATCTTTGAAGAGGCGGC	NiV N mRNA
NiV N 501 rev	CTCCTCTACCTTCCGAACTACTC	
NiV 5716 RT rev	GGCAATGGCATTGTTTCTCCTG	
NiV M 5405 fwd	GCCTCTCATCCCCAAGATCTTCTGGAG	NiV M mRNA
NiV M 5641 rev	GATACTGACAAAAAATATTCTCAGAGC	
NiV 9850 RT rev	GGGTCTCCAACAGTTGACACTGCAC	
NiV G 9273 fwd	CTGATTGACACATCCAGTACCATTA	NiV G mRNA
NiV G 9709 rev	AGTACCTCTCCAACTCCTATTATTC	
NiV 2097 RT fwd	CTAAGCTACTGTCTTTGCACTGG	
NiV N 2182 fwd	CTTAACTTGACCAAGGTCTACCAGG	NiV N/P vRNA
NiV P 2561 rev	GGTGCACTGCAGAAAATCTTCCCAGGC	
NiV 6069 RT fwd	GCCGAGTAGCAGCTGTGTTGCAGCC	
NiV M 6137 fwd	GACAATACAGGGAGAATTCTAAAGGG	NiV M/F vRNA
NiV F 6600 rev	CACTTCTTGAAATGAATCTTCCGATGG	
NiV 8499 RT fwd	CTCTCACAGGAGCGCTAACCTATACAC	
NiV F 8585 fwd	GGATTATGATATAGTTTCATACTACAATAGC	NiV F/G vRNA
NiV G 8797 rev	CTCCAAAAAACGATTCAATCTCTCC	

NiV RT-PCR primer sets were designed and named as described for the HeV RT-PCR primers in Table 7.

Table 9: Buffer components for the RTase reactions for the generation of cDNA

Component	Amount
10X Buffer RT	2 µL
5 mM dNTPs	2 µL
RT primer (10 µM)	2 µL
RTase	1 µL
Template RNA	1 µL
H_2O	12 µL
Total	**20 µL**

Reverse transcription reactions were incubated for 1 hour at 37˚C to generate cDNA. The RTase in the reactions was then inactivated by incubation for 5 min at 93˚C.

Table 10: Buffer components for the amplification of NiV and HeV nucleic acid from cDNA templates

vRNA & mRNA PCR	
2X Master Mix	25 µL
fwd primer (10 µM)	3 µL
rev primer (10 µM)	3 µL
Template cDNA	1 µL
H_2O	18 µL
Total	**50 µL**

PCR reactions were performed with the Qiagen Taq PCR MasterMix kit using 1 µL of cDNA generated by a separate RTase reaction.

Table 11: Cycling parameter for the amplification of viral nucleic acid by PCR

vRNA & mRNA PCR parameters		
95˚C	5 min	
95˚C	1 min	35X
55˚C	1 min	
72˚C	1 min	
72˚C	7 min	
4˚C	hold	

Table 12: Reaction components for the amplification of fGAPDH mRNA

fGAPDH PCR reactions	
Component	**Amount**
5X buffer	10 µL
10 mM dNTPs	2 µL
fGAPDH fwd (10 µM)	3 µL
fGAPDH fwd (10 µM)	3 µL
Enzyme mix	2 µL
Template RNA	1 µL
H_2O	29 µL
Total	**50 µL**

Components for feline GAPDH (fGAPDH) RT-PCR reactions. All fGAPDH RT-PCR reactions were performed with the Qiagen One-Step RT-PCR kit.

Table 13: Cycling parameter for the amplification of fGAPDH mRNA

fGAPDH RT-PCR reaction parameters		
50˚C	30 min	
95˚C	15 min	
95˚C	1 min	35X
50˚C	1 min/30 sec	
72˚C	1 min	
72˚C	10 min	
4˚C	hold	

Fluorescent fusion inhibition assay

The expression plasmids used in all experiments up to this point contain a GFP ORF whose expression is driven by an IRES element downstream of the NiV glycoprotein ORF (Fig. 9A). For the fluorescent fusion inhibition assay, non-fluorescent versions of the plasmids pHITBE-NiV F and pHITBE-NiV G were developed. Briefly, the GFP ORF was excised by digestion with *Age* I and *Not* I (New England Biolabs) for 2 hours at 37˚C, yielding fragments for pHITBE-NiV F of ~700 bp and ~7850 bp, and fragments for pHITBE-NiV G of ~700 bp and ~7900 bp. The vector backbone (larger fragment) containing the NiV glycoprotein ORF was gel extracted, filled in with Klenow fragment (New England Biolabs) at 37˚C for 30 minutes, and then ligated back together with T4 DNA ligase to give the plasmids pHITΔGFP-NiV F and pHITΔGFP-NiV G.

293T cells were seeded into 6-well dishes. When the cells were approximately 80-90% confluent, they were transfected with 4 µg each of either pczCFG5 IEGZ, pczCFG5-NiV F, pczCFG5-NiV G, or pHITΔGFP-NiV F+pHITΔGFP-NiV G. After 8 hours, the cells were trypsinized and the pczCFG5 IEGZ-, pczCFG5-NiV F-, and pczCFG5-NiV G-transfected cells were mixed separately in a 1:1 ratio with pHITΔGFP-NiV F+pHITΔGFP-NiV G transfected cells in a fresh 12-well dish. The cells were incubated overnight, fixed with 3.7% PBS-buffered formaldehyde, and then examined for green fluorescent syncytia.

The presence of fluorescent syncytia indicates that cells are participating in fusion, while the absence of fluorescent syncytia indicates that cells are not participating in fusion.

FACS analysis of cell surface ephrin-B2 and ephrin-B3 expression

In order to assess the effect of NiV glycoprotein expression of cell surface ephrin-B2 and ephrin-B3, cell surface staining was performed and stained cells were analyzed by fluorescence-activated cell sorting (FACS) on a FACSCalibur flow cytometer (BD, California). Rabbit polyclonal antibodies against human ephrin-B1, human ephrin-B2, and human ephrin-B3 were purchased either from Genex Biosciences (Hayward, California) or Santa Cruz Biosciences (Santa Cruz, California). Antibodies from Genex Biosciences were diluted in Mg^{2+}/Ca^{2+}-free PBS (Gibco) to a concentration of 1 mg/mL, and then used as described below. Polyclonal goat anti-rabbit R-PE conjugate was purchased from Jackson ImmunoResearch (West Grove, Pennsylvania). Mock and transfected cells were removed from 75 cm^2 flasks using 10 mL/flask of Versene (Invitrogen) and resuspended into a single-cell suspension by pipetting. The resuspended cells were fixed with an equal volume of PBS-buffered 4% PFA overnight at 4˚C. The cells were centrifuged the following day for 5 minutes at 500x*g*, the supernatant was discarded, and the cells were resuspended in 5 mL of fresh Mg^{2+}/Ca^{2+}-free

PBS (Gibco). For FACS staining, $2.5x10^5$ cells/tube were blocked with 10 μL of human γ-globulin (Sigma) for 10 minutes at room temperature. Primary antibody diluted in Mg^{2+}/Ca^{2+}-free PBS (100 μL/tube) was added to cells, mixed, and incubated at 4°C for 30 minutes. Cells were washed twice with Mg^{2+}/Ca^{2+}-free PBS (1 mL/tube) with centrifuging for 5 minutes at 500x*g* between each wash. Secondary antibody diluted in Mg^{2+}/Ca^{2+}-free PBS was added to cell pellets, mixed, and incubated for 30 minutes at 4˚C, followed by two washes with Mg^{2+}/Ca^{2+}-free PBS (1 mL/tube). Final cell pellets were resuspended in 1 mL of Mg^{2+}/Ca^{2+}-free PBS and then fixed overnight at 4˚C with an equal volume of 4% PBS-buffered PFA. Primary antibody dilutions were generally 1:100, while secondary antibody dilutions ranged from 1:100 to 1:400.

Construction and cloning of NiV G and CDV H chimeric attachment glycoprotein genes

The NiV G and CDV H ORFs were aligned and divided into four sections: Cytoplasmic tail, transmembrane domain, and stalk (CTS); β-propeller blades 1 and 2; β-propeller blades 3 and 4; and β-propeller blades 5 and 6 (Fig. 10). Six different chimeras were constructed, three having the N-terminal region of NiV G and three having the N-terminal region of CDV H. For chimeras with the N-terminal region of NiV G, the segments were designed such that the NiV G CTS

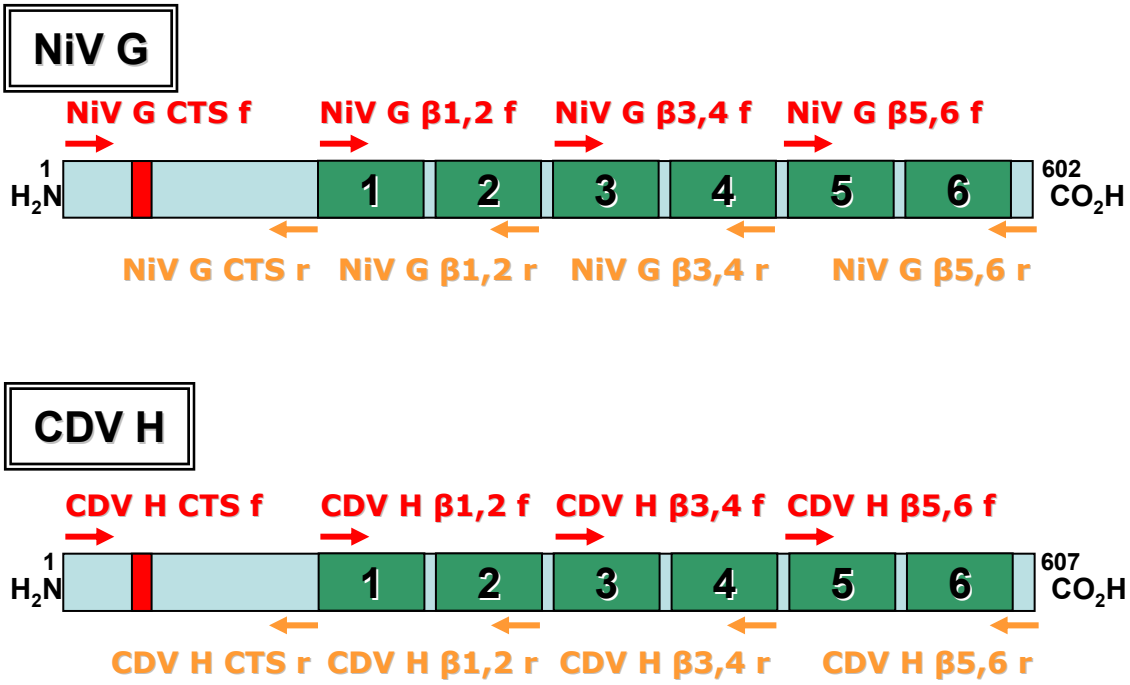

Figure 10: Strategy for the construction of chimeric CDV H-NiV G glycoproteins. See text for details on PCR amplification and assembly.

domain would be joined to the CDV H β-propeller blades 1-6, the NiV G CTS and β-propeller blades 1 and 2 joined to CDV H β-propeller blades 3-6, and NiV G CTS and β-propeller blades 1-4 joined with CDV H β-propeller blades 5 and 6. For chimeras with the N-terminal region of CDV H, the segments were designed such that the CDV H CTS domain would be joined to the NiV G β-propeller blades 1-6, the CDV H CTS and β-propeller blades 1 and 2 joined to NiV G β-propeller blades 3-6, and CDV H CTS and β-propeller blades 1-4 joined with NiV G β-propeller blades 5 and 6. The common 5' primer was designed with an *Asi*S I site immediately flanking the ORF and the common 3' primer was designed with a *Sac* II site immediately flanking the ORF. The primers used to amplify each segment are shown in Table 14. Each segment was individually amplified by PCR with *Pfu* DNA polymerase (Stratagene or Fermentas) and PCR purified using the QIAquick PCR purification kit (Qiagen). Segment PCR cycling parameters are shown in Table 15, and the reaction conditions are shown in Table 16. To facilitate assembly of the individual chimera PCR segments, the N-terminal amplicon was engineered to have a 25-bp region at its 3' end that is complementary to the 5' end of the C-terminal amplicon. Purified PCR segments were mixed in a new PCR reaction (the amounts of each segment are shown in Table 17), initially without primers. After primerless extension, the 5' and 3' common primers for each chimera set were added to the reaction and the

Table 14: Primers for the generation of chimeric glycoprotein clones

Primer name	Sequence (5'->3')
NiV G CTS *Asi*S I fwd	GATCGCGATCGCATGCCGGCAGAAACAAGAAAGTTAGATTCG
NiV G β1,2 fwd	TGCAAATTCACACTGCCTCCCTTG
NiV G β3,4 fwd	AGTATCGAGAAAGGGAGGTATGATAAAG
NiV G β5,6 fwd	TGTCCAGAGATCTGCTGGGAAGGAG
NiV G CTS rev	AGTAATTAGTAAAATTCACCTTGATTTTTTCATTCACATTCTCATTTATACTTGC
NiV G β1,2 rev	TTGTTATATGTATTTTTTCTACTGATCGTAGGGCAAGTTGATGTTGATTGTAACCC
NiV G β3,4 rev	CATCTTTATCCATAATCTGGGATGTTGTATTGAATCTAGGGCATTGTGATTGCCC
NiV G β5,6 *Sac* II rev	GATCCCGCGGTATTTAGCTTTTTATGTACATTGCTCTGG
CDV H CTS *Asi*S I fwd	GATCGCGATCGCATGCTCTCCTACCAAGACAAGGTGGGTGCC
CDV H β1,2 fwd	ATCAAGGTGAATTTTACTAATTACTGCG
CDV H β3,4 fwd	TCAGTAGAAAAAATACATATAACAAATCACCG
CDV H β5,6 fwd	ACATCCCAGATTATGGATAAAGATG
CDV H CTS rev	TCAAGGGAGGCAGTGTGAATTTGCACTTACTAGGTGGATTAATGCACCAGTGG
CDV H β1,2 rev	TATCATACCTCCCTTTCTCGATACTTGGGTGAGCGACAGGTATCACTTCTTCAAC
CDV H β3,4 rev	CTCCTTCCCAGCAGATCTCTGGACATTGAATAGGTAAATAACAATTTCCACTTGATT CCC
CDV H β5,6 *Sac* II rev	GATCCCGCGGATTTCAAGGTTTTGAACGGTTACATGAG

See text for details on primer naming and design.

Table 15: PCR cycling parameters for the amplification of chimeric glycoprotein segments and joining reactions

Segment PCR cycling parameters			Joining PCR cycling parameters		
94˚C	2 min		94˚C	2 min	
94˚C	10 sec	26X	40˚C	3 min	
52˚C*†	30 sec		68˚C	2 min 30 sec	
72˚C	2 min‡		94˚C	10 sec	26X
72˚C	7 min		54˚C	30 sec	
4˚C	hold		68˚C	2 min 30 sec	
			68˚C	7 min	
			4˚C	hold	

* - All annealing temperatures used a touchdown (dT) of -0.2˚C/cycle.
† - For PCR segment #1, an annealing temperature of 55˚C was used. For PCR segment #5, an annealing temperature of 60˚C was used.
‡ - For PCR segment #1, an extension time of 1 min 30 sec was used. For PCR segment #5, an extension time of 3 min was used.

Table 16: Reaction components for the amplification of chimeric glycoprotein segments

Chimeric glycoprotein segment PCR reactions			
	Segments 2-4, 6-12	**Segment 1**	**Segment 5**
10X buffer	5 µL	5 µL	5 µL
2.5 mM dNTPs	4 µL	4 µL	4 µL
Pfu polymerase	0.5 µL	0.5 µL	0.5 µL*
25 mM $MgSO_4$	–	2 µL	2 µL
		H2O to a final volume of 50 µL	

* - For amplification of chimera segment #5, a mix of 0.5 µL each of *Pfu* polymerase (Fermentas) and Expand HiFidelty polymerase (Roche) was used.
For all PCR reactions, a small amount of template was added and primers were during the initial 94˚C denaturation step (hot-start method).

Table 17: Reaction components for the amplification of full-length chimeric glycoprotein ORFs

Chimera joining PCR reactions				
	5' segment	**Amount**	**3' segment**	**Amount**
G_{145}/H_{463}	#1	1 µL	#2	2 µL
G_{338}/H_{268}	#3	1 µL	#4	2 µL
G_{498}/H_{113}	#5	1 µL	#6	1 µL
H_{145}/G_{458}	#7	1 µL	#8	1 µL
H_{340}/G_{265}	#9	1 µL	#10	1 µL
H_{495}/G_{105}	#11	2 µL	#12	1 µL

Chimera joining PCR reactions were run as described in Table N. For chimeras G_{145}/H_{463}, G_{338}/H_{268}, and G_{498}/H_{113}, the 5' primer was NiV G CTS *Asi*S I fwd and the 3' primer was CDV H β5,6 *Sac* II rev. For chimeras H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105}, the 5' primer was CDV H CTS *Asi*S I fwd and the 3' primer was NiV G β5,6 *Sac* II rev.

chimera ORFs were amplified. Joining PCR cycling parameters are shown in Table 15. The chimera amplicons were cloned into the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's instructions. Colonies were picked and mini-prepped with the QIAprep Spin Mini-Prep Kit (Qiagen) and were then sequenced. Mutation-free clones of all chimeras (G_{145}/H_{463}, G_{338}/H_{268}, H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105}) were obtained, except for clones G_{498}/H_{113}. However, one clone was digested with *Kpn* I and *Eco*R I (New England Biolabs) and a mutation-free segment with a size of 659 bp was purified. Similarly, another clone was digested with *Kpn* I and *Xho* I (New England Biolabs) and a mutation-free segment with a size of 1242 bp was purified. These two segments were ligated with *Eco*R I/*Xho* I-digested pCR2.1-TOPO vector backbone to generate a mutation-free 5+6 clone. Chimeras were amplified from the TOPO vectors using either primers NiV G *Bam*H I fwd (5'-GATCGGATCCGCCACCATGCCGGCAGAAAACAAG-3') and CDV H *Sph* I rev (5'-GATCGCATGCATTTCAAGGTTTTGAACGGTTACATGAG-3') or CDV H *Bam*H I fwd (5'-GATCGGATCCATGCTCTCCTACCAAGACAAGGTGGG-3') and NiV G *Sph* I rev (5'GATCGCATGCTATTTAGCTTTTTATGTACATTGCTCTGG-3'). The reaction components and PCR cycling parameters are shown in Table 18. In parallel, NiV F was also amplified from pczCFG5-NiV F using the primers NiV F *Bam*H I fwd

Table 18: Reaction components and PCR cycling parameters for the amplification of full-length chimeric glycoprotein ORFs and insertion into pCG1-IRESzeomut

Chimera cloning into pCG1-IRESzeomut				
Component	**Amount**	**PCR cycling parameters**		
10X *Pfu* buffer	5 µL	94˚C	2 min	
2.5 mM dNTPs	4 µL	94˚C	10 sec	
fwd primer	1 µL	52˚C	30 sec	26X
rev primer	1 µL	72˚C	2 min	
Pfu Turbo	0.5 µL	72˚C	7 min	
H2O to 50 µL		4˚C	hold	

For chimeras G_{145}/H_{463}, G_{338}/H_{268}, and G_{498}/H_{113}, the 5' primer was NiV G *Bam*H I fwd and the 3' primer was CDV H *Sph* I rev. For chimeras H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105}, the 5' primer was CDV H *Bam*H I fwd and the 3' primer was NiV G *Sph* I rev. The template for each reaction was the corresponding TOPO clone for each chimera.

(5'-GATCGGATCCGCCACCATGGTAGTTATACTTGAC-3') and NiV F *Sph* I rev (5'-GATCGCATGCCTATGTCCCAATGTAGTAGAGATCCCC-3'), and NiV G was amplified from pczCFG5-NiV G using the primers NiV G *Bam*H I fwd and NiV G *Sph* I rev. These amplicons were gel-purified and digested with *Bam*H I and *Sph* I and then ligated into the vector pCG1-IRESzeomut (a kind gift of Dr. Veronika von Messling) that had been digested with *Bam*H I and *Sph* I (New England Biolabs) and dephosphorylated with Antarctic Phosphatase (New England Biolabs). Clones of these plasmids (pCG-NiV F, pCG-NiV G, pCG-G_{145}/H_{463}, pCG-G_{338}/H_{268}, pCG-G_{498}/H_{113}, pCG-H_{145}/G_{458}, pCG-H_{340}/G_{265}, and pCG-H_{495}/G_{105}) were prepped for further experiments.

Expression and detection of CDV H-NiV G chimeric glycoproteins

Following successful cloning of the chimeric glycoproteins into the pCG1-IRESzeomut expression vector, it was necessary to verify that the clones expressed the glycoproteins. 293T cells were seeded into 6-well plates and then transfected with 4 μg of a chimeric glycoprotein clone using 5 μL/well of Lipofectamine 2000 (Invitrogen). At 24 hours post-transfection, the OptiMEM transfection medium was removed and replaced with 2.5 mL/well of fresh DMEM (Sigma) containing 10% FBS (Wisent). At 48 hours post-transfection, medium was removed and the cells were resuspended non-enzymatically using

1 mL/well of Versene (Gibco) to remove cells from the plate. Cells were centrifuged at 500x*g* for 10 minutes, and the pellet was lysed in 250 µL of 1X SDS-gel loading buffer (without BME). SDS-PAGE gels and Western blots were run as described earlier. Membranes were first probed with swine anti-NiV G serum at a dilution of 1:1,000, followed by incubation with an HRP-conjugated goat anti-swine secondary antibody (Sigma) at a dilution of 1:10,000. The membranes were stripped and then re-probed with a rabbit polyclonal antiserum raised against the cytoplasmic tail of CDV H (a kind gift of Dr. Veronika von Messling) at a dilution of 1:1,000-1:2,000, followed by incubation with an HRP-conjugated goat-anti rabbit secondary antibody (Sigma) at a dilution of 1:10,000.

Assessment of fusion promotion ability of CDV H-NiV G chimeric glycoproteins

After verification of successful expression of the chimeric glycoproteins by Western blots, their functional ability to promote fusion was assessed by co-transfection with NiV F and CDV F. The chimera expression plasmids (pCG-G_{145}/H_{463}, pCG-G_{338}/H_{268}, pCG-G_{498}/H_{113}, pCG-H_{145}/G_{458}, pCG-H_{340}/G_{265}, and pCG-H_{495}/G_{105}) were all separately co-transfected with NiV F or CDV H as described in previous sections in either 293T or Vero dogSLAMtag cells. 293T cells were used because they are easily transfected, although they are only able to

support fusion with NiV G since 293T cells lack the canine SLAM receptor for CDV H. Vero dogSLAMtag cells (a kind gift of Dr. Veronika von Messling) were used because they support fusion with both NiV G and CDV H, since they express the recombinant canine SLAM receptor for CDV H. For the assay, 293T and Vero dogSLAMtag cells were seeded in 12-well plates and were transfected the following day with 1 μg each of either pCG-NiV F or pCG-F5804PZeo. All CDV expression plasmids were kindly provided by Dr. Veronika von Messling. An additional 1 μg of either pCG-G_{145}/H_{463}, pCG-G_{338}/H_{268}, pCG-G_{498}/H_{113}, pCG-H_{145}/G_{458}, pCG-H_{340}/G_{265}, or pCG-H_{495}/G_{105} was added. Co-transfections of pCG-NiV F+pCG-NiV G, pCG-F5804PZeo+pCG-H5804PZeo, pCG-NiV F+pCG-H5804PZeo, and pCG-F5804PZeo+pCG-NiV G were used as controls for cell-cell fusion. Transfections of NiV+NiV G should fuse in both 293T and Vero dogSLAMtag cells, while CDV F+CDV H should only fuse in Vero dogSLAMtag cells and not in 293T cells. Since the glycoproteins of NiV and CDV are functionally incompatible, co-transfections of NiV F+CDV H and CDV F+NiV G should not induce fusion either cell line.

Influence of NiV M expression on NiV and CDV glycoprotein-mediated fusion

NiV M is presumed to be an important protein in the assembly of infectious virus. The matrix protein of MeV has been shown to have a modulatory role in fusion promotion (Cathomen *et al.*, 1998a; Reuter *et al.*, 2006). Co-transfection of NiV M whether either NiV F or CDV H and one of the chimeric glycoproteins was attempted to determine whether NiV M could force fusion. For this assay, 293T or Vero dogSLAMtag cells were seeded into 12-well dishes and the following day were transfected with varying combinations of NiV F or CDV F with each homologous attachment protein (NiV G or CDV H) or chimeric glycoprotein, and either with or without NiV M. After 24 hours, the OptiMEM transfection medium was removed and replaced with fresh DMEM containing 10% FBS. At 48 hours post-transfection, cells were fixed in PBS-buffered 4% paraformaldehyde and examined under phase contrast microscopy for evidence of fusion.

Immunofluorescence assay to detect cell surface expression of CDV H-NiV G chimeric glycoproteins

293T cells were seeded to 96-well tissue culture plates coated with poly-D-lysine. The next day, cells were transfected with 0.25 μg of pczCFG5-NiV G,

pCG-NiV G, pCG-H_{145}/G_{458}, pCG-H_{340}/G_{265}, or pCG-H_{495}/G_{105} and 0.25 μL of Lipofectamine 2000 per well in a total volume of 50 μL/well of OptiMEM. Cells were incubated at 37˚C for 48 hours. For IFA staining, cells were washed once with 100 μL/well PBS and then blocked with 50 μL/well of PBS+1% BSA at 37˚C for 30 minutes. After incubation, the blocking buffer was removed and the primary antibody diluted in PBS+1%BSA was added. Either pig 36 anti-NiV serum or pig 38 anti-NiV serum were used as primary antibodies at dilutions of 1:320 in a total volume of 50 μL/well. Primary antibody incubations were performed at room temperature for 1 hour. After incubation, the primary antibody mixture was removed and the cells were washed three times with 100 μL/well of PBS and then fixed in 50 μL/well of 10% PBS-buffered formalin (final concentration of 3.7% formaldehyde) at 37˚C for 30 minutes. Following fixation, the cells were washed three times with 100μL/well of PBS. Secondary antibody and fluorophore staining was performed as for the primary antibodies, except that the secondary antibody was a biotin anti-swine conjugate (Sigma) and the fluorophore was a streptavidin-PE conjugate (KPL, Sigma, or Jackson Immunoresearch), both used at a dilution of 1:100. Staining with the streptavidin-PE conjugate was performed in the dark. Stained cells were visualized on a Zeiss Axiovert inverted microscope in the Cy3 channel.

Assessment of chimeric CDV H-NiV G glycoprotein interaction with ephrin-B2 and ephrin-B3 by FACS

The cellular expression of NiV G does not result in the down-regulation of either NiV receptor, ephrin-B2 and ephrin-B3, from the cell surface (Sawatsky *et al.*, 2007). However, the chimeric glycoproteins have different combinations of cytoplasmic, transmembrane, and extracellular domains, which may lead to different phenotypes with regard to the modulation of ephrin-B2 and ephrin-B3 cell surface expression. For this experiment, 293T cells were seeded in 6-well dishes (three in total) and the following day were transfected with 4 μg per well of either pCG1-IRESzeomut, pCG-NiV G, pCG-H5804PZeo, pCG-G_{145}/H_{463}, pCG-G_{338}/H_{268}, pCG-G_{498}/H_{113}, pCG-H_{145}/G_{458}, pCG-H_{340}/G_{265}, and pCG-H_{495}/G_{105}. At 24 hours post-transfection, the transfection medium was removed and the cells were detached from the plate using 1 mL/well of Versene. The cells were resuspended with an additional 1 mL of Mg^{2+}/Ca^{2+}-free PBS and then centrifuged at 500x*g* for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 500 μL of fresh Mg^{2+}/Ca^{2+}-free PBS. The cells were fixed with an equal volume of PBS-buffered 4% PFA, then centrifuged at 500x*g* for 10 minutes to pellet the cells, which were then resuspended in 500 μL of Mg^{2+}/Ca^{2+}-free PBS. For FACS staining, approximately $2.5x10^5$ cells were used per tube. Cells were stained with either secondary antibody alone (goat anti-

rabbit PE at 1:200, KPL), rabbit anti-ephrin-B2 at 1:100 (Santa Cruz Biotechnologies) with secondary antibody, or rabbit anti-ephrin-B3 at 1:100 (Santa Cruz Biotechnologies) with secondary antibody. The protocol for FACS staining was described earlier. Stained cells were fixed with an equal volume of PBS-buffered 4% PFA, centrifuged at 500x*g* for 10 minutes, and then resuspended in 500 µL of Mg^{2+}/Ca^{2+}-free PBS before being run on a FACSCalibur flow cytometer.

Recombinant CDV and NiV genomes and RNP protein expression plasmids

Plasmids containing the full-length antigenomic sequence of CDV strain 5804P and the associated expression plasmids for CDV N, P, and L (the ribonucleoprotein complex, or RNP, proteins) were kindly provided by Dr. Veronika von Messling (Laval, Québec). Two wild-type CDV 5804P genomic plasmids were provided: one carrying the eGFP ORF as an ATU between the M and F genes (named rCDV 5804P eGFPM) (Fig. 11) and the other carrying the eGFP ORF as an ATU between the H and L genes (named rCDV 5804P eGFPH) (Fig. 11) (von Messling *et al.*, 2004). All CDV RNP complex ORFs were cloned into the pTM1 vector, which contains a T7 RNA polymerase (RNAP) promoter and allows for expression of these proteins when cells are co-infected with MVA-T7. Although it is attenuated, MVA-T7 is still capable of substantial host gene

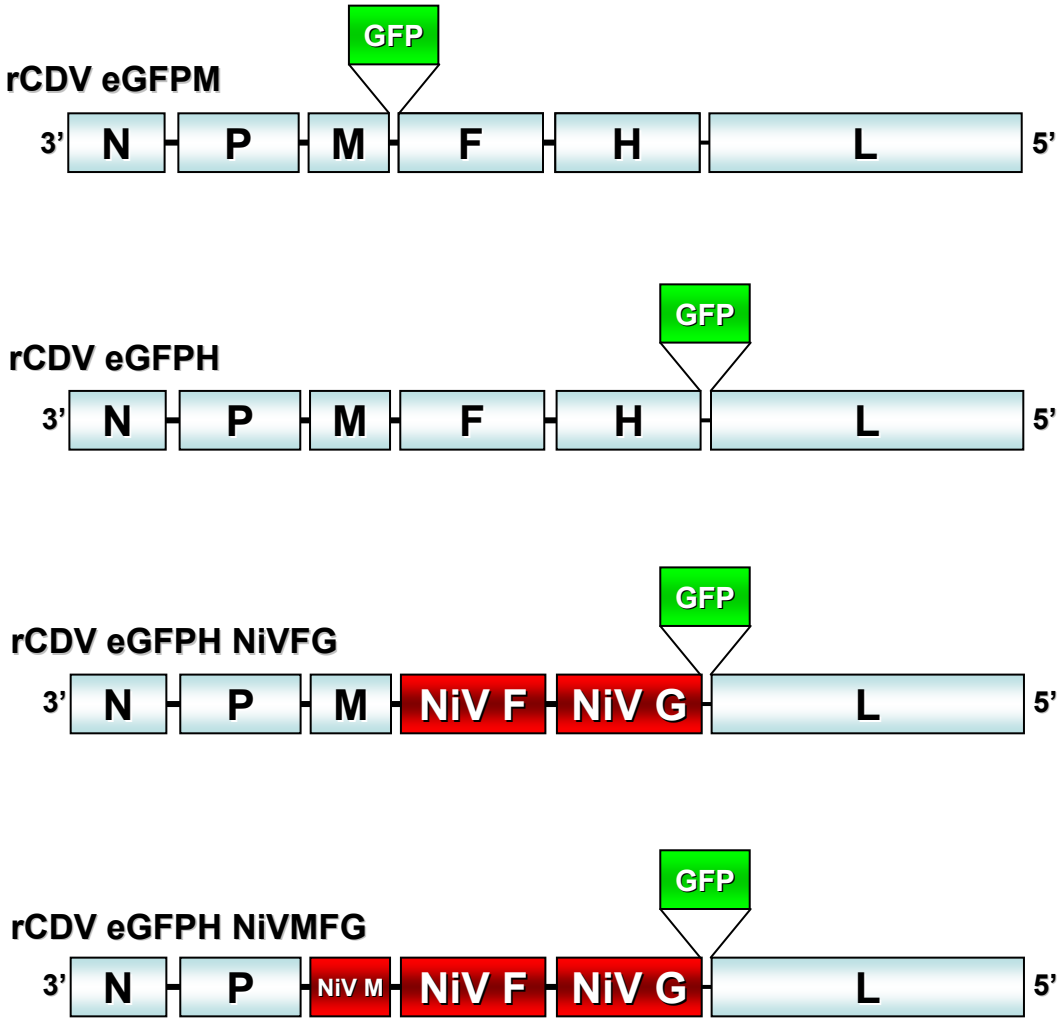

Figure 11: Recombinant CDV genomes. Schematic representations are shown for all recombinant wild type CDV genomes and the chimeric rCDV eGFPH NiVFG and rCDV eGFPH NiVMFG viruses.

shut-off function, so the use of a promoter recognized by an endogenous RNAP (i.e. RNAP I or II) would not produce high amounts of transcript. Genomic plasmids for chimeric CDVs bearing the NiV M, F, or G proteins were again generated and kindly provided by Dr. Veronika von Messling. CDV genomes with the F and H ORFs substituted for those of NiV F and G was named rCDV eGFPH NiVFG (Fig. 11) and CDV with the M, F, and H ORFs substituted for those of NiV M, F, and G was named rCDV eGFPH NiVMFG (Fig. 11). rCDV NiVFG and rCDV NiVMFG both use rCDV 5804P eGFPH as their genomic backbones, so all of these viruses are capable of expressing eGFP upon infection. Recombinant NiV genomes were generated by Dr. Veronika von Messling. The genomes were cloned into the pBR322 plasmid to ensure relatively low copy number in order to minimize bacterial elimination of the plasmids. Two T7 RNAP-driven NiV genomes were generated: one with the full wild-type viral sequence (named rNiV) (Fig. 12) and the other with eGFP as an ATU between the G and L genes (named rNiV eGFPG) (Fig. 12). These same genomes were also cloned into pBR322 under the transcriptional control of the CMV_{IE} promoter to allow for MVA-T7-free rescue of virus. The NiV RNP proteins (N, P, and L) were cloned into pTM1 for use with the MVA-T7-driven rescue system. The eukaryotic expression plasmid pBK-CMV was used to express the NiV N, P, and L plasmids for use with the CMV-driven rescue system. Since we were unsure of

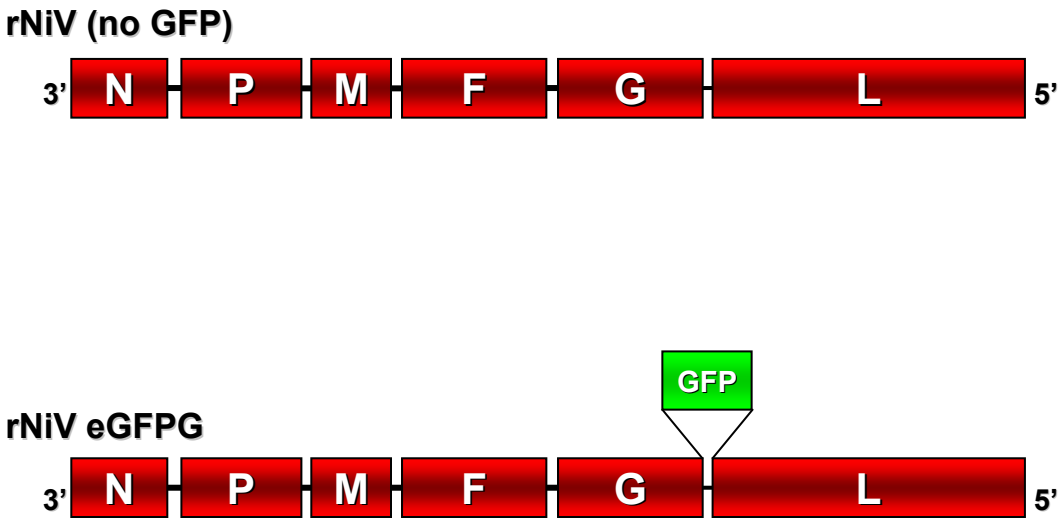

Figure 12: Recombinant NiV genomes. Schematic representations are shown for all recombinant wild type rNiV genomes.

the functionality of the NiV L-CMV clone, pTM1-NiV L was also used with the CMV-driven rescue system, but T7 RNAP had be supplied on an additional eukaryotic expression plasmid (pCAGGS-T7 RNAP).

CDV and NiV rescues

MVA-T7 (a kind gift of Dr. Bernard Moss, New Haven, CT) was grown in BHK-21 cells to a titer of approximately 10^7 $TCID_{50}$/mL. The MVA-T7 supernatant was centrifuged for 10 minutes at 1,000x*g* to pellet cell debris, and the resulting supernatant was stored at -80˚C in 1 mL aliquots. For virus rescues, confluent 293T cells in a 150 cm^2 flask were resuspended using 10 mL per flask of Versene (Gibco). Resuspended 293T cells were centrifuged for 5 minutes at 500x*g*, the supernatant was discarded, and the cell pellet was resuspended in 5 mL of fresh DMEM containing 10% FBS. Cells were counted into a hemacytometer chamber and 10^6 cells per well were added to six-well dishes. MVA-T7 virus stock was diluted $^1/_{10}$ in OptiMEM and 250-500 µL of diluted virus per well was used in all rescues. Diluted virus was added to the 293T cells and OptiMEM was added to bring the volume to 1 mL. The cells and virus were incubated for 1 hour at 37˚C. During this incubation period, the transfection mixture was prepared. For rCDV eGFPM, rCDV eGFPH, rCDV eGFPH NiVFG, and rCDV eGFPH NiVMFG rescues, 1 µg of pTM1-CDV N, 1 µg of pTM1-CDV

P, and 0.5 μg of pTM1-CDV L was mixed with 5-10 μg of genomic plasmid in 200 μL of OptiMEM. For rNiV and rNiV eGFPG rescues, pTM1-NiV N, pTM1-NiV P, and pTM1-NiV L were used. After 5 minutes at room temperature, 200 μL of OptiMEM containing 8 μL of Lipofectamine 2000 (Invitrogen) was added, and the resulting mixture was incubated at room temperature for a further 30 minutes. The transfection mixture was then added to the MVA-T7-infected 293T cells and a further 1 mL of DMEM containing 10% FBS was added. For rescues with the genomic plasmids for rCDV eGFPH NiVFG, rCDV eGFPH NiVMFG, rNiV, and rNiV eGFPG, the transfected cells were immediately brought into the BSL4 laboratory and all further manipulation of these cultures were done in BSL4 containment. These cells were then incubated overnight at 37˚C. The following day, the medium was changed and replaced with 2.5 mL per well of fresh DMEM containing 10% FBS and 1% penicillin/streptomycin (Gibco). After a further 24-48 hours of incubation at 37˚C (48-72 hours post-transfection), the 293T cells were resuspended with the medium in the well and then overlaid onto Vero E6 or Vero dogSLAMtag cells in 10-cm dishes. Vero E6 cells were used for the rescue of rCDV NiVFG, rCDV NiVMFG, rNiV, and rNiV eGFPG viruses, while Vero dogSLAMtag cells were only used for the rescue of rCDV eGFPM and rCDV eGFPH viruses. The 10-cm dishes were examined every 1-2 days for the development of CPE (i.e. syncytia). If, after 5-6 days, the cells in the 10-cm

dishes became too crowded, these cells were split at a ratio of 1:2 into two new 10-cm dishes to allow the development of syncytia.

If CPE was identified in any of the 10-cm dishes, the supernatant was collected and centrifuged at 500x*g* for 10 minutes to remove debris, and 500 µL was applied to confluent monolayers of Vero E6 or Vero dogSLAMtag cells in six-well dishes. If CPE was observed in any of these wells, the culture supernatant was transferred to a 150 cm^2 flask to grow virus stock. Stocks were grown until 80-90% CPE was observed. Flasks were subjected to one freeze-thaw cycle and the medium was harvested, centrifuged for 10 minutes at 1,000x*g*, and then frozen at -80˚C in 1 mL aliquots.

Four separate stocks of rCDV NiVFG were obtained. The stocks were titrated in BSL4 as follows. Virus stock was serially diluted from 10^{-1} until 10^{-8}, and 1 mL of each of the dilutions from 10^{-3} until 10^{-8} was applied to Vero dogSLAMtag cells in 24-well dishes. Virus was allowed to adsorb for 1 hour at 37˚C, at which point the inoculum was removed and replaced with 1 mL per well of fresh DMEM containing 2% FBS. The cells were checked every 1-2 days for the development of CPE. Cells were left for approximately one week, at which point it was determined that maximal CPE had been attained.

RT-PCR analysis of rCDV NiVFG virus stocks

The nucleic acids from each of the four virus stocks were isolated from the supernatants using the Qiagen Viral RNA Mini kit as per the manufacturer's instructions. This kit should only isolate viral RNA from supernatant samples, but it does isolate DNA from supernatants as well. The primers CDV N 540 fwd (5'-GGCTGGTTGGAGAATAAGGA-3') and CDV N 961 rev (5'-CCAAGAGCCGGATACATAGT-3') were used to amplify segments of the CDV N ORF. CDV H was amplified using the primers CDV H β1,2 fwd and CDV H β3,4 rev (Table 14). NiV N was amplified using the primers NiV N 115 fwd and NiV N 501 rev (Table 7). NiV M was amplified using the primers NiV M 5405 fwd and NiV M 5641 rev (Table 7). NiV G was amplified using the primers NiV G 9273 fwd and NiV G 9709 rev (Table 7). The primers SPox HA 5' (5'-ATGCCGGTACTTATGTATGTGC-3') and SPox HA 3' (5'-TCTTGTCTGTTGTGGATTCT-3') (kindly provided by Allen Grolla) were used to amplify a segment of the vaccinia virus HA gene. The primers SPox crmB 5' (5'-TACCGGTCTCAGCGAATC-3') and SPox crmB 3' (5'-GACGCTAGATAGACAGTC-3') or SPox crmB 3'-2 (5'-ACCGTGTCCGAATGCGGCAT-3') (kindly provided by Allen Grolla) were used to amplify a segment of the vaccinia virus crmB gene (Carletti *et al.*, 2005).

Assessment of virus release from BHK-21 and CHO-K1 cells

In order to assess the effect of CDV M on the release of virus from BHK-21 and CHO-K1 cells (ATCC), six-well plates of Vero E6, BHK-21, and CHO-K1 were infected with dilutions of 10^{-1}, 10^{-2}, and 10^{-3} of NiV or rCDV NiVFG. Viral dilutions of NiV corresponded to 10^5, 10^4, and 10^3 TCID50 per well, while the dilutions of rCDV NiVFG corresponded to 10^4, 10^3, and 10^2 $TCID_{50}$ per well. Virus was applied to the cells and allowed to adsorb for 1 hour at 37˚C. The inoculum was then removed and the medium was replaced with 2.5 mL per well of fresh DMEM containing 2% FBS. The cells were incubated for 3-4 days, after which the culture supernatants were harvested and centrifuged for 10 minutes at 1,000x*g* to remove cell debris. 1 mL of the clarified NiV and rCDV NiVFG supernatants were then added to six-well dishes of Vero dogSLAMtag cells and incubated for 5-6 days to assess whether CPE developed. Cell monolayers were fixed in 4% PBS-buffered PFA overnight at 4˚C and then dunked out of BSL4.

Assessment of ephrin-B2 and ephrin-B3 down-regulation by rCDV NiVFG

293T cells were seeded in a 150 cm^2 flask and infected with 7 mL of rCDV NiVFG stock 3 diluted 10^{-1} in OptiMEM. Virus was allowed to adsorb for 1 hour at 37˚C before the inoculum was removed and replaced with fresh DMEM containing 2% FBS. The following day, the cells were detached using 10 mL of

Versene. The cells were pipetted to ensure a single cell suspension and centrifuged for 10 minutes at 500x*g* to pellet the cells. The supernatant was removed and replaced with fresh Mg^{2+}/Ca^{2+}-free PBS. The cells were resuspended and fixed with an equal volume of PBS-buffered 4% PFA overnight at 4˚C. The following day, the cells were centrifuged for 10 minutes at 500x*g*, the supernatant was removed, and 5 mL of fresh Mg^{2+}/Ca^{2+}-free PBS was added, followed by an equal volume of PBS-buffered 4% PFA. The cells were then dunked out of BSL4 and stained for ephrin-B2 and ephrin-B3 for FACS analysis as described in previous sections.

RESULTS

Cloning of NiV F and NiV G and assessment of fusogenic behaviour

The NiV F and NiV G were successfully cloned into the retroviral expression vectors pczCFG5 IEGZ and pHITBE (Figs. 13 and 14). Furthermore, the orientation was verified for pczCFG5-NiV F and pczCFG5-NiV G, indicating that NiV F and NiV G were cloned in the sense direction (Fig. 13).

Transfection of pczCFG5-NiV F, pczCFG5-NiV G, pHITBE-NiV F, or pHITBE-NiV G individually did not result in fusion (Fig. 15, NiV F and NiV G panels). However, co-transfection of pczCFG5-NiV F and pczCFG5-NiV G or pHITBE-NiV F and pHITBE-NiV G resulted in extensive fusion in the cell monolayer (Fig. 15, NiV F+NiV G panel). This also occurred with pczCFG5-NiV F and pHITBE-NiV G or pHITBE-NiV F and pczCFG5-NiV G were co-transfected. This indicates that NiV F and NiV G were both successfully expressed in both expression vector systems. When cells singly transfected with NiV F or NiV G were mixed in a co-culture experiment, no fusion resulted (Fig. 15, co-culture panel). This indicated that in order for fusion to occur, both NiV F

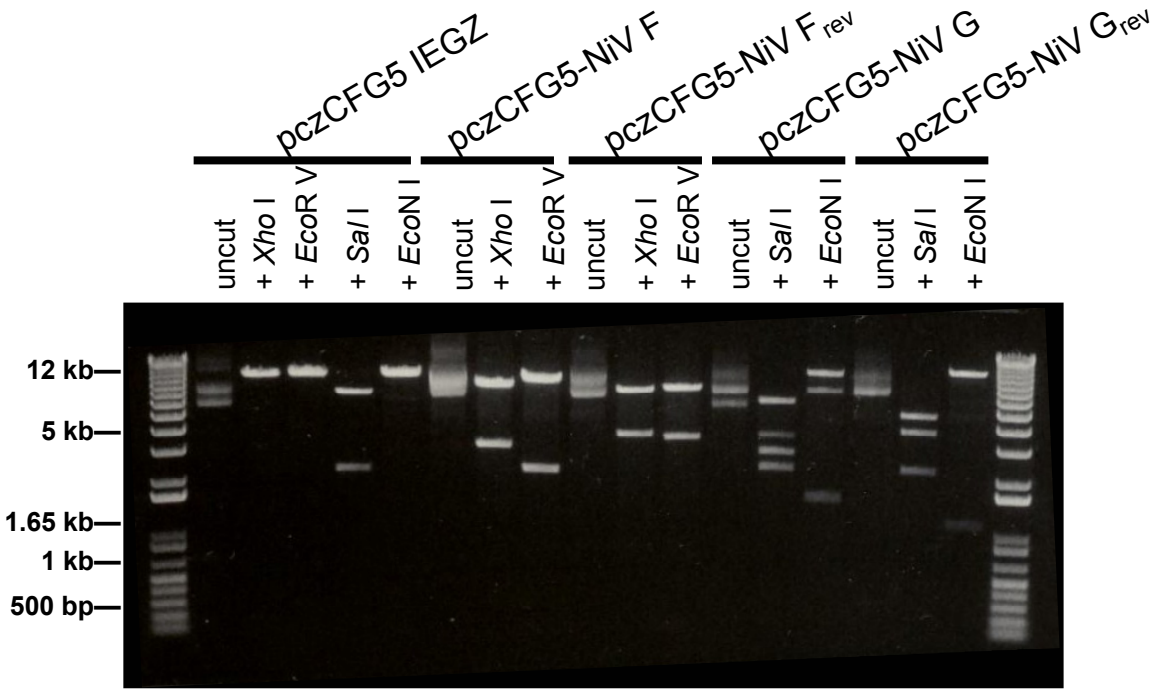

Figure 13: Sample digests for each pczCFG5 IEGZ-based expression plasmid are shown. Because the NiV F and G ORFs were cloned into pczCFG5 IEGZ using blunt-ended cloning, two restriction enzymes were used to verify the orientation of the inserts. The molecular weight markers are the 1 kb Plus DNA Ladder (Invitrogen).

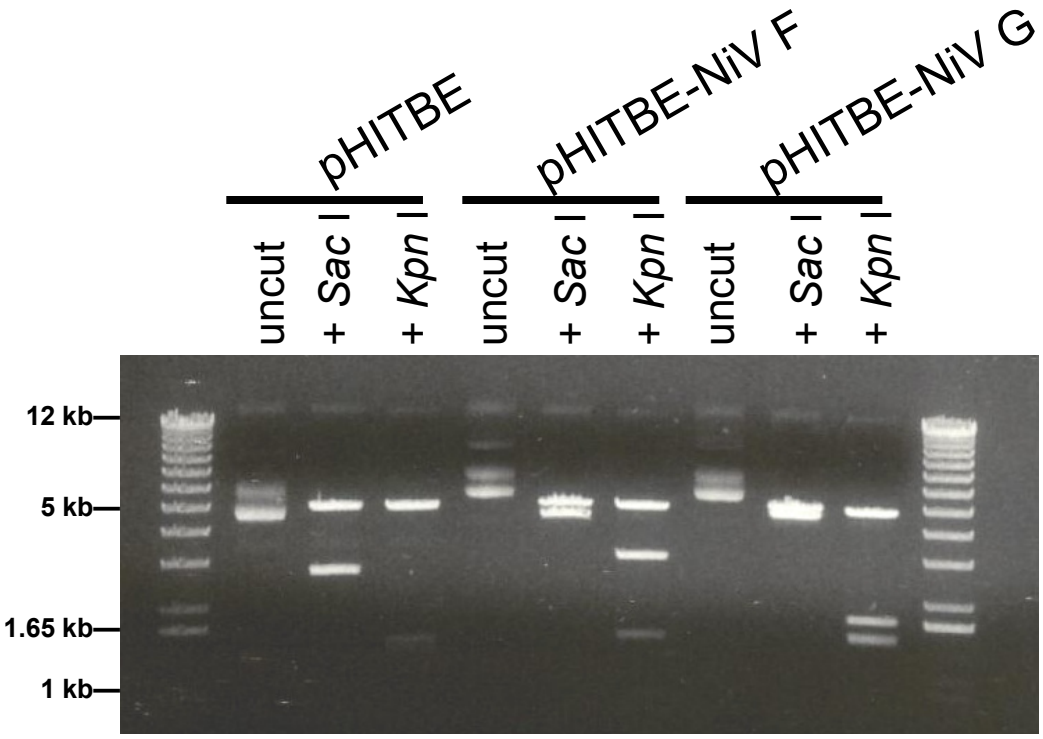

Figure 14: Sample digests for each pHITBE-based expression plasmid are shown. Two restriction enzymes were used to verify the orientation of the inserts. The molecular weight markers are the 1 kb Plus DNA Ladder (Invitrogen).

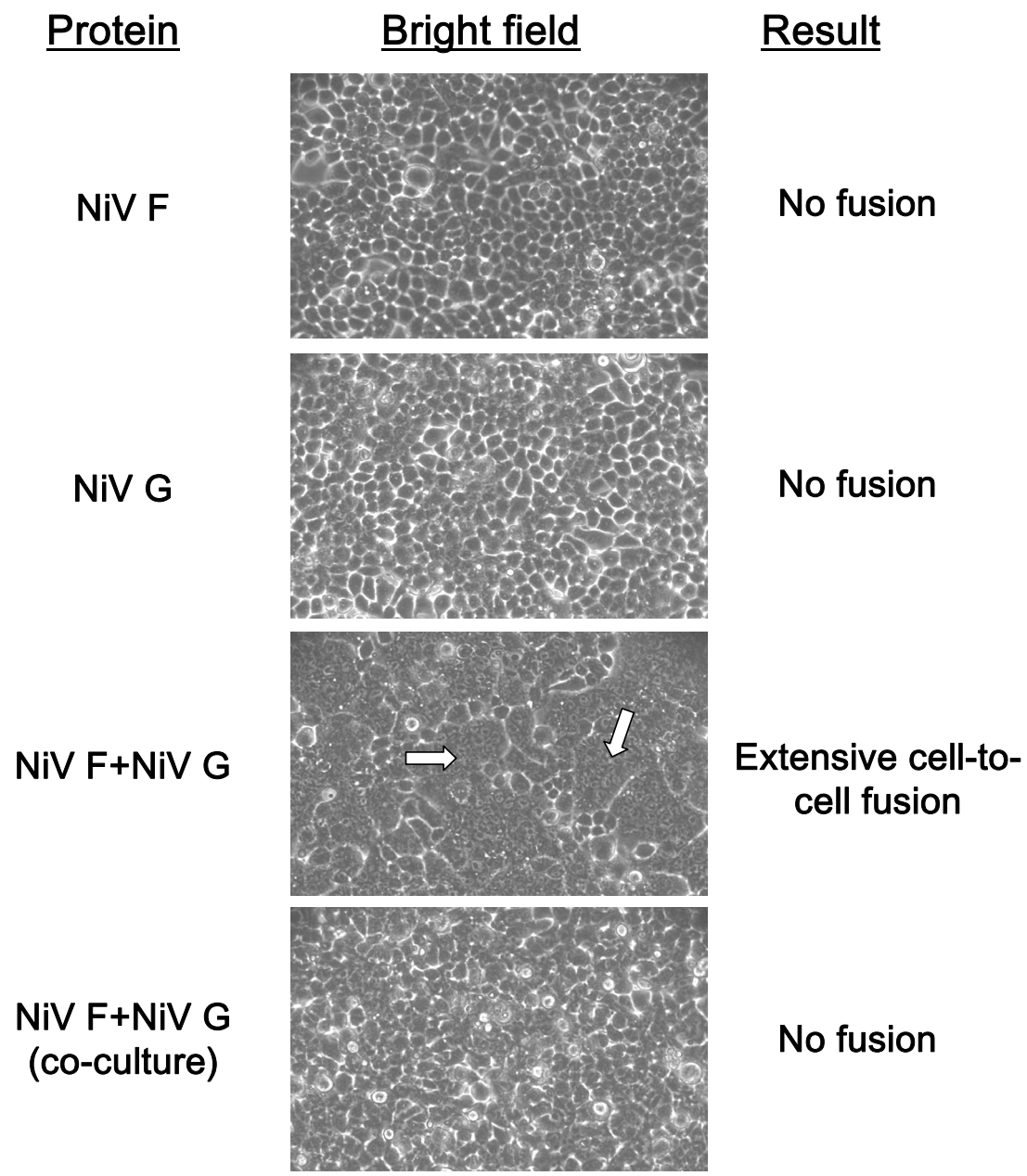

Figure 15: Co-expression of NiV F and NiV G in 293T cells. NiV F and NiV G were expressed either separately or together in 293T cells. Syncytia are indicated by white arrows in the NiV F+NiV G panel. In the co-culture panel, separate populations of NiV F- and NiV G-transfected cells were mixed. Reproduced from Sawatsky *et al.* (2007) *J. Gen. Virol.* **88**(2):582-591.

and NiV G had to be expressed in the same, identical cell. NiV is therefore similar to other paramyxoviruses in its glycoprotein requirements for fusion.

Detection of NiV F and NiV G by Western blot

Although successful expression of NiV F and NiV G had been demonstrated by functional assay (i.e. fusion), it was also necessary to detect the proteins by Western blot to directly verify expression. The Western blot for NiV F, as detected by a guinea pig anti-NiV serum, shows the uncleaved F_0 precursor at approximately 60 kDa and the cleaved F_1 fragment at approximately 50 kDa, which is consistent with the predicted molecular weight (Fig. 16, left panel) (Tamin *et al.*, 2002). The small cleavage fragment of F_0 (F_2) with a molecular weight of approximately 19 kDa (Bossart *et al.*, 2002), is too small to be seen on this blot. The Western blot for NiV G, as detected by a swine anti-NiV G serum, is observed as one band at approximately 75-80 kDa on the blot (Fig. 16, right panel), which is also consistent with its predicted molecular weight (Bossart *et al.*, 2002).

Production of retroviral particles and generation of transgenic cells

Retroviral particles carrying replication genomes were successfully generated and pseudotyped with the VSV G glycoprotein. Three types of particles were produced: pczCFG5 IEGZ, pczCFG5-NiV F, and pczCFG5-NiV G.

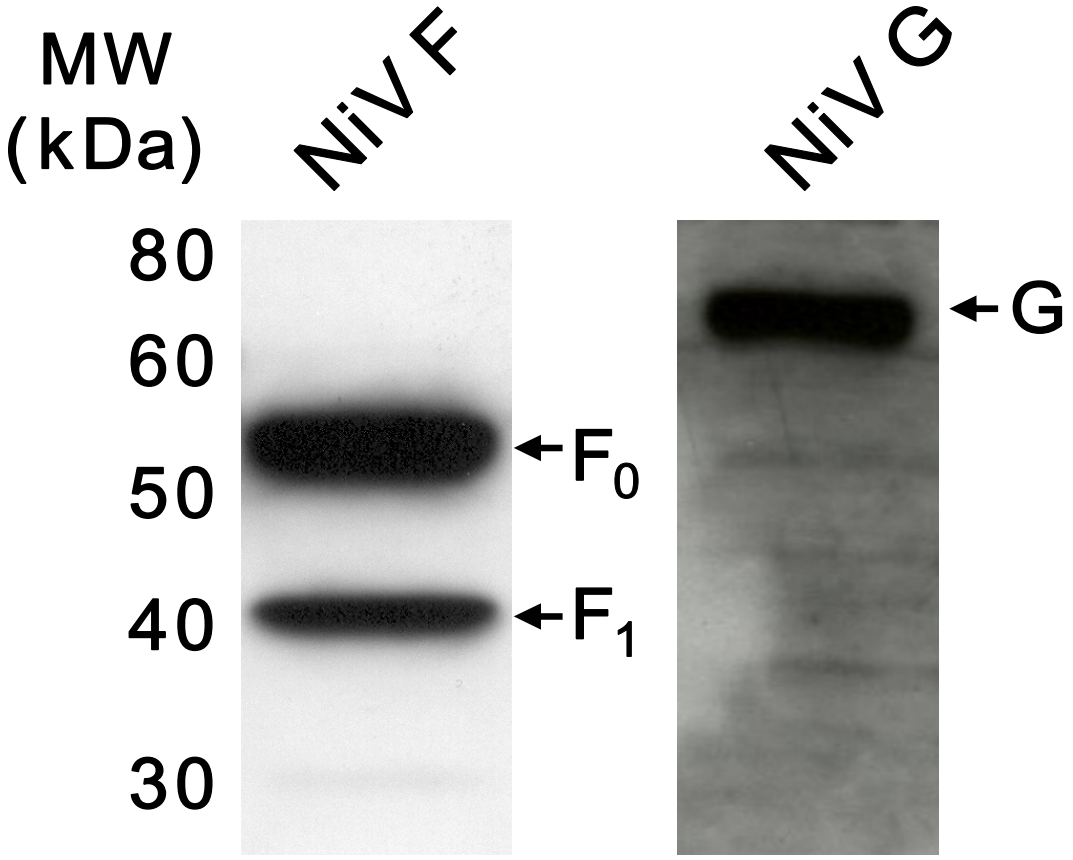

Figure 16: Western blots of 293T whole cell lysates transfected with either pczCFG5-NiV F or pczCFG5-NiV G. The NiV F blot was performed with guinea pig anti-NiV serum and goat anti-guinea pig HRP-conjugated secondary antibody. The NiV G blot was performed with swine anti-NiV G serum (Weingartl *et al.,* 2006) and goat anti-swine HRP-conjugated secondary antibody. Reproduced from Sawatsky *et al.* (2007) *J. Gen. Virol.* **88**(2):582-591.

These retroviral particles were then used to generate three transgenic cell populations by transduction: CRFK-pcz, CRFK-NiV F, and CRFK-NiV G. Functional expression of the NiV glycoproteins in these transgenic cells were verified by transduction with particles carrying the complementary glycoprotein (i.e. CRFK-NiV F were transduced with particles carrying NiV G). The development of syncytia demonstrated that NiV F and NiV G were successfully expressed in the transgenic cells (Fig. 17A, bottom left and right). The expression of GFP was also examined through FACS analysis. Overall GFP levels were somewhat weaker than expected, and the lower levels of GFP-expressing cells were thought to be due to relatively low titers of retroviral particles used for transduction. Enrichment of these cell populations was attempted by treatment with Zeocin. After Zeocin treatment, CRFK-NiV F cells had GFP expression levels of approximately 93% (Fig. 17B, left panel) and CRFK-NiV G cells had GFP levels of almost 89% (Fig 17B, right panel), indicating that expression of the transgene cassette was quite high. While treatment with Zeocin resulted in enhanced levels of GFP expression, it also seemed to decrease NiV glycoprotein expression somewhat. The reason for this is unclear. It was determined that repeated transduction would be used to increase NiV glycoprotein expression in the transgenic cells rather than Zeocin treatment, since this appeared to be the

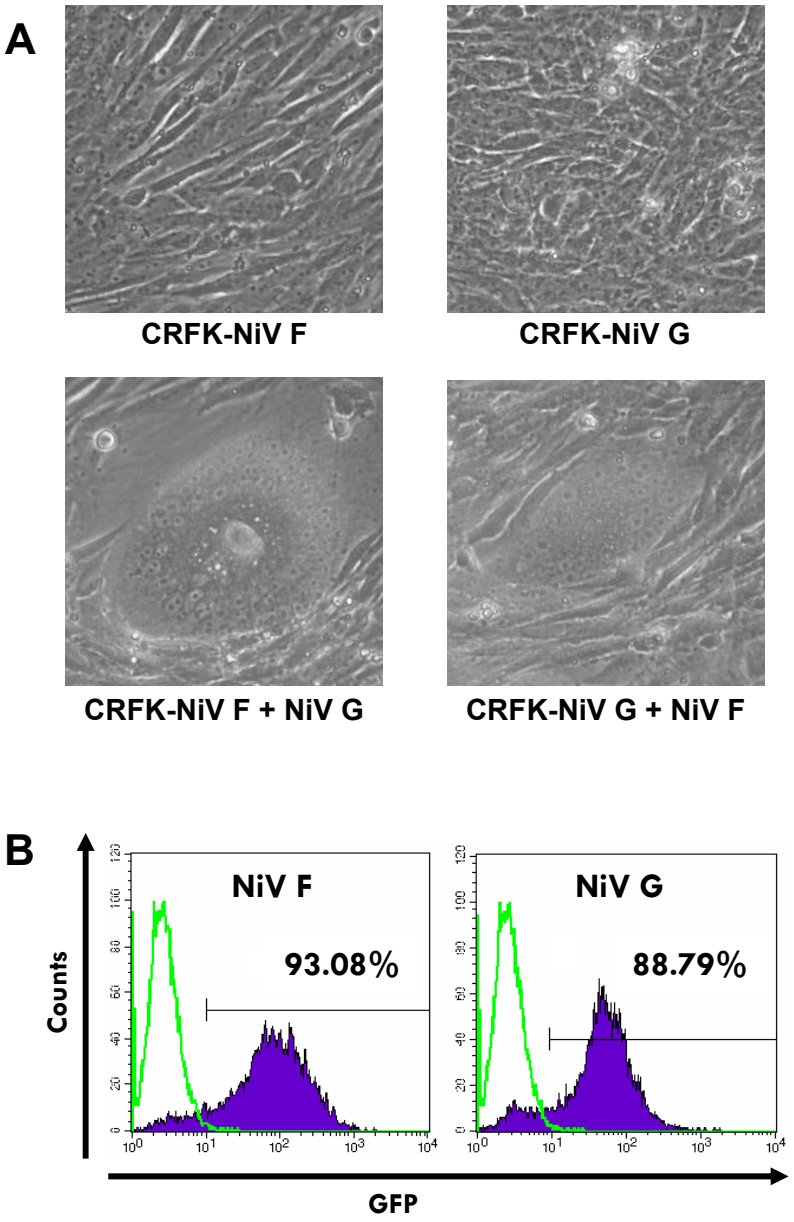

Figure 17: Transgenic cells expressing NiV F and NiV G. A) CRFK-NiV F (top left) and CRFK-NiV G (top right) show normal cellular morphology. The addition of retroviral particles carrying NiV G to CRFK-NiV F cells (bottom left) and particles carrying NiV F to CRFK-NiV G cells (bottom right) demonstrate the formation of syncytia in transgenic cells. B) GFP levels in CRFK-NiV F (left panel) and CRFK-NiV G (right panel) cells after enrichment by treatment with Zeocin. Reproduced from Sawatsky *et al.* (2007) *J. Gen. Virol.* **88**(2):582-591.

best way to ensure that expression of the NiV glycoprotein transgene was not substantially diminished.

Exposure of transgenic cells to NiV and HeV

The transgenic cells were exposed to NiV and HeV in BSL4 containment. After 5 to 7 days of exposure to NiV and HeV, all control cell populations (CRFK wt [Fig. 18, left column], CRFK-pcz, and CRFK-NiV F) showed extensive cytopathic effect (CPE) at all virus doses, while CRFK-NiV G cells were resistant to CPE caused by both NiV and HeV at all doses of virus (Fig. 18, middle and right columns). CRFK-NiV G cells did begin to show some CPE toward 5 days post-exposure; the reason for this is unclear. These same results were also observed when 293T cells were transfected with either pczCFG5 IEGZ, pczCFG5-NiV F, or pczCFG5-NiV G and exposed to NiV and HeV (not shown).

RT-PCR of NiV- and HeV-exposed transgenic cells

Since CRFK-NiV G cells did not show CPE when exposed to low and intermediate doses of NiV and HeV, it appeared that these cells were resistant to virus infection. However, it was not clear whether virus had entered these cells or not based on the lack of CPE. In order to clarify this issue, total cell lysate from unexposed and NiV- and HeV-exposed transgenic cells (CRFK wt, CRFK-

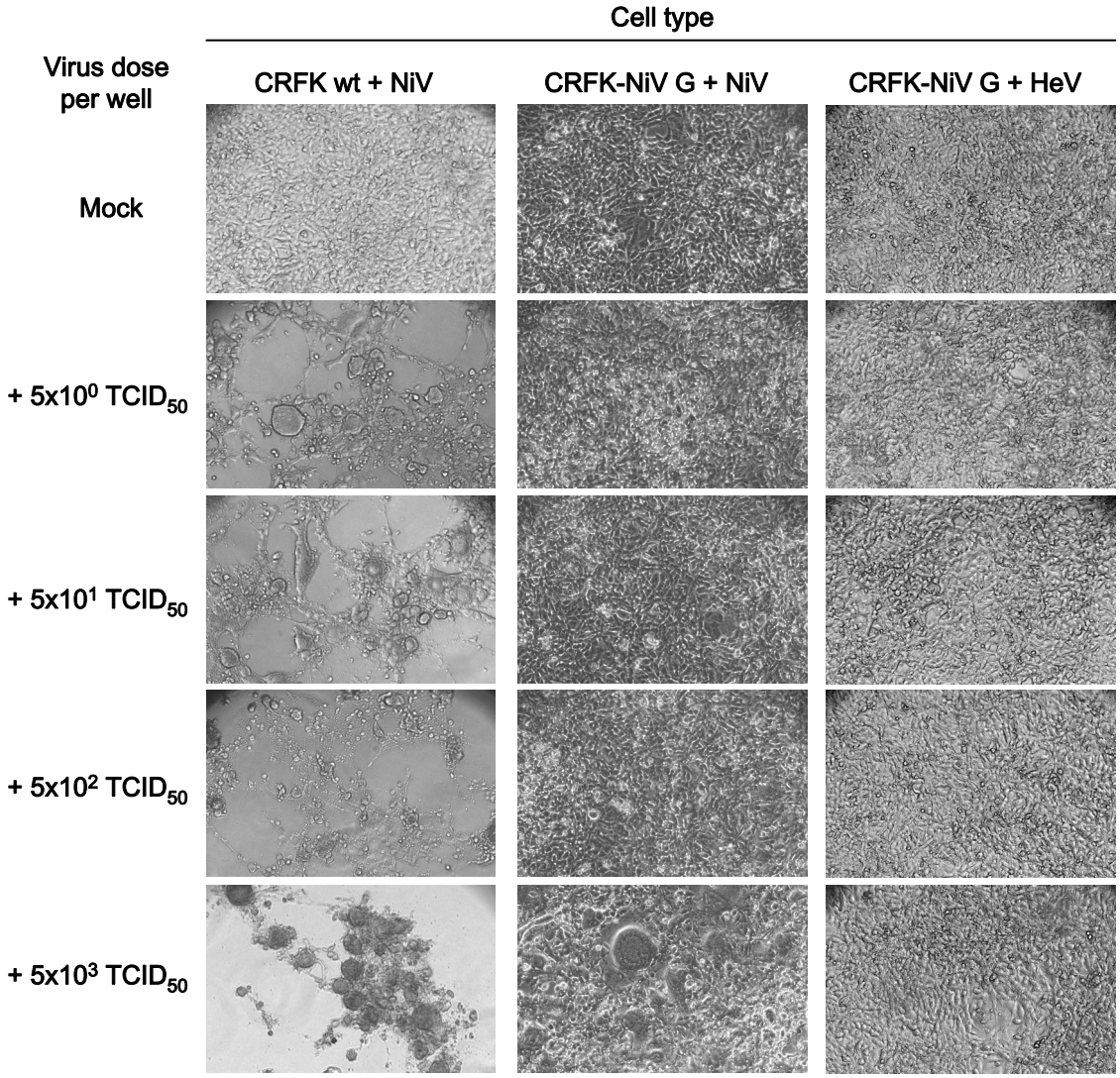

Figure 18: Resistance of CRFK-NiV G transgenic cells to NiV- and HeV-induced CPE. Wild-type and transgenic cells were exposed to NiV and HeV as described in the Methods section. Cells were fixed in 7.4% PBS-buffered formaldehyde at 5 days post-exposure and examined for CPE. Reproduced from Sawatsky *et al.* (2007) *J. Gen. Virol.* **88**(2):582-591.

pcz, CRFK-NiV F, and CRFK-NiV G) cells was harvested at five days post-exposure for RNA analysis. Two-step RT-PCR reactions were performed in order to generate strand-specific amplicons from either (+)-sense mRNA or (-)-sense viral genomic RNA (vRNA). Mock-infected cells did not have any viral nucleic acid (Fig. 19, left and middle panels). mRNA and vRNA were consistently detected in control cells (CRFK wt, CRFK-pcz, and CRFK-NiV F) (Fig. 20, Fig. 21, and Fig. 22), while mRNA and vRNA were not detected in CRFK-NiV G cells that had survived low doses (5 $TCID_{50}$/ well) of HeV (Fig. 21, CRFK-NiV G+HeV) and NiV (Fig. 22, CRFK-NiV G+NiV). A band was always detected for NiV G mRNA in CRFK-NiV G, confirming production of the NiV G transcript (Fig. 22, CRFK-NiV G, mock and +NiV "NiV G" lanes). Cellular RNA extracts were tested for the presence of fGAPDH cellular RNA, and all were strongly positive by RT-PCR (Figs. 19 and 21).

Assessment of the effect of NiV G expression in a recombinant fusion assay

The lack of viral nucleic acid in CRFK-NiV G cells following exposure to NiV and HeV seemed to indicate the lack of CPE in these cells was due to lack of viral entry. In order to further confirm this observation, a recombinant fluorescent fusion inhibition assay was developed based on the retroviral expression plasmids, using the plasmids pHITΔGFP-NiV F and pHITΔGFP-NiV

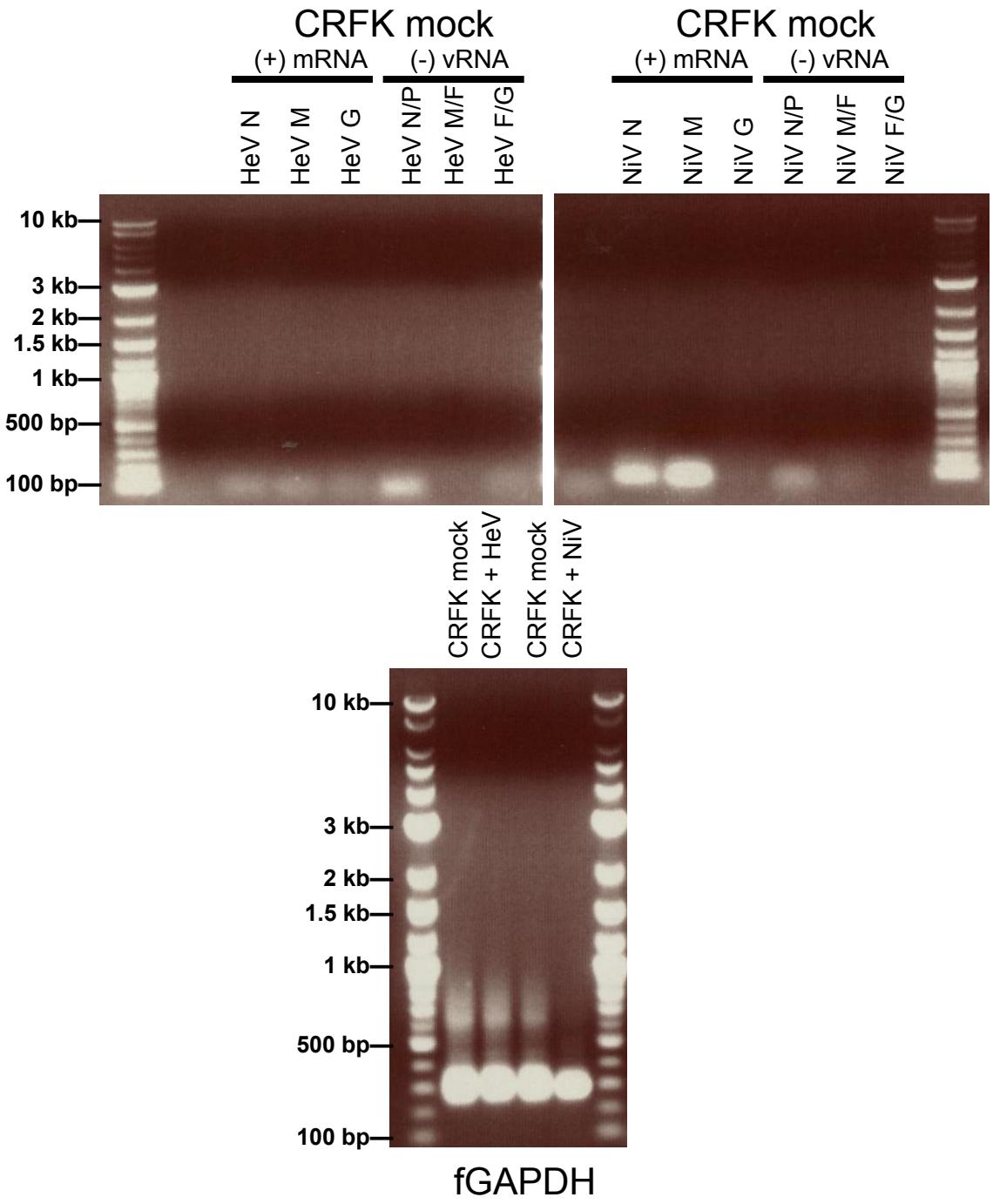

Figure 19: Control RT-PCR reactions for the detection of viral nucleic acid in HeV- and NiV-exposed cells. Top left panel: RT-PCR reactions for CRFK wt cells exposed to HeV. Top right panel: RT-PCR reactions for CRFK wt cells exposed to NiV. Bottom panel: Control RT-PCR for fGAPDH mRNA. Expected amplicon size for fGAPDH RT-PCR is 294 bp. The molecular weight markers in all gels is the 2-Log DNA Ladder (New England Biolabs).

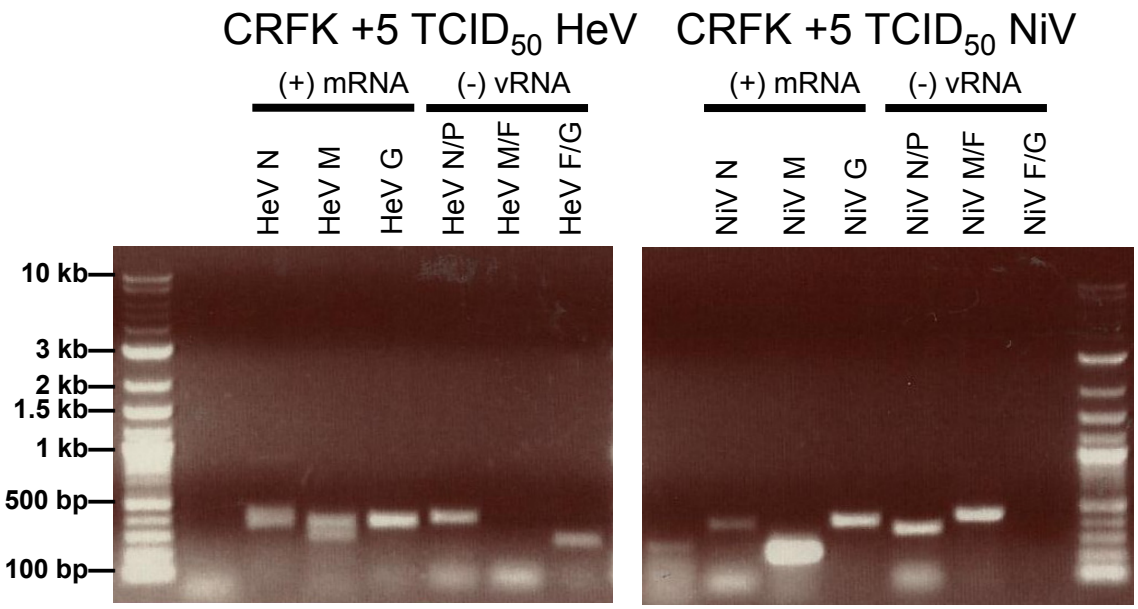

Figure 20: Detection of HeV and NiV nucleic acid in infected CRFK wt cells. Left panel: RT-PCR reactions for CRFK wt cells exposed to HeV. Expected amplicon sizes are 398 bp (HeV N), 317 bp (HeV M), 401 bp (HeV G), 424 bp (HeV N/P), 251 bp (HeV M/F), and 298 bp (HeV F/G). Right panel: RT-PCR reactions for CRFK wt cells exposed to NiV. Expected amplicon sizes are 387 bp (NiV N), 237 bp (NiV M), 437 bp (NiV G), 380 bp (NiV N/P), 464 bp (NiV M/F), and 213 bp (NiV F/G). The molecular weight markers in all gels is the 2-Log DNA Ladder (New England Biolabs).

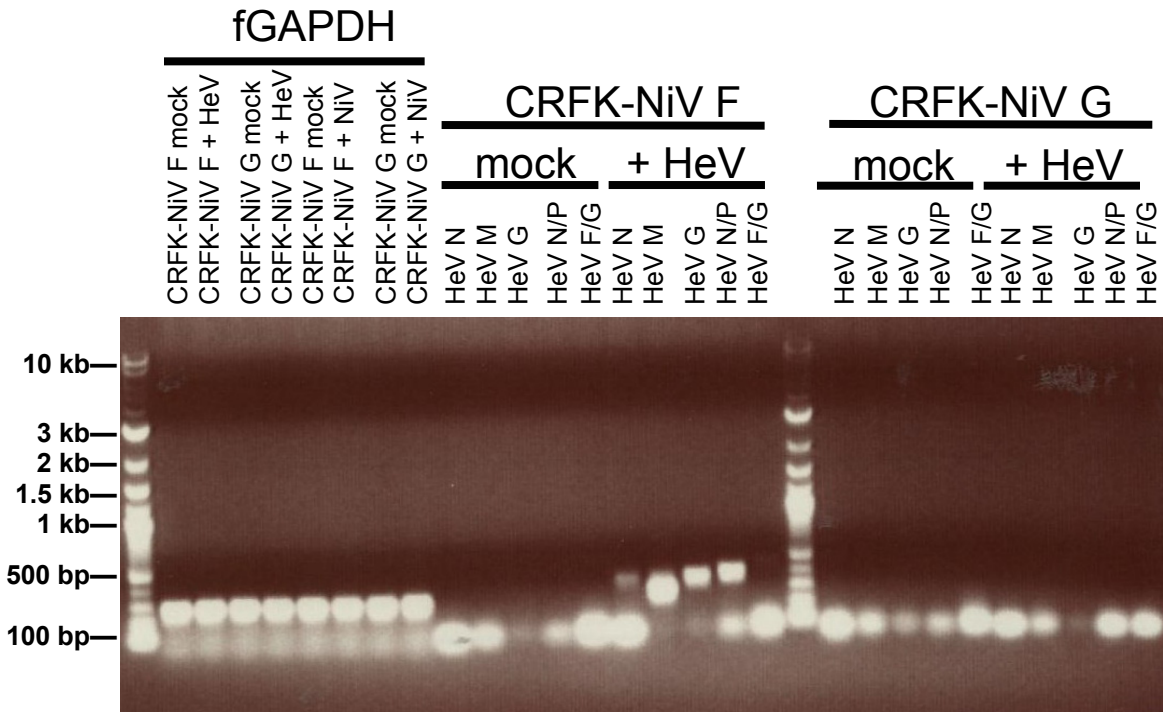

Figure 21: Detection of HeV nucleic acid in HeV-exposed CRFK-NiV F and CRFK-NiV G transgenic cells. Left panel: fGAPDH control RT-PCR reactions for CRFK-NiV F and CRFK-NiV G cells. Middle panel: RT-PCR for HeV nucleic acid in mock and HeV-exposed CRFK-NiV F cells. Right panel: RT-PCR for NiV nucleic acid in mock and HeV-exposed CRFK-NiV G cells. The molecular weight markers in all gels is the 2-Log DNA Ladder (New England Biolabs).

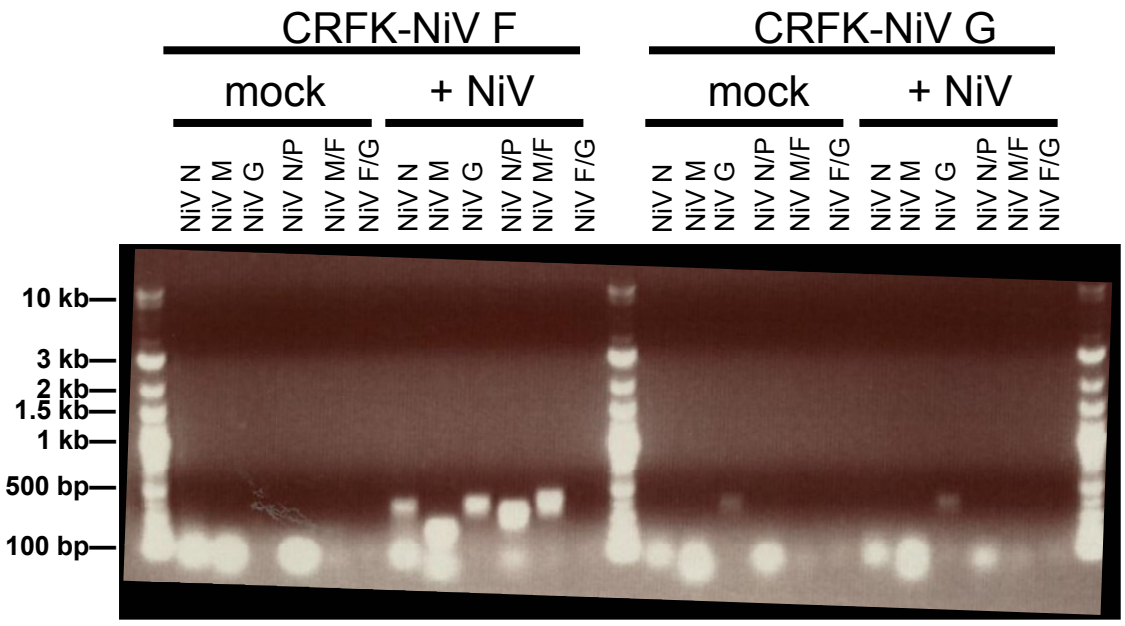

Figure 22: Detection of NiV nucleic acid in NiV-exposed CRFK-NiV F and CRFK-NiV G transgenic cells. Left panel: RT-PCR for NiV nucleic acid in mock and NiV-exposed CRFK-NiV F cells. Right panel: RT-PCR for NiV nucleic acid in mock and NiV-exposed CRFK-NiV G cells. The molecular weight markers in all gels is the 2-Log DNA Ladder (New England Biolabs).

G, from which the GFP ORF had been successfully removed (Fig. 23). In this system, cells transfected with pczCFG5 IEGZ (GFP^+, NiV F/G^-) (Fig. 24A, left panel) or pczCFG5-NiV F (GFP^+, NiV F^+) (Fig. 24A, middle panel) were able to fuse with a population of cells that had been co-transfected with pHITΔGFP-NiV F and pHITΔGFP-NiV G (GFP^-, NiV F^+/G^+), thereby generating fluorescent syncytia (Fig. 24B, left and middle panels). However, cells transfected with pczCFG5-NiV G (GFP^+, NiV G^+) (Fig. 24A, right panel) did not fuse with cells co-transfected with pHITΔGFP-NiV F and pHITΔGFP-NiV G (GFP^-, NiV F^+/G^+), which resulted in the formation of syncytia with individual green cells throughout the monolayer (Fig. 24B, right panel). Taken together, these results indicate that expression of NiV G is specifically able to inhibit the ability of its cellular receptor to interact with NiV G on opposing cells.

Assessment of cell surface ephrin-B2 and ephrin-B3 expression

According to most models of receptor interference, expression of an attachment protein results in the down-regulation of cell surface expression of the cognate cellular receptor (Fig. 8). With the identification of ephrin-B2 (Negrete *et al.*, 2005; Bonaparte *et al.*, 2005) and ephrin-B3 (Negrete *et al.*, 2006) as receptors for NiV and HeV, we therefore endeavoured to determine whether this phenomenon was occurring in 293T cells expressing NiV G. Control cells stained

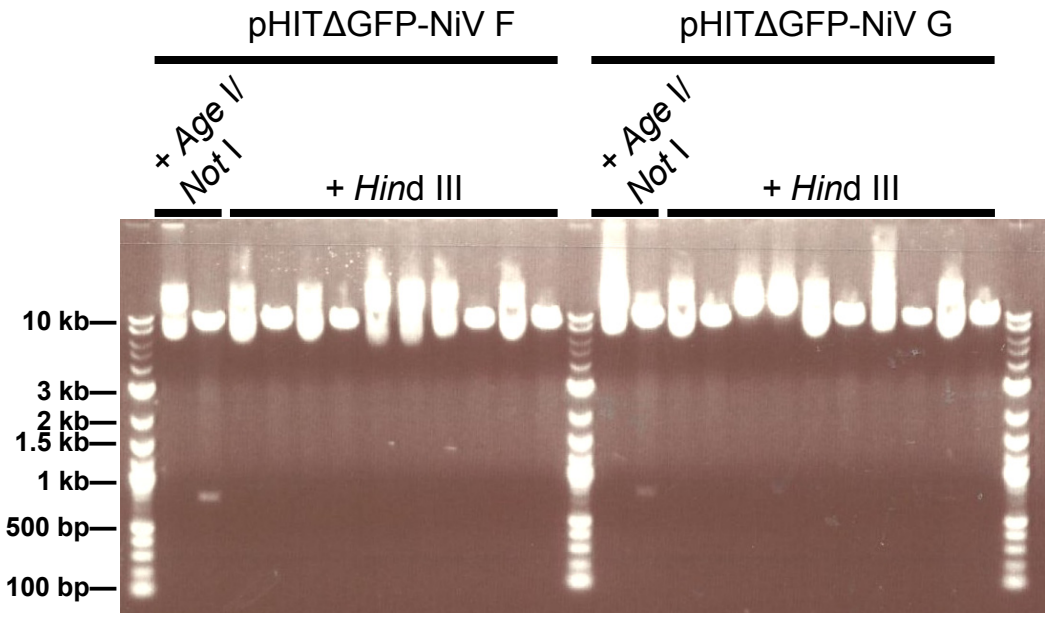

Figure 23: Creation of non-fluorescent pHITΔGFP-NiV F and pHITΔGFP-NiV G. For both plasmids, the GFP ORF was removed by digestion with *Age* I and *Not* I. The upper band consisting of the vector backbone and the glycoprotein ORF was gel extracted, filled in with Klenow fragment, and then re-ligated. Re-ligated plasmids were digested with *Hin*d III to ensure that no GFP ORF insert was present. The molecular weight markers in all gels is the 2-Log DNA Ladder (New England Biolabs).

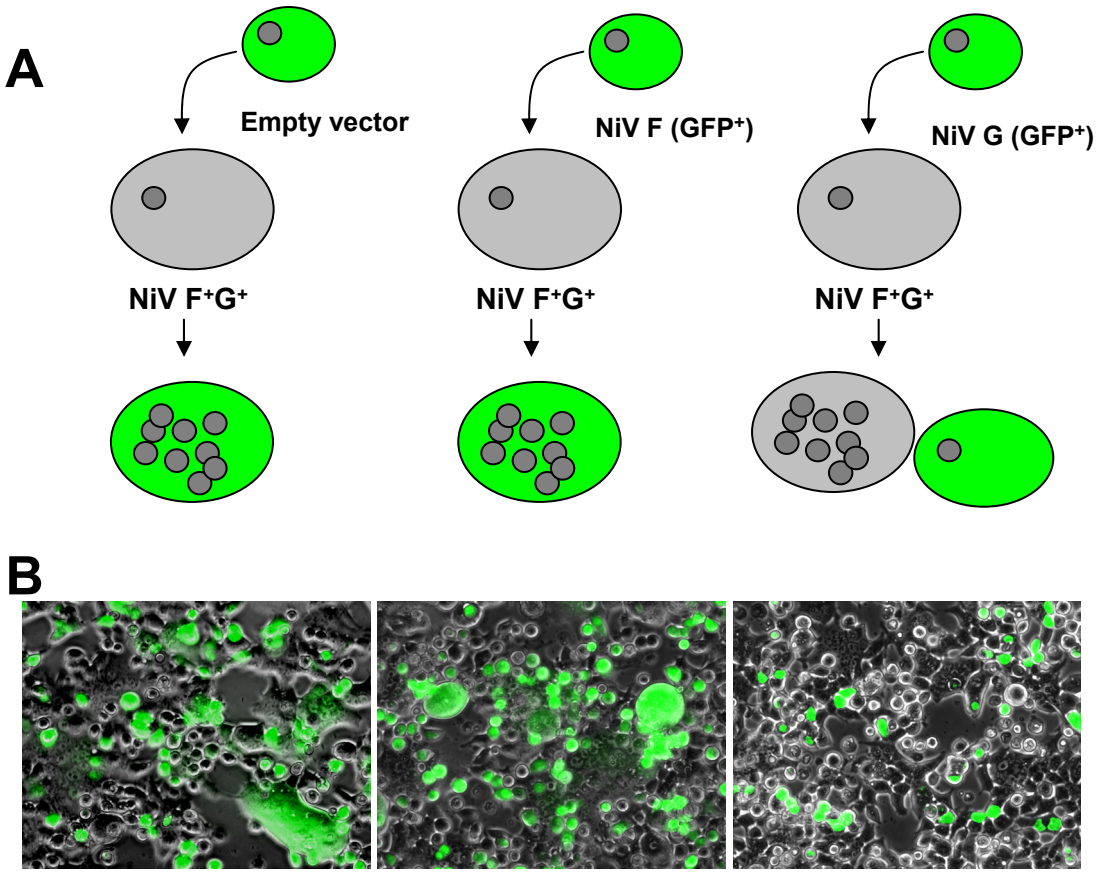

Figure 24: Expression of NiV G in target cells induces resistance to NiV F/G-mediated fusion. A) Cells transfected with either pczCFG5 IEGZ, pczCFG5-NiV F (NiV F), or pczCFG5-NiV G (NiV G) were separately mixed with a population of cells which had been co-transfected with the non-fluorescent expression plasmids pHITΔGFP-NiV F and pHITΔGFP-NiV G. B) The presence or absence of fluorescent syncytia was observed 12 hours after mixing. The brightfield view is overlaid with the green fluorescence emission view. Reproduced from Sawatsky *et al.* (2007) *J. Gen. Virol.* **88**(2):582-591.

positive for cell surface expression of ephrin-B1, ephrin-B2, and ephrin-B3 (Figs. 25 and 26, green lines). Ephrin-B1 staining was included as a control since it is known to not bind NiV G (Negrete *et al.*, 2006). In 293T cells transfected with pczCFG5-NiV G, there were no differences in cell surface levels of ephrin-B1, ephrin-B2, or ephrin-B3 (Figs. 25 and 26, blue lines). Cells transfected with pczCFG5 IEGZ (empty vector) or pczCFG5-NiV F showed no differences in the proportion of cells with cell surface ephrin-B1, ephrin-B2, or ephrin-B3 staining, indicating that expression of NiV F has no effect on cell surface levels of the ephrin-B2 and ephrin-B3 ligands (not shown in the figures). The same FACS staining also revealed that cell surface ephrin-B2 and ephrin-B3 levels were also unchanged by NiV infection (Fig. 25, brown lines). This indicates that NiV G does not have the ability in either the context of recombinant protein expression or virus infection to down-regulate ephrin-B2 and ephrin-B3.

Cloning of chimeric glycoproteins

The chimeric CDV H-NiV G glycoproteins represent the second approach in defining the NiV G functional domains. The chimeras were each amplified as separate segments by NiV G- or CDV H-gene specific primers and were then assembled in a PCR joining reaction which took advantage of specific 25-bp complementarity between the amplicons to be joined. All individual segments

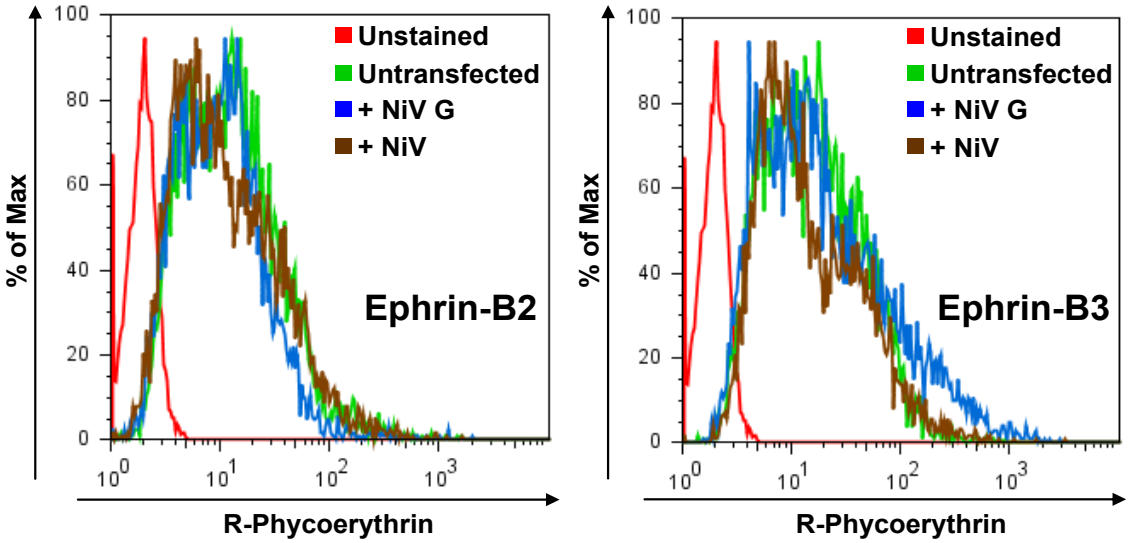

Figure 25: Ephrin-B2 and ephrin-B3 cell surface expression in the presence and absence of NiV G expression or NiV infection. Cells were fixed in 2% PBS-buffered PFA and stained with polyclonal rabbit antibodies against ephrin-B2 (left panel) or ephrin-B3 (right panel), followed by goat anti-rabbit PE conjugate. Unstained cells (red line) are shown together with untransfected (green line), NiV G-transfected (blue line), and NiV-infected (brown line) cells stained for ephrin-B2 or ephrin-B3 ligand. Reproduced from Sawatsky *et al.* (2007) *J. Gen. Virol.* **88**(2):582-591.

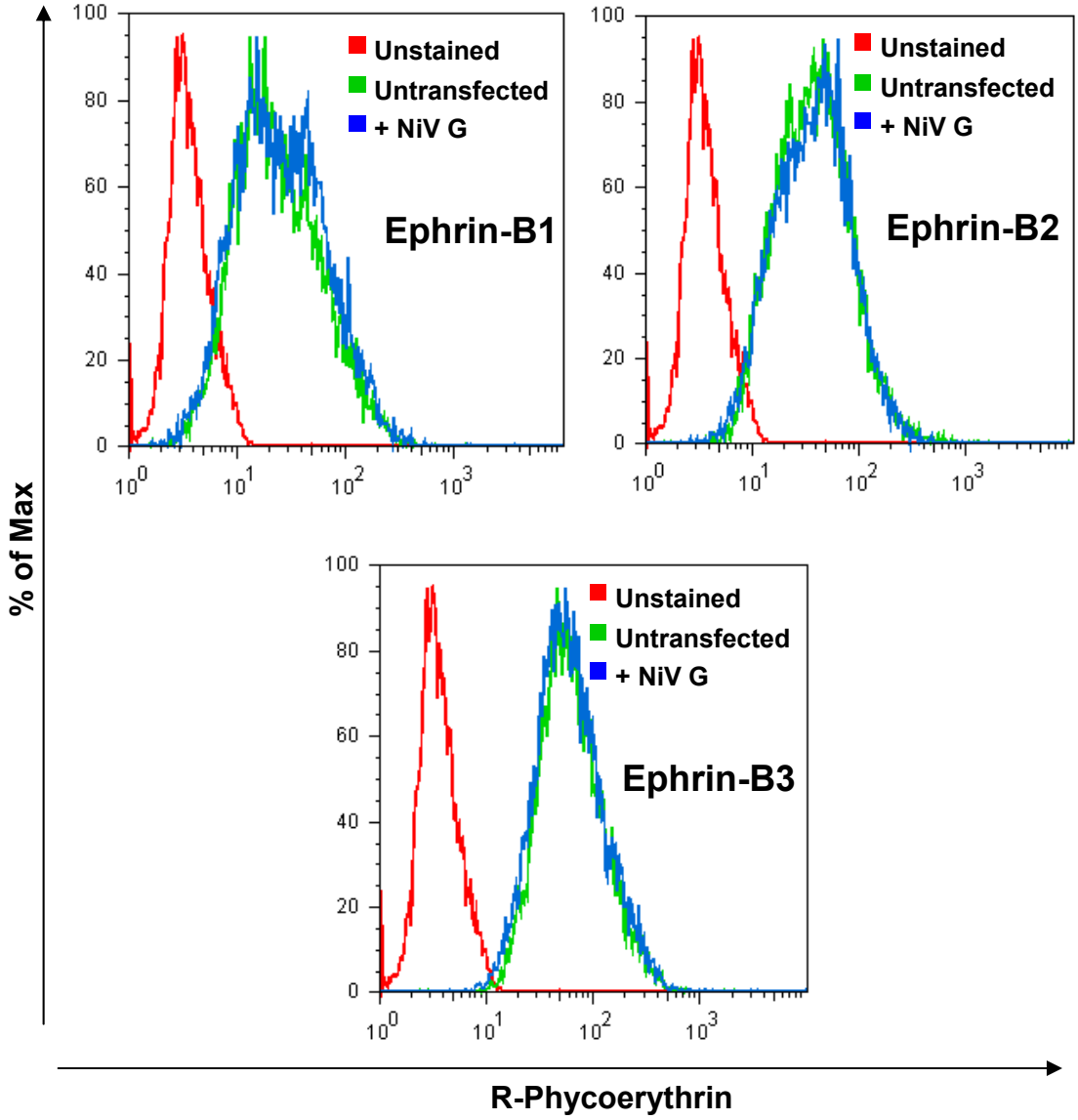

Figure 26: Ephrin-B1, ephrin-B2, and ephrin-B3 cell surface expression in the presence and absence of NiV G expression. Cells were fixed in 2% PBS-buffered PFA and stained with polyclonal rabbit antibodies against ephrin-B1 (left panel), ephrin-B2 (centre panel), or ephrin-B3 (right panel), followed by goat anti-rabbit PE conjugate. Unstained cells (red line) are shown together with untransfected (green line) and NiV G-transfected (blue line) cells stained for ephrin-B1, ephrin-B2, or ephrin-B3 ligand.

were successfully amplified (Fig. 27) and subsequently joined (Fig. 28). The full-length chimeras were then cloned into the pCR2.1-TOPO vector, generating the plasmids designated as 1+2-TOPO, 3+4-TOPO, 5+6-TOPO, 7+8-TOPO, 9+10-TOPO, and 11+12-TOPO (Fig. 29). After sequencing, mutation-free clones were picked for each chimera and the chimeras were amplified using primers to allow for cloning using the *Bam*H I and *Sph* I sites into the expression vector pCG1-IRESzeomut (Cathomen *et al.*, 1995). pCG1-IRESzeomut was selected because it has been used by many other laboratories to express paramyxovirus glycoproteins. pCG1-IRESzeomut contains a β-globin splice acceptor immediately upstream of the CMV immediate early promoter, allowing for enhanced transcript stability. The cloning was successfully performed for all chimeras, thereby generating the expression plasmids pCG-G_{145}/H_{463}, pCG-G_{338}/H_{268}, pCG-G_{498}/H_{113}, pCG-H_{145}/G_{458}, pCG-H_{340}/G_{265}, and pCG-H_{495}/G_{105} (Fig. 30). In parallel, NiV F and NiV G were both amplified from pczCFG5-NiV F and pczCFG5-NiV G and cloned into pCG1-IRESzeomut to give the expression plasmids pCG-NiV F and pCG-NiV G (Fig. 31).

Detection of chimeric glycoprotein expression

After successful cloning, the first step was to assess the expression of the chimeric glycoproteins by Western blot. The chimeras G_{145}/H_{463}, G_{338}/H_{268}, and

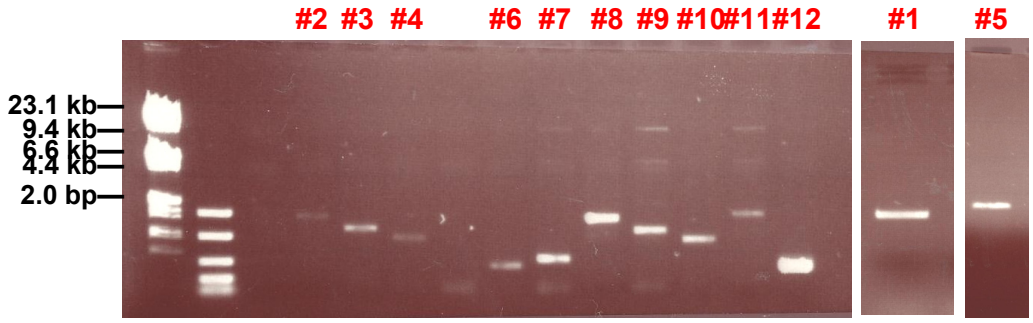

Figure 27: PCR reactions for each individual chimeric glycoprotein segment. See text for details. Additional optimization was performed for the reactions generating segments #1 and #5. The molecular weight marker is the λ phage *Hin*d III digest DNA marker (Roche).

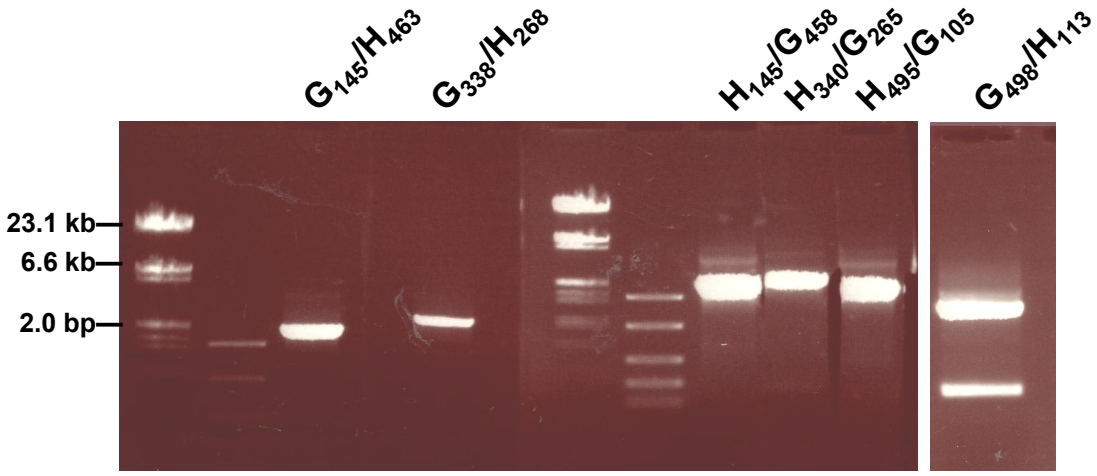

Figure 28: Joining PCR reactions showing the successful assembly of the chimeric glycoproteins. For G_{498}/H_{113}, the top band in the gel was gel extracted. The molecular weight marker is the λ phage *Hin*d III digest DNA marker (Roche).

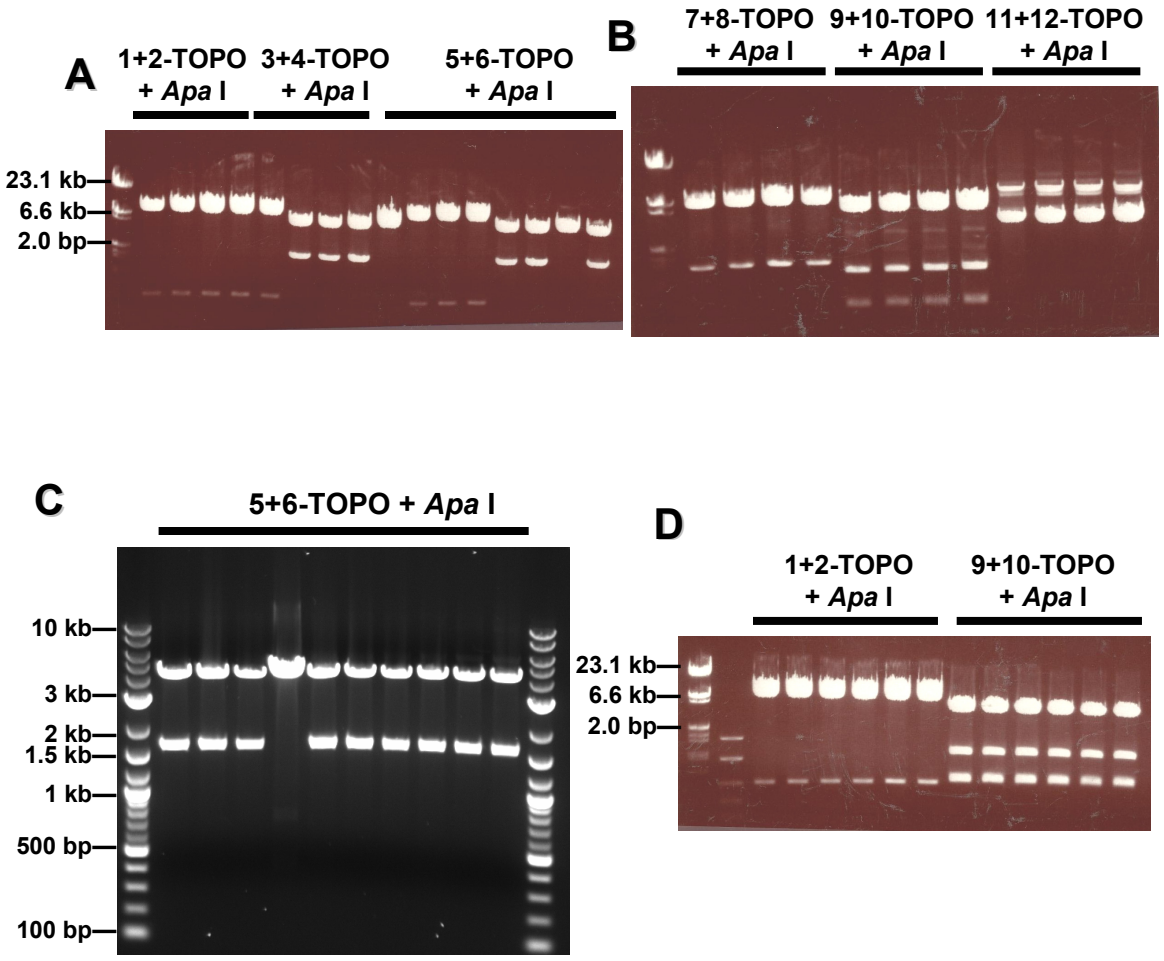

Figure 29: Gels showing the successful TOPO cloning of chimeric glycoprotein amplicons. See text for details.

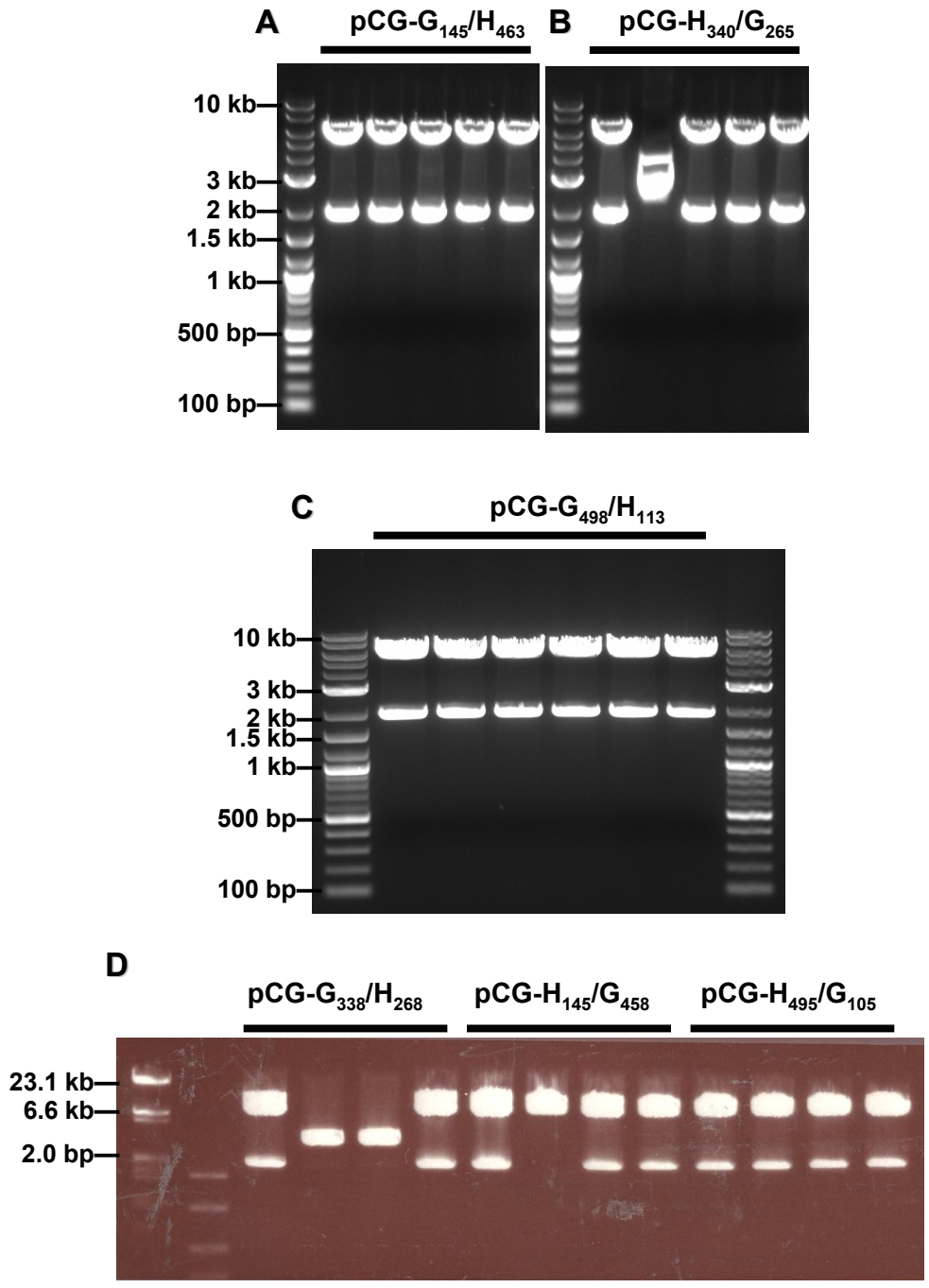

Figure 30: Cloning of chimeric glycoproteins into pCG1-IRESzeomut. The chimeric glycoprotein ORFs were amplified from the TOPO clones and cloned into the *Bam*H I and *Sph* I sites of pCG1-IRESzeomut. The gels show mini-preps of each chimeric glycoprotein clone digested with *Bam*H I and *Sph* I to confirm the presence of the insert.

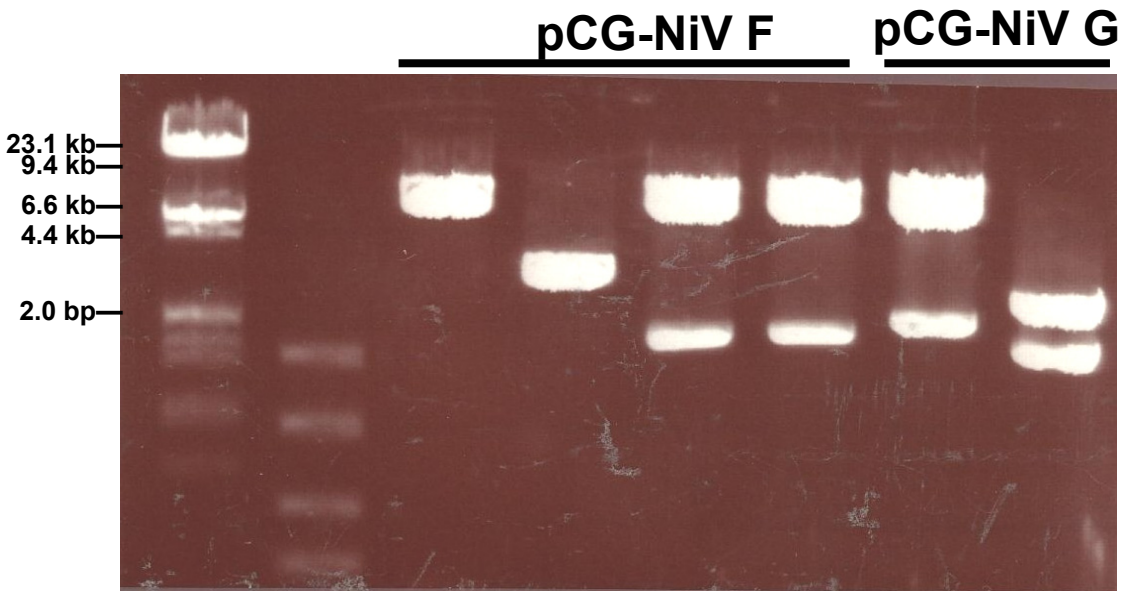

Figure 31: Cloning of NiV F and NiV G ORFs into pCG1-IRESzeomut. The NiV F and NiV G ORFs were amplified from pczCFG5-NiV F and pczCFG5-NiV G, respectively, and cloned into the *Bam*H I and *Sph* I sites of pCG1-IRESzeomut. The gel shows pCG-NiV F and pCG-NiV G mini-preps digested with *Bam*H I and *Sph* I to confirm the presence of the insert.

G_{498}/H_{113}, all bearing the N-terminal region (cytoplasmic, transmembrane domain, and stalk) and varying portions of the globular head of NiV G, were all successfully detected by the swine anti-NiV G serum, indicating cellular expression of these proteins (Fig. 32A). These chimeras were not detected by the rabbit anti-CDV H_{cyt} antibody, since this antibody was raised against the cytoplasmic tail of CDV H (Fig. 32B). The chimeras H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105}, all bearing the N-terminal region and varying portions of the globular head of CDV H, were all detected by the rabbit anti-CDV H_{cyt} antibody (Fig. 32B). Surprisingly, the swine anti-NiV G serum, which had successfully recognized the N-terminus of NiV G, was unable to detect any of the chimeras with the CDV H C-terminus (Fig. 32A). Nevertheless, all chimeras appeared to be expressed and were of approximately the correct size (~70 kDa).

Co-expression of chimeric glycoproteins with NiV F and CDV F

Having successfully demonstrated that the chimeric glycoproteins were expressed by Western blot, it was necessary to determine whether they retained some of their native functions (i.e. fusion promotion, receptor binding, interaction with NiV F or CDV F). First, each chimera was co-expressed with pCG-NiV F or pCG-F5804PZeo. Combinations of pCG-NiV F+pCG-NiV G and pCG-F5804PZeo+pCG-H5804PZeo were included as positive controls for fusion,

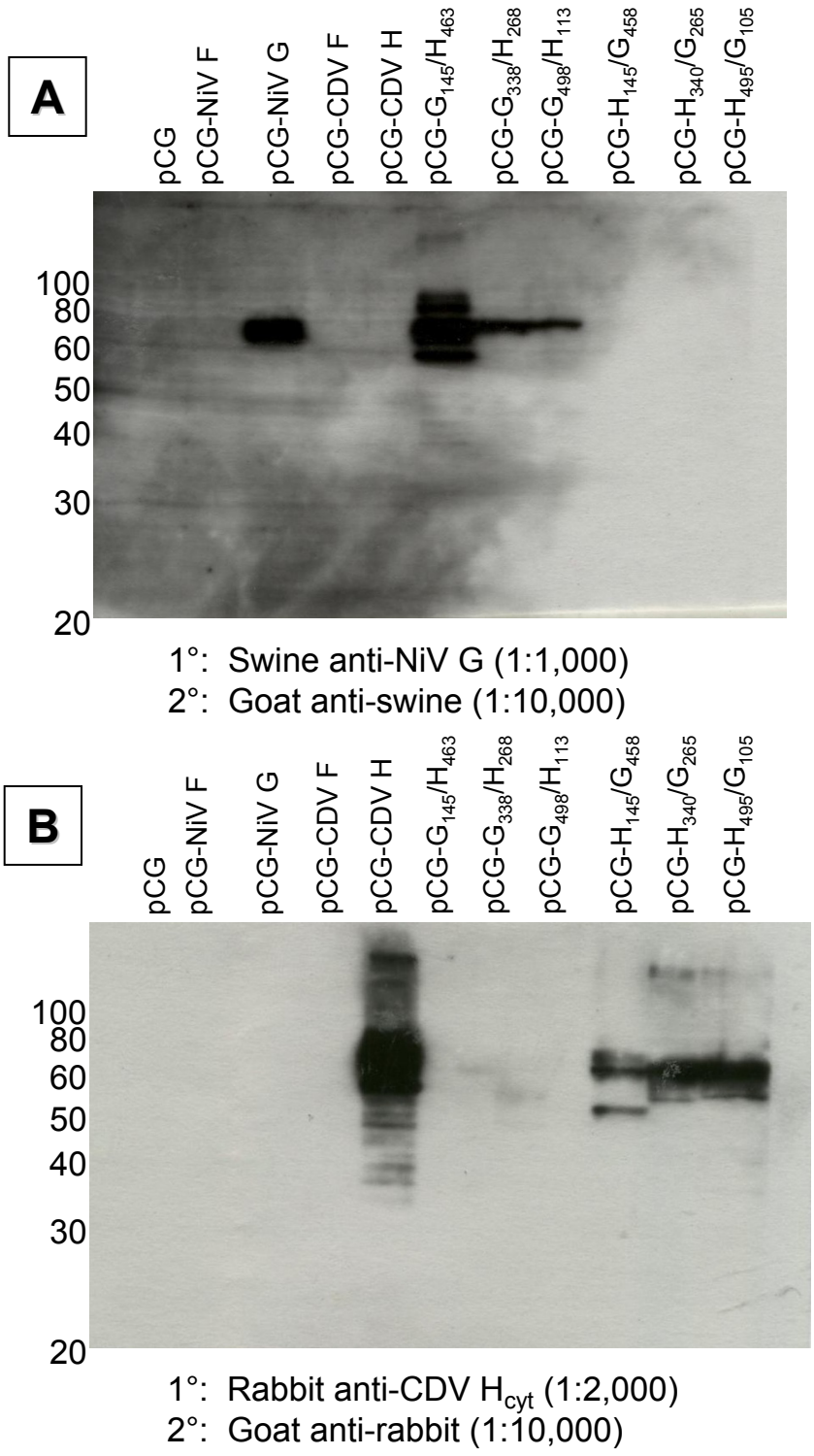

Figure 32: Expression of chimeric glycoproteins. Chimeric glycoprotein expression plasmids were transfected into 293T cells and total cell lysates were harvested at 48 hours post-transfection. A) Western blot using swine anti-NiV G serum. B) Western blot using rabbit anti-CDV H_{cyt} peptide antiserum.

while combinations of pCG-NiV F+pCG-H5804PZeo and pCG-F5804PZeo+pCG-NiV G were included as negative fusion controls when the heterotypic glycoproteins are expressed. While the positive controls of NiV F+NiV G and CDV F+CDV H generated extensive fusion after 48 hours (Fig. 33), none of the chimeras was able to complement either NiV F or CDV F and induce fusion (Fig. 33). As expected, neither combination of NiV F+CDV H nor CDV F+NiV G induced fusion (Fig. 33), indicating that the heterologous glycoprotein combinations are not functional with respect to fusion.

Influence of NiV M on fusion

The matrix proteins of paramyxoviruses play an important role in the assembly of virions during infection. Co-expression of NiV M with each combination of CDV F or NiV F and one of the chimeric glycoproteins was attempted to determine whether NiV M could influence the fusion activity of the chimeras. In all experiments that were attempted, the additional expression of NiV M could not force fusion from any of the chimeric glycoproteins (Fig. 34).

Cell surface IFA staining of H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105}

Results show that H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105} are not recognized by the pig 30 anti-NiV serum in a Western blot. Additionally, although the

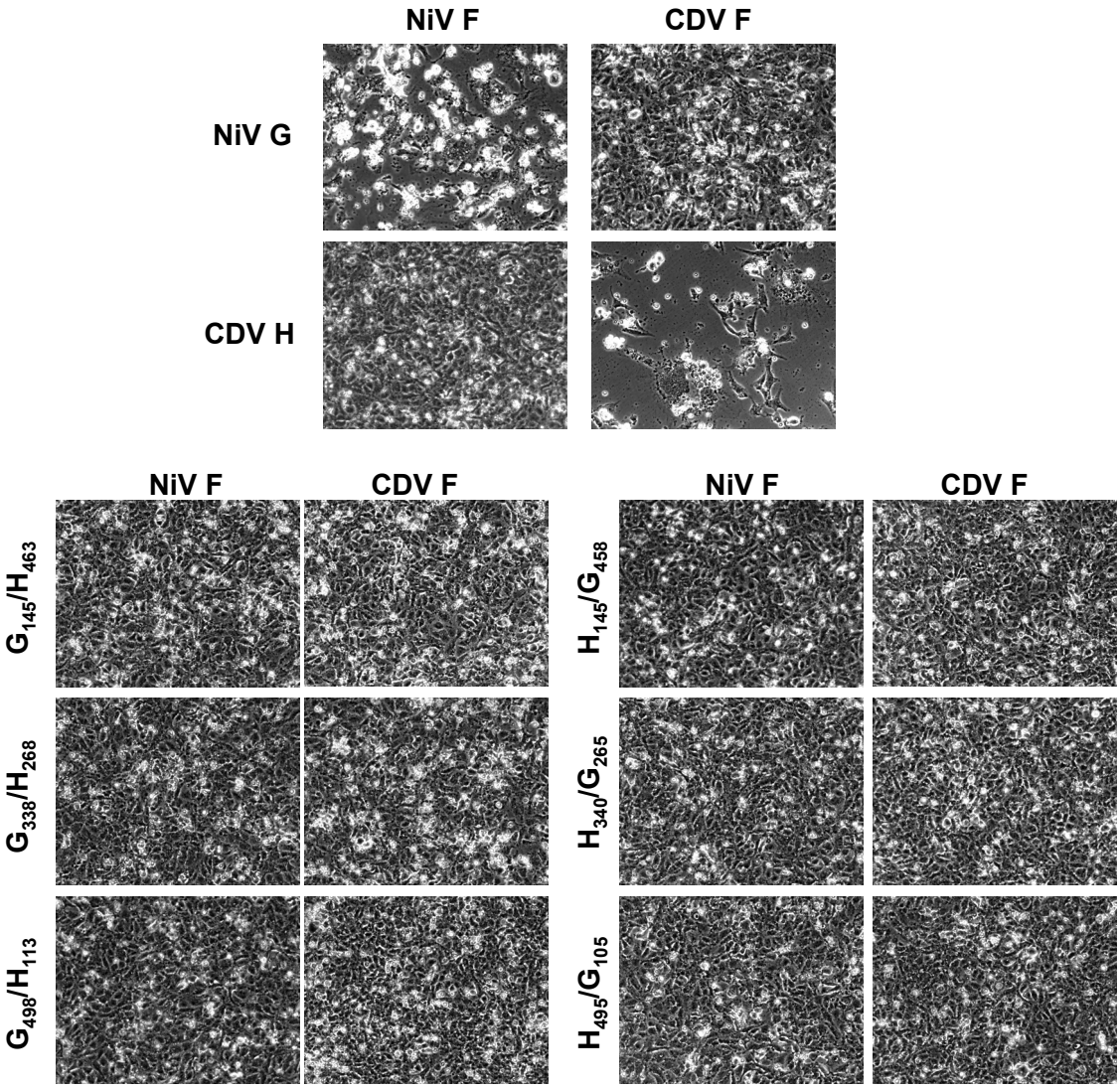

Figure 33: Chimeric glycoproteins are unable to induce fusion when co-expressed with NiV F or CDV F. Chimeric glycoproteins were co-expressed with either NiV F or CDV F in Vero dogSLAMtag cells and examined by light microscopy for evidence of fusion. The homologous combinations of NiV F+NiV G and CDV F+CDV H were included as positive controls for fusion, while the heterologous combinations of NiV F+CDV H and CDV F+NiV G were included as negative controls for lack of fusion.

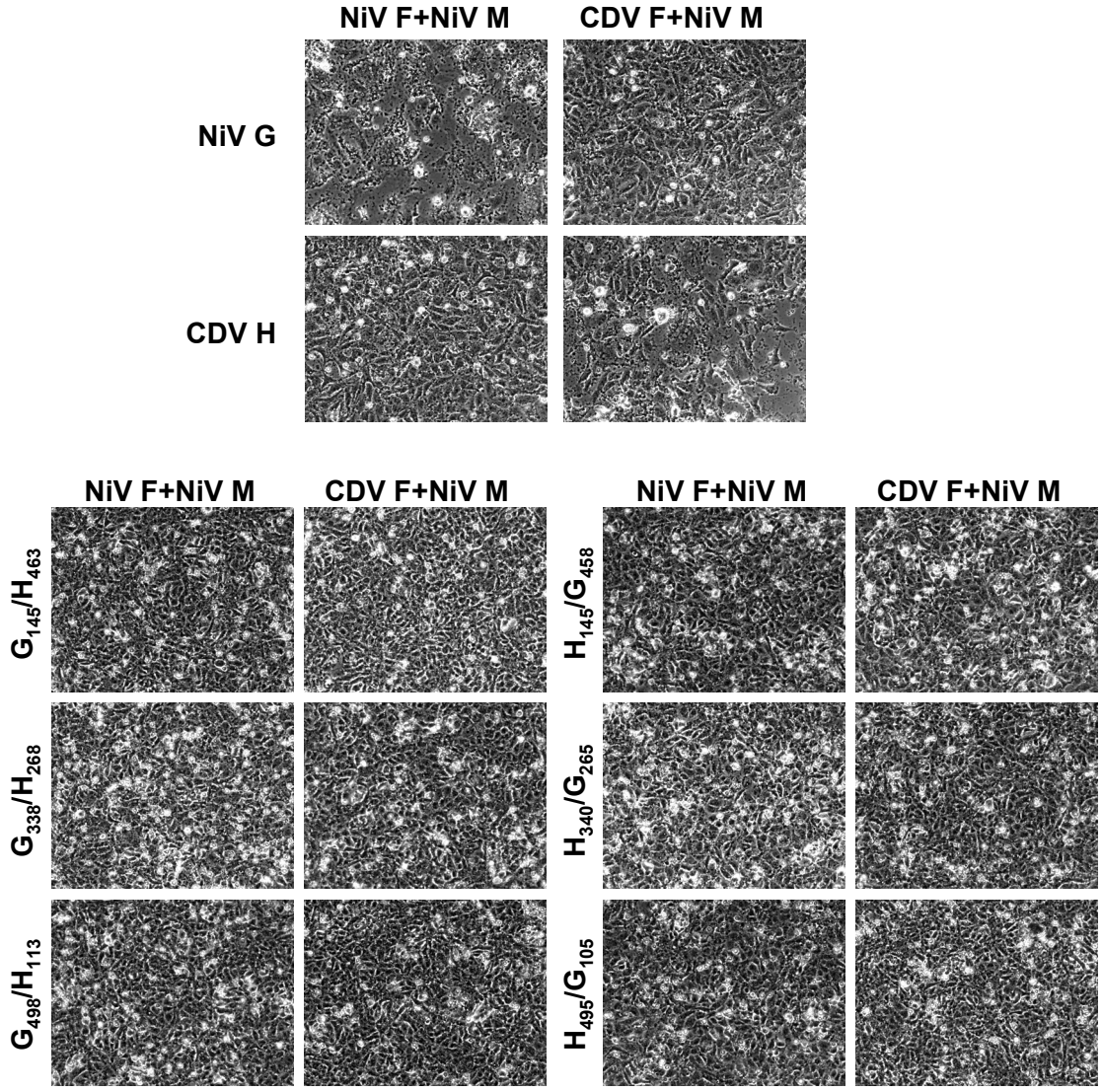

Figure 34: Chimeric glycoproteins are unable to induce fusion when co-expressed with NiV F or CDV F and NiV M. Chimeric glycoproteins were co-expressed with either NiV F or CDV F and NiV M in Vero dogSLAMtag cells and examined by light microscopy for evidence of fusion. The homologous (NiV F+NiV G and CDV F+CDV H) and heterologous (NiV F+CDV H and CDV F+NiV G) combinations were included positive and negative fusion controls, respectively. pCG-NiV M was included in all transfections to determine whether NiV M has an effect on fusion of the chimeric glycoproteins.

glycoproteins are expressed, none of them is able to mediate cell-cell membrane fusion in either the presence or absence of NiV M. In an attempt to determine whether H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105} are expressed on the cell surface, cells were transfected with expression plasmids for these chimeric glycoproteins and IFA was performed using two other swine anti-NiV sera from pigs 36 and 38. H_{145}/G_{458} was detected on the cell surface by IFA using both anti-NiV sera from pigs 36 and 38 (Fig. 35, bottom left and right panels), whereas H_{340}/G_{265} and H_{495}/G_{105} were not detected on the cell surface (not shown). Control staining with NiV G also showed comparable cell surface expression when detected with anti-NiV sera from pigs 36 and 38 (Fig. 35, top left and right panels). However, all of these chimeric glycoproteins were detected when fixed cells were permeabilized with 0.1% Triton X-100 which opens pores in the membranes of fixed cells allowing for intracellular staining (not shown).

Assessment of cell surface ephrin-B2 and ephrin-B3 expression

Finally, transfected 293T cells were stained for cell surface ephrin-B2 and ephrin-B3 to determine whether the chimeric glycoproteins had different receptor down-regulation properties than full-length NiV G. As shown earlier, expression of full-length membrane anchored NiV G does not result in the down-regulation of NiV G from the cell surface, but the replacement of different

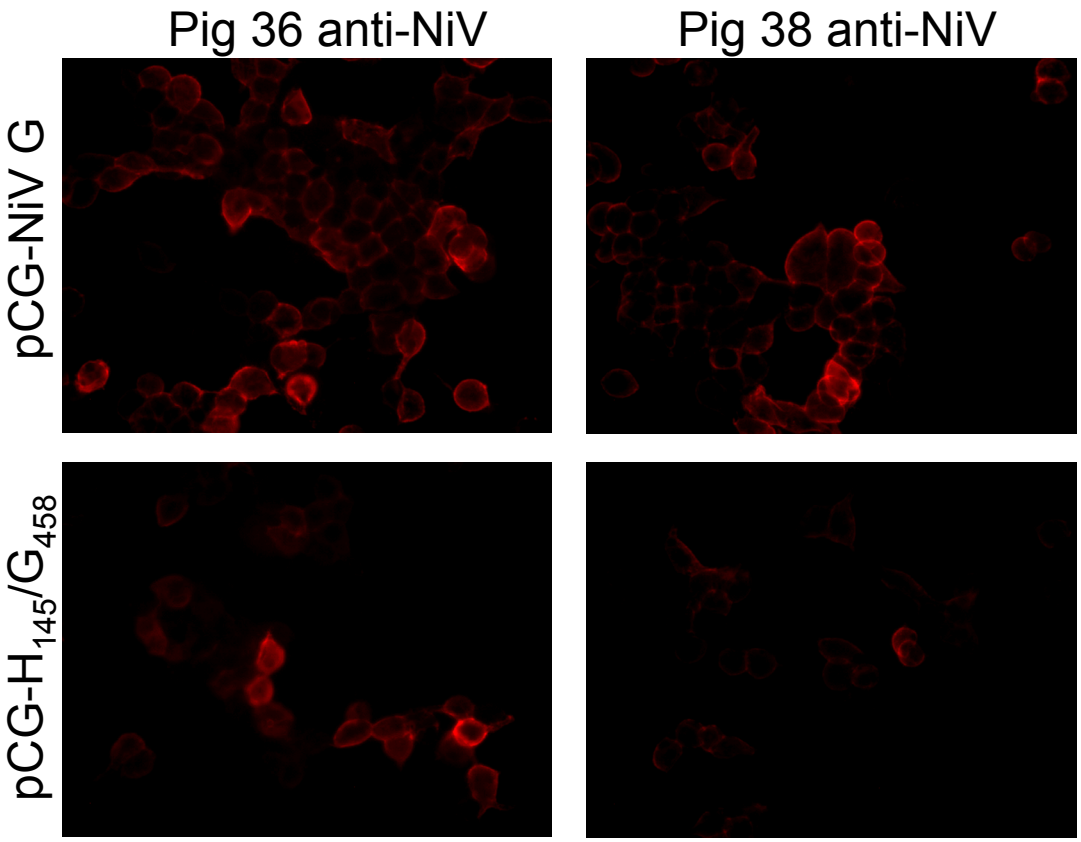

Figure 35: H_{145}/G_{458} is found on the cell surface by immunofluorescence assay (IFA). 293T cells were transfected with the expression plasmids pCG-NiV G or pCG-H_{145}/G_{458} and then fixed in 2% PBS-buffered PFA and stained with anti-NiV sera from either pig 36 (left top and bottom panels) or pig 38 (right top and bottom panels), followed by an anti-swine biotin conjugate and a streptavidin-PE conjugate fluorophore. Cells transfected with H_{340}/G_{265} and H_{495}/G_{105} were negative for cell surface staining with anti-NiV sera from both pigs 36 and 38.

functional domains of NiV G with those from CDV H may influence that behaviour and therefore whether the chimeric glycoproteins would down-regulate cell surface levels of viral receptor. For this experiment, 293T cells were transfected with one of the chimeric glycoproteins or pCG1-IRESzeomut as a negative vector control, pCG-NiV G as a positive control for NiV G expression, or pCG-H5804PZeo as a negative control for glycoprotein specificity. After cell surface staining for ephrin-B2 and ephrin-B3 expression, cells were analyzed on a BD FACSCalibur flow cytometer. Cell surface levels of ephrin-B2 and ephrin-B3 were unchanged whether cells expressed NiV G or CDV H. In this respect, the chimeric glycoproteins seem to behave in a similar manner to the full-length NiV G glycoprotein. Expression of NiV F, NiV G, or CDV H had not effect on ephrin-B2 and ephrin-B3 (Fig. 36). Expression of chimeric glycoproteins with the C–terminus of NiV G (G_{145}/H_{463}, G_{338}/H_{268}, and G_{498}/H_{113}) had no effect on ephrin-B2 and ephrin-B3 (Fig. 37), and neither did expression of chimeric glycoproteins with the N-terminus of CDV H (H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105}) (Fig. 38).

Rescue of rCDV eGFPH NiVFG

Many attempts were made to recover rCDV eGFPH, rCDV eGFPH NiVFG, and rCDV eGFPH NiVMFG, while fewer attempts were made to recover

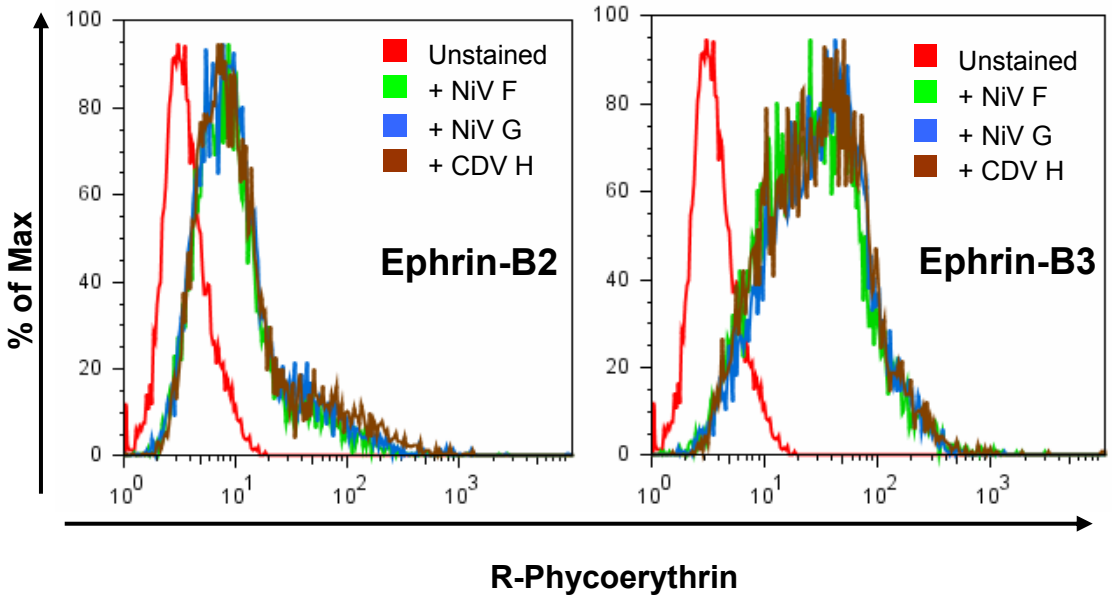

Figure 36: Ephrin-B2 and ephrin-B3 cell surface expression in the presence and absence of CDV H expression. Cells were fixed in 2% PBS-buffered PFA and stained with polyclonal rabbit antibodies against ephrin-B2 (left panel) or ephrin-B3 (right panel), followed by goat anti-rabbit PE conjugate. Unstained cells (red line) are shown together with NiV F-transfected (green line), NiV G-transfected (blue line), and CDV H-transfected (brown line) cells stained for ephrin-B2 or ephrin-B3 ligand.

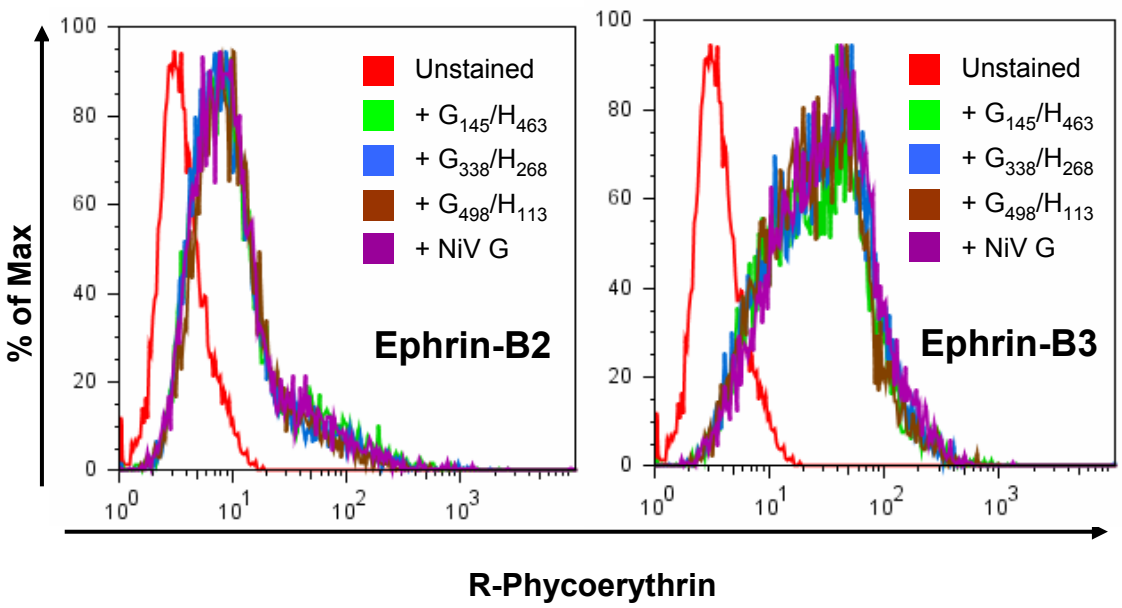

Figure 37: Ephrin-B2 and ephrin-B3 cell surface expression in the presence and absence of chimeric glycoprotein expression. Cells were fixed in 2% PBS-buffered PFA and stained with polyclonal rabbit antibodies against ephrin-B2 (left panel) or ephrin-B3 (right panel), followed by goat anti-rabbit PE conjugate. Unstained cells (red line) are shown together with G_{145}/H_{463}-transfected (green line), G_{338}/H_{268}-transfected (blue line), or G_{498}/H_{113}-transfected (brown line) cells stained for ephrin-B2 or ephrin-B3 ligand. Cells transfected with NiV G (purple line) are shown as controls for both ephrin-B2 and ephrin-B3 staining.

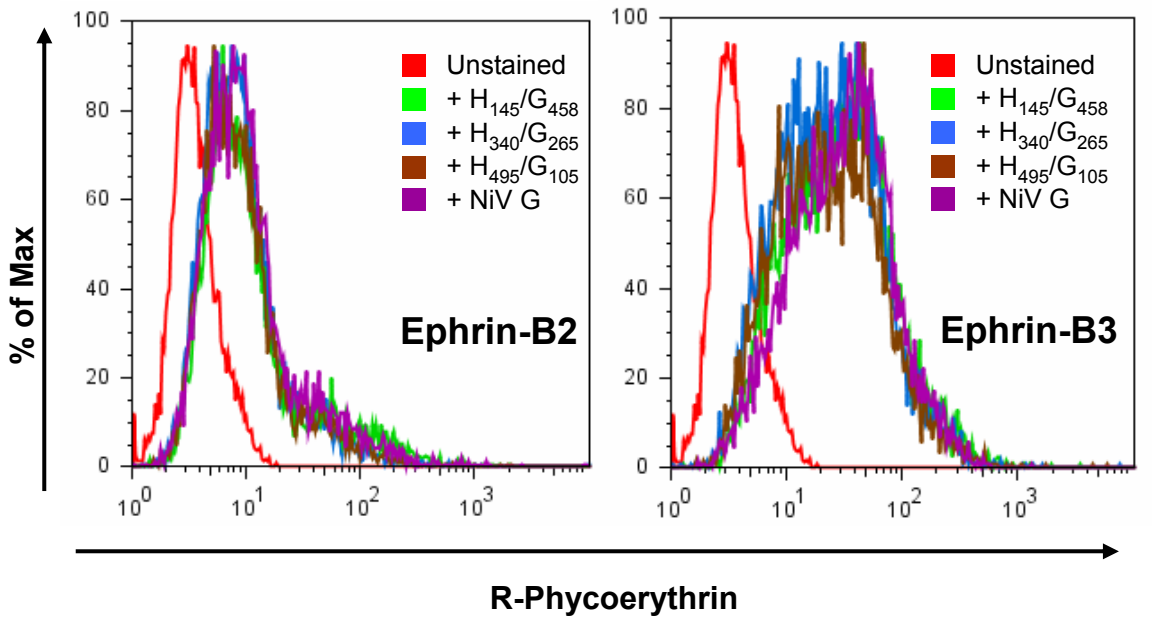

Figure 38: Ephrin-B2 and ephrin-B3 cell surface expression in the presence and absence of chimeric glycoprotein expression. Cells were fixed in 2% PBS-buffered PFA and stained with polyclonal rabbit antibodies against ephrin-B2 (left panel) or ephrin-B3 (right panel), followed by goat anti-rabbit PE conjugate. Unstained cells (red line) are shown together with H_{145}/G_{458}-transfected (green line), H_{340}/G_{265}-transfected (blue line), or H_{495}/G_{105}-transfected (brown line) cells stained for ephrin-B2 or ephrin-B3 ligand. Cells transfected with NiV G (purple line) are shown as controls for both ephrin-B2 and ephrin-B3 staining.

rCDV eGFPM, rNiV, and rNiV eGFPG. The genomic plasmids digests with *Bam*H I for p5804P eGFPH (7 bp, 455 bp, 1377 bp, 2493 bp, 2875 bp, 4893 bp, and 7999 bp), p5804P eGFPH NiVFG (7 bp, 455 bp, 1377 bp, 2875 bp, 7026 bp, and 7999 bp), and p5804P eGFPH NiVMFG (7 bp, 455 bp, 1377 bp, 1627 bp, 2875 bp, 5453 bp, and 7999 bp) all gave the correct band pattern (Fig. 39). To date, attempts to rescue rCDV eGFPM, rCDV eGFPH, and rCDV eGFPH NiVMFG have been unsuccessful. rNiV and rNiV eGFPG rescues were attempted with both CMV-driven and MVA-T7 RNAP-driven systems, but with no success thus far. The combination of 1 μL of pTM1-CDV N, 1 μL of pTM1-CDV P, and 0.5 μL of pTM1-CDV L along with 7 μg of rCDV eGFPH NiVFG genome resulted in a successful virus rescue. Vero E6 cells with the 293T overlay were incubated at 37˚C for 6 days. At this point, there were some smaller syncytia-like structures visible in the cell monolayer by light microscopy, but there was no obvious CPE. The cells were split 1:2 into two new 10-cm dishes, and after 2 days extensive syncytia had developed. Vero E6 cells were not ready for virus isolation, so the cell cultures were frozen at -80˚C and then thawed when cells were ready for isolation. Inoculation of supernatants from these freeze-thawed cells resulted in the development of extensive syncytia in Vero E6 cells, thus confirming that the CPE-causing material could be transferred to new cultures. These cells were fixed and brought out of BSL4 to check for eGFP expression. Large syncytia

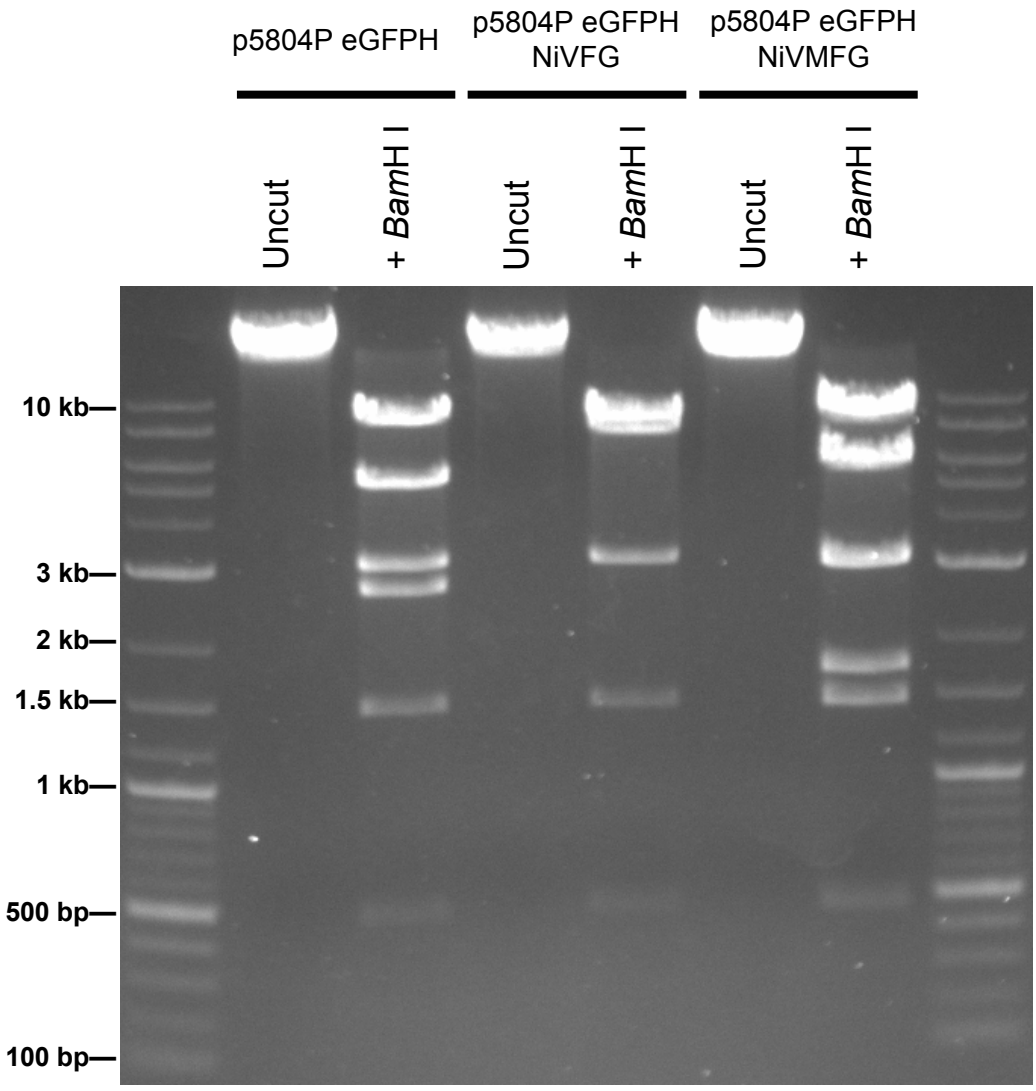

Figure 39: Restriction digests of recombinant CDV genome plasmids. The plasmids p5804P eGFPH, p5804P eGFPH NiVFG, and p5804P eGFPH NiVMFG were digested with *Bam*H I to check for genome integrity.

Expected digestion fragments:

p5804P eGFPH 7 bp, 455 bp, 1377 bp, 2493 bp, 2875 bp, 4893 bp, and 7999 bp

p5804P eGFPH NiVFG 7 bp, 455 bp, 1377 bp, 2875 bp, 7026 bp, and 7999 bp

p5804P eGFPH NiVMFG 7 bp, 455 bp, 1377 bp, 1627 bp, 2875 bp, 5453 bp, and 7999 bp.

developed (Fig. 40, top row) that also expressed high levels of eGFP (Fig. 40, middle row), indicating that these regions of CPE were due to a virus that induced fusion and also expressed eGFP. The culture supernatants from Vero E6 these cells were used to grow virus stocks of the chimeric rCDV eGFPH NiVFG virus. Four separate stocks were grown from these initial seed stocks. Stocks 1, 2, and 4 all had titers of between 10^4 and 10^5 as determined by $TCID_{50}$, while stock 3 had a titer of 10^5 $TCID_{50}$/mL.

RT-PCR of rCDV eGFPH NiVFG stock supernatants

The viral stocks were tested for the presence of the appropriate nucleic acids that would indicate the presence of rCDV eGFPH NiVFG. To that end, RT-PCR reactions were performed on RNA isolated from the viral stocks. Specific primers sets were used to check for the presence of CDV N, CDV H, NiV N, NiV M, and NiV G RNA. The RT-PCR reactions for CDV N and NiV G should be positive, while those for CDV H, NiV N, and NiV M should all be negative, since these genes are not present in the genomic clone. Indeed, amplicons of the correct sizes were observed by RT-PCR for both CDV N and NiV G (Fig. 41, CDV N and NiV G lanes for Stocks 1-4), but no specific amplicons were generated by primers directed against CDV H, NiV N, or NiV M (Fig. 41). There was some non-specific product generated by the NiV N primer set, but the PCR product

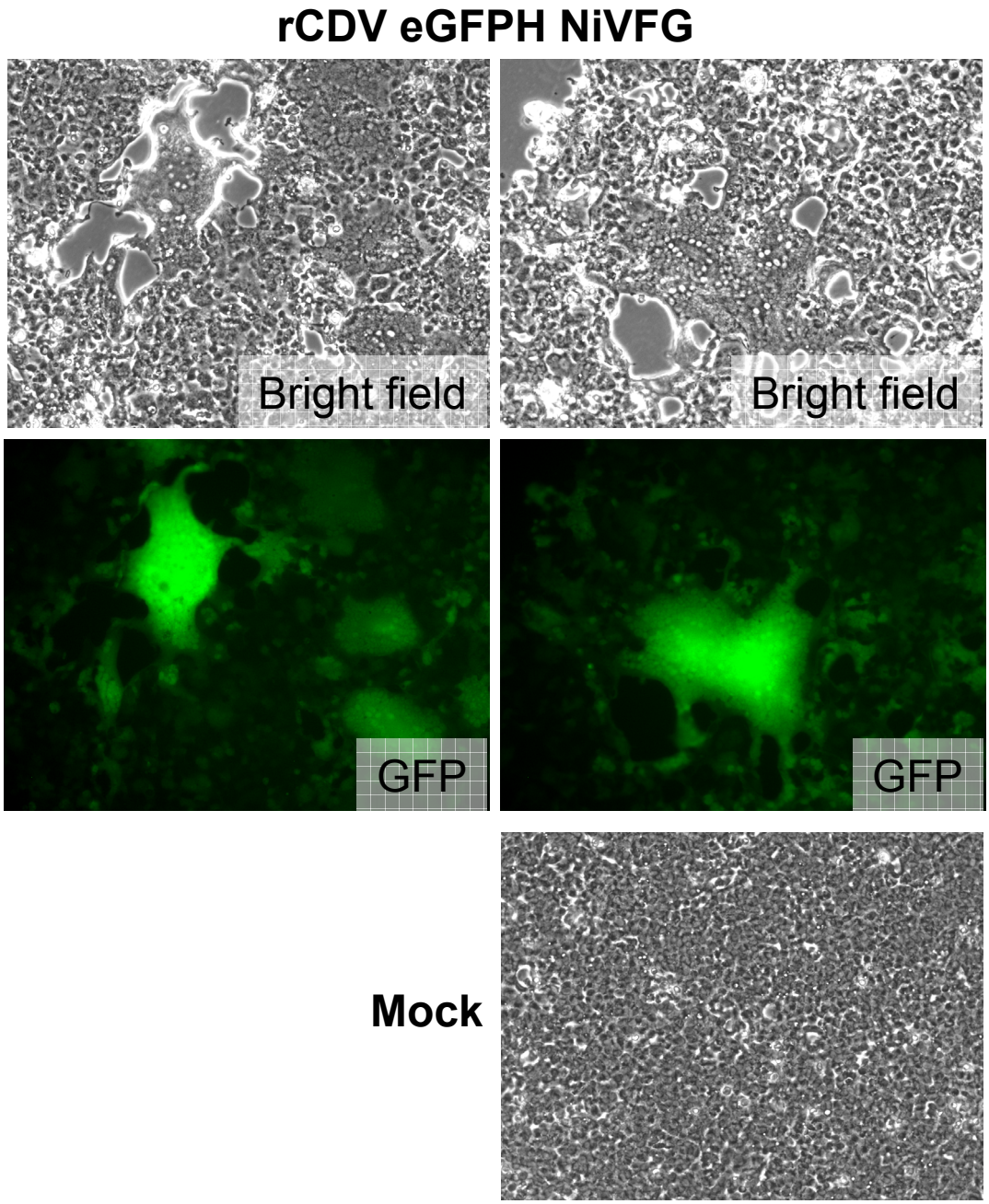

Figure 40: Infection of Vero E6 cells by rCDV eGFPH NiVFG. Vero E6 cells were infected with rCDV eGFPH NiVFG and fixed in 2% PBS-buffered PFA after six days. Cells were examined for CPE by bright field microscopy (top left and right panels) as well as by fluorescence microscopy (middle left and right panels). Mock-infected cells (bottom panel) are shown as a control.

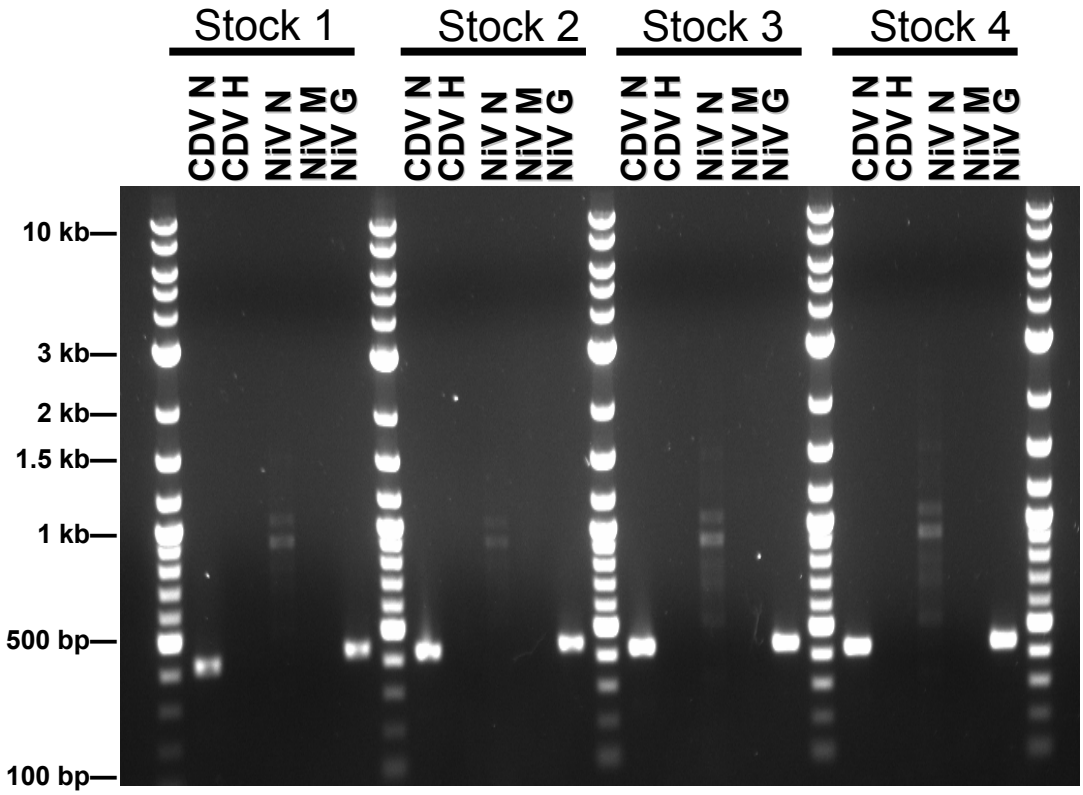

Figure 41: RT-PCR of rCDV eGFPH NiVFG stock supernatants. Total RNA was extracted from the superantant of each rCDV eGFPH NiVFG stock (#1-4) and assessed by RT-PCR for the presence of CDV H, NiV N, and NiV M nucleic acid (negative controls) and CDV N and NiV G nucleic acid to confirm the presence of chimeric virus in the supernatant.

was far larger than the expected specific NiV N product. This clearly confirms that the infectious agent that induced the GFP-expressing syncytia in Vero E6 cultures was rCDV eGFPH NiVFG, and not another contaminating virus.

Detection of orthopoxvirus DNA in rCDV eGFPH NiVFG supernatants

The viral stocks were also assayed for the presence of MVA-T7 DNA using primers directed against the HA and crmB genes of orthopoxviruses. These primers are able to discriminate between HA and crmB sequences of orthopoxviruses based on both the size of the amplified fragment and the restriction fragment length polymorphism (RFLP) pattern when digested with specific restriction enzymes (Carletti *et al.*, 2005). The purpose for this experiment, however, was simply to determine whether poxvirus DNA was present in the viral stocks that had been prepared. Stocks 1, 3, and 4 had very strong poxvirus crmB signals, while the crmB signal of stock 2 was slightly weaker (Fig. 42). Stock 4 had a very strong signal for poxvirus HA, while the poxvirus HA signals of stocks 1 and 3 were considerably weaker (Fig. 42). No poxvirus HA amplicon could be detected in stock 2 (Fig. 42). It is unclear, however, whether this is contaminating MVA-T7 in the supernatant or simply DNA carryover during viral passages.

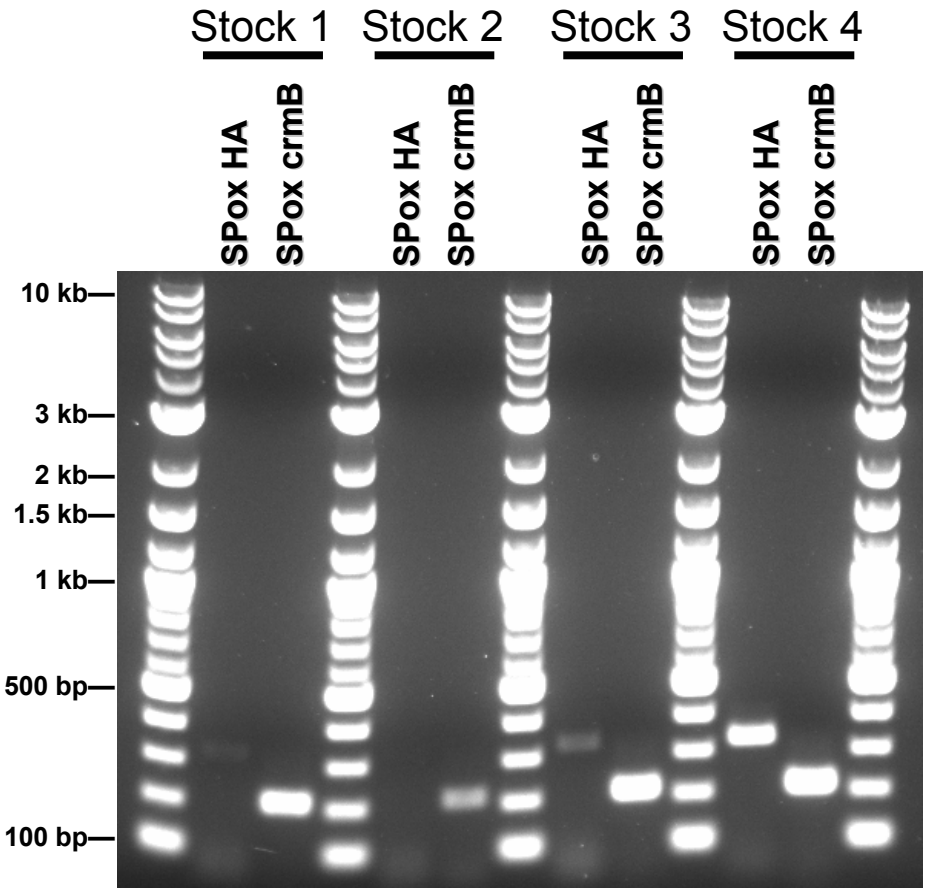

Figure 42: PCR of rCDV eGFPH NiVFG stock supernatants to test for poxvirus DNA. Total RNA was extracted from the supernatant of each rCDV eGFPH NiVFG stock (#1-4) and assessed by PCR for the presence of poxvirus HA and crmB nucleic acid, which are both present in MVA-T7 during virus rescue.

Assessment of rCDV NiVFG release from infected cells

We next sought to determine whether the release of rCDV eGFPH NiVFG is impaired in different cell lines. This might be due to the inability of CDV M to interact with the cellular budding machinery in BHK-21 cells. NiV is able to infect BHK-21 cells very efficiently and produce infectious progeny virus which can then be used in further rounds of infection. We took advantage of the fact that rCDV eGFPH NiVFG has the exterior glycoproteins of NiV and the interior protein of CDV, including CDV M. Therefore, rCDV eGFPH NiVFG will be able to infect BHK-21 cells, but if CDV M impairs virus release from BHK-21 cells due to its inability to mediate budding, there should be very little progeny virus released from these infected cells. Vero dogSLAMtag, BHK-21, and CHO-K1 cells were all efficiently infected by NiV and all of the supernatants from these cell lines contained infectious virus which could be transferred to fresh cell cultures. Over the course of the experiment, all of the NiV-infected wells were completely infected and totally destroyed. Vero dogSLAMtag, BHK-21, and CHO-K1 cells were all able to be infected with rCDV eGFPH NiVFG and produced extensive CPE manifested as syncytia. None of the supernatants from rCDV eGFPH NiVFG-infected Vero dogSLAMtag, BHK-21, and CHO-K1 cells, however, were able to induce syncytia after 5-6 days of incubation with Vero dogSLAMtag cells (Fig. 43, left column). There was no evidence of eGFP

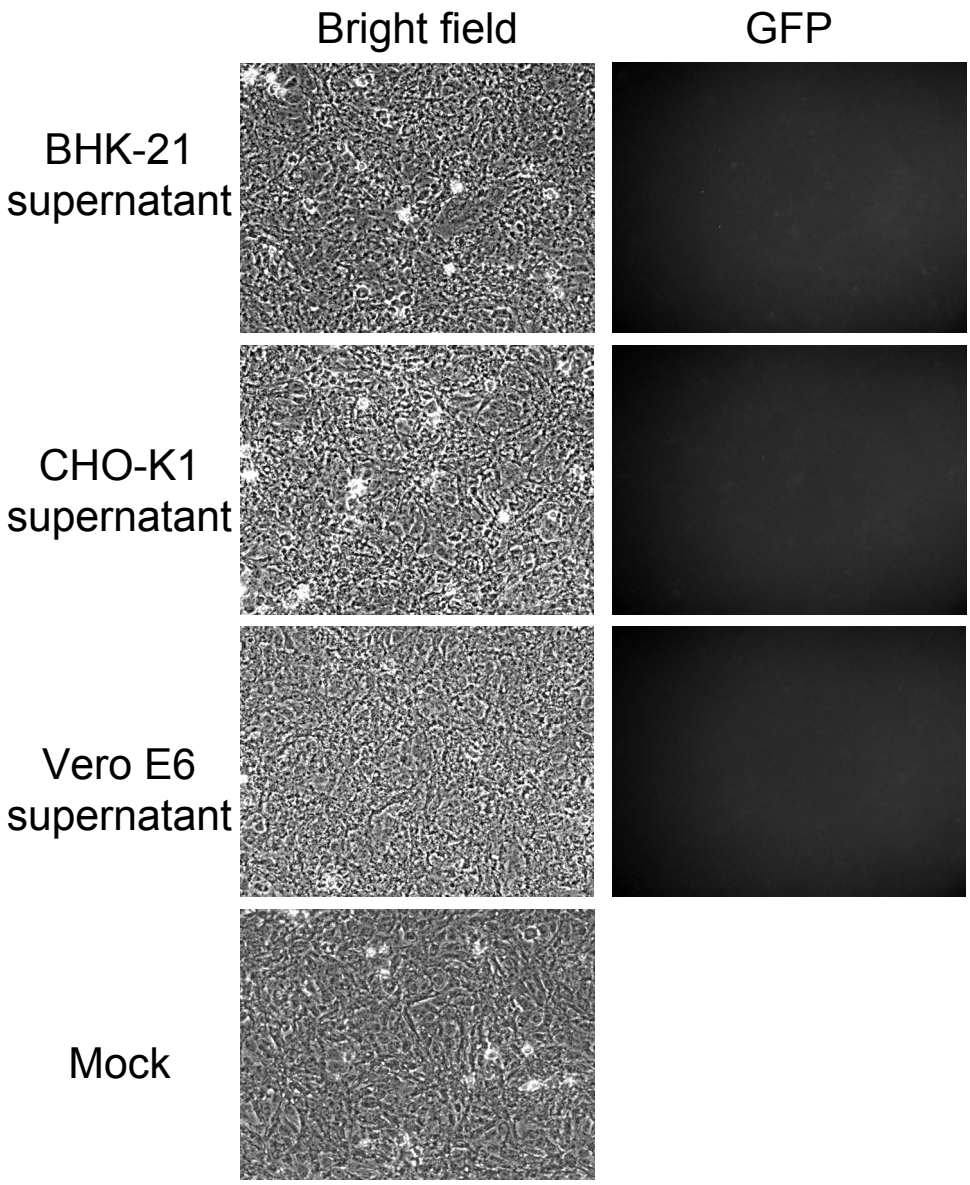

Figure 43: Assessment of rCDV eGFPH NiVFG release. BHK-21, CHO-K1, and Vero E6 cells were infected with rCDV eGFPH NiVFG and the cells were allowed to proceed to complete CPE. The supernatants from each infected cell line were harvested and applied to Vero dogSLAMtag cells and incubated for one week, at which time the cells were fixed with 2% PBS-buffered PFA and examined by bright field microscopy for CPE (top row) and by fluorescence microscopy for GFP expression (middle row), which would be indicative of rCDV eGFPH NiVFG infection. A bright field image of mock-infected Vero dogSLAMtag cells is shown as a control.

expression that would indicate an infection in any of the Vero dogSLAMtag wells that had been exposed to supernatants from rCDV eGFPH NiVFG-infected Vero dogSLAMtag, BHK-21, or CHO-K1 cells (Fig. 43, right column). While this is perhaps not surprising for BHK-21 supernatants, it is quite surprising for Vero dogSLAMtag and CHO-K1 cells. These results would seem to indicate that release of rCDV eGFPH NiVFG from all of these cells is generally impaired to quite a high degree. Further experiments with rCDV eGFPH NiVMFG would clarify the role of CDV M and NiV M in the release of chimeric virus from infected cells, while experiments with either rCDV eGFPM or rCDV eGFPH would clarify whether CDV can in fact enter BHK-21 or CHO-K1 cells. Unfortunately, none of these viruses have been rescued successfully, so the experiments cannot currently be performed.

Down-regulation of ephrin-B2 and ephrin-B3 by rCDV eGFPH NiVFG

When infected 293T cells were tested for differential expression of ephrin-B2 and ephrin-B3, staining revealed that cells infected with rCDV eGFPH NiVFG significantly down-regulated both cell surface receptors (Fig. 44, blue lines), hen compared with mock infected 293T cells (Fig. 44, green lines).

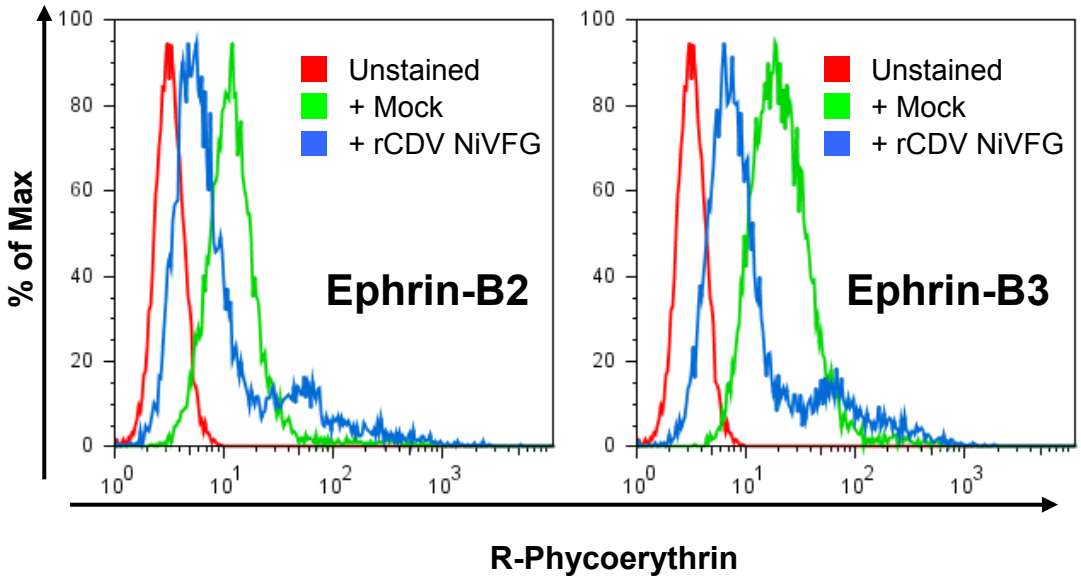

Figure 44: Ephrin-B2 and ephrin-B3 cell-surface down-regulation by rCDV eGFPH NiVFG. 293T cells were infected with rCDV eGFPH NiVFG, harvested in a single-cell suspension at 24 hours post-infection, and fixed with 4% PBS-buffered PFA. Rabbit anti-ephrin-B2 (left panel) or rabbit anti-ephrin-B3 (right panel) antibodies and an anti-rabbit R-PE-conjugated secondary antibody were used to detect cell surface ephrin-B2 and ephrin-B3 levels.

DISCUSSION

Fusion mediated by NiV F and G

After successfully cloning both NiV F and NiV G glycoprotein ORFs into the retroviral expression vectors (pczCFG5 IEGZ and pHITBE), successful expression of the glycoproteins was observed through both recombinant fusion assay and Western blot. The positive result on the Western blot shows that both proteins are produced and appear to be glycosylated. The N-linked glycosylation patterns of NiV F and HeV F are known (Moll *et al.*, 2004; Carter *et al.*, 2005), but which N-linked glycosylation sites are used by NiV G and HeV G is unknown. The positive result observed in the recombinant fusion assay shows that both glycoproteins are also functional, and that they both mediate cell-cell fusion. This also confirms the correct processing and glycosylation of the proteins, since incorrect processing or glycosylation would likely result in intracellular retention.

The initial study presented in this thesis confirms previous observations on the expression and behaviour of the NiV glycoproteins when expressed recombinantly in cell culture. In and of themselves, NiV F and NiV G are not

fusogenic, although they are highly fusogenic when expressed in the same cell (Tamin *et al.*, 2002; Bossart *et al.*, 2002). The expression of NiV F and NiV G in separate cell populations followed by mixing in culture does not result in the development of syncytia. This observation implies that NiV F and NiV G must be expressed in the same cell and that the interaction between the proteins is important for function, likely at the intracellular level. A similar result has been observed in co-cultures of cells separately expressing the F and HN glycoproteins of HPIV-4a (Nishio *et al.*, 1994). In contrast, cell-cell fusion has been observed in similar experiments in which cells separately expressing the F and HN glycoproteins of HPIV-2 were mixed together (Hu *et al.*, 1992), although this has been contradicted by another study (Heminway *et al.*, 1994). This last finding implies that the HN protein of HPIV-2 is able to trigger HPIV-2 F-mediated fusion regardless of whether the proteins are present in the same membrane, that is to say a parallel orientation, or in opposing membranes, or an antiparallel orientation. This is clearly not the case with either the HPIV-4a or the NiV glycoproteins. It is interesting to note that HPIV-2 and HPIV-4a are both in the genus *Rubulavirus* and yet they seem to display very different behaviours with regard to fusion promotion.

The fact that some paramyxovirus attachment glycoproteins are able to promote fusion when expressed in separate co-cultured cells seems to indicate

that the fusion promotion process is somewhat plastic. This could be due to either flexibility in the fusion glycoprotein or the attachment glycoprotein. This phenomenon could also be due to plasticity in the interaction between the HPIV-2 F and HN glycoproteins, since this is the only system in which this type of fusion has been observed. Contradictory reports in the literature would also suggest that opposite-orientation HPIV-2 F+HN-mediated fusion is highly dependent on the conditions under which the experiment is conducted and therefore might be an artifact rather than a reflection of real viral glycoprotein behaviour. However, most paramyxovirus fusion and attachment glycoprotein interactions appear to be of the conventional same-cell type. The NiV glycoproteins fall within this latter category. The NiV F and G glycoproteins also display sufficient relation to the HeV glycoproteins which allows the F and G glycoproteins to complement each other in the fusion process (Bossart *et al.*, 2002; Tamin *et al.*, 2002).

Some paramyxovirus fusion proteins are also able to promote fusion in the absence of the attachment glycoprotein. It is well known that the HRSV fusion protein is able to induce cell-cell fusion when expressed in cells (Branigan *et al.*, 2005). The HRSV G glycoprotein does not contribute noticeably to the process of fusion promotion, nor does it have a role in cell tropism for HRSV, which is a function assumed by most other paramyxovirus attachment

glycoproteins (Schlender *et al.*, 2003). Among members of the subfamily *Paramyxovirinae*, only SV5 and PPRV have F proteins which are capable of mediating membrane fusion in the absence of their homologous attachment proteins (Seth & Shaila, 2001; Horvath *et al.*, 1992; Dutch *et al.*, 1998). It is clear from all available data that the attachment protein-independent fusion by members of the subfamily *Paramyxovirinae* is the exception rather than the rule, whereas members of the subfamily *Pneumovirinae*, such as HRSV and HMPV, have F proteins which independently mediate fusion with minimal contributions from the attachment protein (Branigan *et al.*, 2005; Schowalter *et al.*, 2006).

Heterotypic fusion seems to occur only among members of the same genus. For members of the *Morbillivirus* genus, the combinations of MV F+CDV H and CDV F+MV H are able to induce cell-cell fusion (Bar-Lev Stern *et al.*, 1995), as is the combination of RPV F+MeV H (Tamin *et al.*, 2002). The combinations of HPIV-2 F+MuV HN and SV41 F+HPIV-2 HN are able to induce fusion, while the combinations of HPIV-2 F+SV41 HN and SV41 F+MuV HN do not (Tsurudome *et al.*, 1998). SV41, HPIV-2 and MuV are all members of the *Rubulavirus* genus, so this observation is perhaps not so surprising. Likewise, the MeV and CDV F and H glycoproteins (genus *Morbillivirus*) and the combination of SeV F+HPIV-1 HN glycoproteins (genus *Respirovirus*) are able to functionally complement each other, although the reciprocal combination of HPIV-1 F+SeV

HN does not induce fusion (Bousse *et al.*, 1994; Yao *et al.*, 1997). The glycoproteins of HPIV-3 and NDV are not able to complement each other, but the combinations of SV5 F+HPIV-3 HN and SV5 F+NDV HN are able to induce fusion (Bagai & Lamb, 1995). While SV5, HPIV-3 and NDV are all from different genera (*Rubulavirus*, *Respirovirus*, and *Avulavirus*, respectively), SV5 seems to have rather unique requirements for fusion. Additionally, the combinations of NiV F+MeV H, NiV F+CDV H, MeV F+NiV G, and CDV F+NiV G have not been shown in prior studies to complement each other in the fusion process (Bossart *et al.*, 2002). The same study also examined the HeV F and G proteins in combination with the MeV and CDV glycoproteins and obtained similar results. This thesis also shows that the combinations of NiV F+CDV H and CDV F+NiV G are not able to induce fusion. Taken together, the fusion data for NiV F and G co-expression indicates that NiV is a typical paramyxovirus with regard to its fusion requirements.

Viral interference mediated by NiV G

Transgenic cells were successfully created by using the retroviral expression plasmids, which contain the NiV glycoprotein ORFs, as replication-deficient genomes in retroviral particles pseudotyped with VSV G. The transgenic cells were found to express the NiV glycoproteins, which were able to

induce cell-cell fusion in culture. Cellular expression of the NiV G glycoprotein led to a high degree of resistance toward infection with either NiV or HeV.

Several lines of evidence indicate that the protection was specifically conferred by the expression of NiV G. First, the vector control cells (CRFK-pcz) were not resistant to NiV infection and showed the same degree of CPE as CRFK wt cells. This indicates that components of the pczCFG5 IEGZ vector backbone (i.e. GFP-Zeocin resistance fusion protein) do not play a role in resistance to NiV. Second, CRFK-NiV F cells showed cell death upon exposure to virus, indicating that NiV F has no protective effect. The lack of protection seen in CRFK-NiV F cells also demonstrates that resistance is not due to antisense RNA interference from cellular NiV F (+)-sense gene transcripts with the incoming (-)-sense viral genome. If antisense inhibition were the primary mechanism, then it would be expected that both CRFK-NiV F and CRFK-NiV G cells should show approximately the same level of resistance to NiV, which was clearly not the case. Third, viral nucleic acid (mRNA and vRNA) was not detected in CRFK-NiV G cells that had been exposed to 5 $TCID_{50}$ per well of HeV and NiV. Infection and death of CRFK-NiV G cells exposed to high doses of NiV indicate that the protection conferred by NiV G expression can likely be overwhelmed. It is thus possible that this system is "leaky". This could be a result of low numbers of non-transgenic cells supporting replication of NiV to sufficient levels to allow

infection of transgenic cells, or simply that application of sufficient amounts of virus to NiV G-expressing cells can still result in infection. It has also been reported that the affinity of NiV G for ephrin-B3 is approximately 10-fold lower than its affinity for ephrin-B2 (Negrete *et al.*, 2006), implying that lower levels of NiV G expression could perhaps block ephrin-B2, but not totally block ephrin-B3, thus allowing viral binding and entry. Fourth, expression of NiV G renders cells resistant to NiV F- and G-mediated fusion. This indicates that the blockage occurs at the level of virus binding and/or entry. If NiV G inhibits downstream steps in virus replication, one would expect to see fusion occurring with NiV G-expressing cells, which was clearly not observed.

All of the above lines of evidence point toward authentic receptor interference as the predominant mechanism of resistance to NiV and HeV infection in CRFK-NiV G cells, where newly synthesized NiV G in the transgenic cells interacts with the cognate cellular receptor. Since the functional cellular receptor is no longer available to interact with incoming viral glycoprotein, these cells are refractive to infection with NiV. Many viral proteins are known to interact with and down-regulate (Marschall *et al.*, 1997; Breiner *et al.*, 2001), or perhaps even induce degradation of their cellular receptors (Horga *et al.*, 2000), which can lead to the phenomenon of receptor interference.

The lack of CPE in CRFK-NiV G cells exposed to NiV and HeV seemed to indicate a lack of infection. However, it was unclear whether this lack of CPE was due to lack of viral entry or lack of replication after viral entry, since lack of CPE would be observed in either possibility. In light of these possibilities, several approaches were used in an attempt to address the issue of virus entry in CRFK-NiV G cells. First, cells expressing either NiV F or NiV G that had been exposed to NiV or HeV were tested for the presence of viral nucleic acid (mRNA or genomic vRNA). Cells that expressed NiV G did not show any evidence of viral nucleic acid, except for NiV G mRNA, which was expected (Fig. 22). However, cells that expressed NiV F were uniformly positive for NiV or HeV mRNA and genomic vRNA after exposure to virus. These data indicate that NiV and HeV were not able to gain entry into cells that expressed NiV G. It is still possible that low levels of virus were present in the cells and viral nucleic acid in these samples was below the detection limit for the RT-PCR assay. It also does not address the issue of whether viral entry (i.e. attachment and fusion) were inhibited. In order to delve further into the mechanism of inhibition, a second recombinant approach was used to assess the level at which viral interaction with the cell was inhibited. A recombinant fusion assay was developed using separate populations of fluorescent cells expressing either of the NiV glycoproteins which were combined with non-fluorescent cells expressing the

glycoproteins. Expression of NiV G resulted in inability of these cells to participate in NiV F/G-mediated cell-cell fusion, while transfection of vector alone or NiV F resulted in fusion, which indicates that NiV G specifically inhibits the ability of cells to fuse with other fusogenic cells that express both NiV F and G. This implies that there is a block at the level of interaction with the cellular receptor. Taken together, these data indicate that the cellular expression of NiV G results in the inability of NiV and HeV to enter cells, and that the interaction between the virus and the cellular receptor is blocked at a pre-fusion step, likely at the level of virus attachment to the receptor. Furthermore, it also confirms data from other studies showing that NiV and HeV share a common receptor. This has been demonstrated by Bossart and colleagues using a soluble secreted HeV G (sG) molecule (Bossart *et al.*, 2005). This study specifically showed that HeV sG could competitively inhibit infection by both NiV and HeV when applied exogenously with the virus inoculum. In a subsequent study by Bonaparte and colleagues, ephrin-B2 was shown to mediate cell entry by both NiV and HeV (Bonaparte *et al.*, 2005).

In classical models of viral receptor interference, expression of the viral attachment glycoprotein results in the surface down-regulation of the receptor. This has been extensively observed for MeV and HIV, where expression of MeV H results in down-regulation of CD46 and SLAM (Lecouturier *et al.*, 1996; Tanaka

et al., 2002; Welstead *et al.*, 2004), and expression of HIV-1 gp160 results in down-regulation of CD4 (Stevenson *et al.*, 1988; Butera *et al.*, 1991; Shea *et al.*, 2004). The results presented here with NiV G expression do not conform to this model. When NiV G is expressed in cells, the cellular receptors ephrin-B2 and ephrin-B3 are still found on the cell surface. Although viral receptor interference has been established in the NiV system, the exact mechanism seems to be different than that seen for MeV. In this model, cells expressing NiV G still have ephrin-B2 and ephrin-B3 on the cell surface. According to the data presented here, the mechanism of NiV and HeV inhibition in these cells involves a hypothesized interaction between NiV G and ephrin-B2 and/or ephrin-B3 on the cell surface.

Viral interference and the fate of the *Henipavirus* receptors ephrin-B2 and ephrin-B3

In prior studies, it had been speculated that NiV and HeV may share a cellular receptor (Bossart *et al.*, 2002), which was recently confirmed by the identification of ephrin-B2 as a receptor for both NiV and HeV (Bonaparte *et al.*, 2005; Negrete *et al.*, 2005). Ephrin-B3 has also been identified as a receptor for NiV (Negrete *et al.*, 2006), and it seems reasonable to expect that ephrin-B3 could also act as a receptor for HeV. The inhibition of NiV and HeV infection by NiV G expression is therefore likely to occur via the same mechanism, namely

interaction with either ephrin-B2 or ephrin-B3, or both. Previous studies have also indicated a common mechanism of inhibition indirectly by binding of a soluble HeV G (sG) to cells which results in competitive inhibition of NiV and HeV binding and infection (Bossart *et al.*, 2005). One intriguing aspect is the result that both NiV receptors, ephrin-B2 and ephrin-B3, are clearly present on the surfaces of cells expressing NiV G. Based on previous studies in which the MeV H glycoprotein induced a down-regulation of the cell surface expression of the MeV receptors CD46 and SLAM (Schneider-Schaulies *et al.*, 1995a; Schneider-Schaulies *et al.*, 1995b; Lecouturier *et al.*, 1996; Galbraith *et al.*, 1998; Tanaka *et al.*, 2002; Welstead *et al.*, 2004), it might be expected that NiV G would act in the same manner. It was therefore hypothesized that cell surface expression of ephrin-B2 and ephrin-B3 would be decreased in NiV G-expressing cells, which was clearly not the case. This was also observed in cells infected with NiV, where there was no change in the cell surface levels of ephrin-B2 and ephrin-B3. Interestingly, infection of 293T cells by rCDV eGFPH NiVFG clearly down-regulates cell surface expression of ephrin-B2 and ephrin-B3. Expression studies with NiV G have indicated that the protein has no inherent receptor down-regulation properties, as indicated the lack of differential ephrin-B2 and ephrin-B3 staining in NiV G-expressing cells. There are several possibilities that might explain why rCDV eGFPH NiVFG might down-regulate ephrin-B2 and ephrin-

B3. First, a component of the CDV backbone of rCDV eGFPH NiVFG could induce the down-regulation of ephrin-B2 and ephrin-B3. The morbillivirus (i.e. MeV and CDV) H glycoproteins have inherent receptor down-regulation properties since they are able to induce down-regulation when expressed in a recombinant system (Moll *et al.*, 2001; Naniche *et al.*, 1993; Welstead *et al.*, 2004). The CDV H protein is missing from rCDV eGFPH NiVFG, so it is possible that another component of the CDV genome is interacting with NiV G to induce down-regulation of the *Henipavirus* receptors. The CDV M protein would be the most likely culprit for this activity. The very low level of virus release in rCDV eGFPH NiVFG-infected cells would seem to contradict this model, since the substitution of the NiV glycoproteins into the CDV backbone seems to impair virus assembly. This would imply that the CDV M protein is not able to interact very efficiently with the NiV glycoproteins. The second possibility is that the processing of the NiV F protein might result in receptor down-regulation. NiV F has a specific internalization signal in its cytoplasmic tail, while NiV G does not, but is still internalized as a result of bulk membrane turnover. As reported by Vogt and colleagues, the endocytosis rate of NiV F reached levels of 60% after 30 minutes, while endocytosis of NiV G reached a peak of 20% after 20 minutes before declining (Vogt *et al.*, 2005). It is possible that through interaction with F, NiV G is internalized in a sort of "bystander" endocytosis. In infected cells, the

viral matrix protein might modulate this activity. In addition to their roles in virus assembly, the matrix proteins of paramyxoviruses also seem to have fusion inhibition properties, which would likely facilitate the process of virion assembly during infection. Studies using recombinant M-deficient MeVs have shown that very little extracellular virus is produced, but extensive fusion is seen in infected cells (Cathomen *et al.*, 1998a). Knock-down of MeV M in infected cells also increases the size of syncytia, which confirms the fusion-inhibitory role of the M protein (Reuter *et al.*, 2006). It is possible that the NiV M protein prevents NiV G endocytosis, although internalization of NiV F and G is seen in infected cells (Vogt *et al.*, 2005). Some reports in the literature indicate that the glycoproteins of MeV are not endocytosed during infection, and yet CD46 and SLAM are down-regulated (Moll *et al.*, 2001). NiV G expression does not induce down-regulation of ephrin-B2 and ephrin-B3, but some sort of interaction is required between NiV F and NiV G is required for the induction of cell-cell fusion. If NiV F internalization were able to drag NiV G inside the cell during the course of its proteolytic processing, it is possible that ephrin-B2 and ephrin-B3 down-regulation could be induced by NiV F and G co-expression. No studies have attempted to determine the fate of either receptor in NiV F and G co-expressing cells. In the context of viral infection, perhaps the NiV M protein prevents receptor down-regulation. This scenario seems to be unlikely, since NiV G is in

and of itself not able to induce receptor down-regulation. It therefore seems more likely that receptor down-regulation by rCDV eGFPH NiVFG is a result of components of the CDV backbone rather than the NiV glycoproteins.

Based on the data, the conclusion is that expression of NiV G and infection by NiV have no effect on the cell surface levels of ephrin-B2 and ephrin-B3, and that the receptors and NiV G are interacting on the cell surface. This is consistent with previous literature reports in which the NiV G protein is endocytosed at baseline levels and is likely slowly degraded or recycled to the cell surface after endocytosis (Vogt *et al.*, 2005). If endocytosed NiV G complexed with either ephrin-B2 or ephrin-B3 is recycled to the surface or slowly degraded following internalization, it is unlikely that this baseline activity would be noticeable in the cell surface FACS staining assay since the cells used for staining have likely achieved steady state levels of cell surface glycoprotein and receptor expression. Infection by rCDV eGFPH NiVFG, however, is able to induce down-regulation of both receptors. Although receptor down-regulation is the classical mechanism of receptor interference, these data indicate that resistance to infection can still occur if the receptor is still present on the cell surface.

Characterization of chimeric glycoproteins

In an attempt to determine the functional domains of NiV G, six chimeric CDV H-NiV G glycoproteins were designed. CDV H and NiV G were aligned and the chimeric glycoproteins were constructed based on the location of CDV H structural features. Only the homologous combinations of NiV F+NiV G and CDV F+CDV H are able to induce cell-cell fusion, while the heterologous combinations of NiV F+CDV H and CDV F+NiV G could not induce fusion. This provides a nice read-out system for any functions relating the fusion that have been altered. By Western blot, it was established that the chimeric CDV H-NiV G glycoproteins were expressed, but were fusion deficient. However, it is still possible that other functions, such as receptor binding were intact. It was therefore necessary to determine whether the chimeric glycoproteins were able to interact with both known *Henipavirus* receptors, ephrin-B2 and ephrin-B3 (Bonaparte *et al.*, 2005; Negrete *et al.*, 2005; Negrete *et al.*, 2006). As seen earlier, NiV G expression does not result in the down-regulation of either ephrin-B2 or ephrin-B3 from the cell surface. All of the chimeric glycoproteins exhibited this behaviour, with ephrin-B2 and ephrin-B3 present on the cell surface in the same proportion as with mock-transfected or NiV G-expressing cells. This could be due to several factors. First, there may not be a sequence inherent in the chimeric glycoproteins or the full-length NiV G that directs internalization from the cell

surface, such as that possessed by MeV H (Moll *et al.*, 2001). The lack of ephrin-B2 and ephrin-B3 down-regulation by either full-length NiV G or the chimeric glycoproteins would seem to indicate that this is the case. Second, the chimeric glycoproteins, due to structural changes as a result of the combination of heterologous domains, might have lost the capacity for receptor binding. Even if correctly expressed, these chimeric glycoproteins would not be able to down-regulate ephrin-B2 and ephrin-B3 from the cell surface because they simply do not interact with the receptor.

It was hoped that some of the chimeras would induce fusion when expressed with either NiV F or CDV F. Unfortunately, this was not the case. None of the chimeric glycoproteins were able to promote fusion when co-expressed with either CDV F or NiV F. Since all chimeric glycoproteins were detected by Western blot, lack of expression seems to be an unlikely explanation for this lack of activity. As before, there are several possible reasons for these observations. First, the chimeric glycoproteins could be fully processed, glycosylated, and transported to the cell surface, but due to structural alterations caused by the substitution of heterologous domains in the protein's primary structure, they lack one or more of either the receptor binding, fusion promotion, or F-interaction functions. Second, the chimeric glycoproteins could be impaired in their processing and retained within an intracellular compartment. This is a

more likely possibility, but endoglyosidase H (Endo H) and peptide-N-glycosidase F (PNGase F) treatment of the chimeric glycoproteins was not attempted, so the question of ER retention remains. Given the extent of structural modification of the chimeric glycoproteins, it would seem more likely that they would be misfolded and retained within the ER by protein folding chaperones. In either scenario, there is some possibility that the chimeric glycoproteins could interact with NiV F either within an intracellular compartment or on the cell surface.

The Western blot data for the chimeric glycoproteins also reveals interesting structural data regarding antibodies directed against NiV G. The swine anti-NiV G serum was only able to recognize chimeras which had the N-terminal ~25% of NiV G (G_{145}/H_{463}, G_{338}/H_{268}, and G_{498}/H_{113}). Chimera H_{145}/G_{458}, despite having the C-terminal ~75% of NiV G only the N-terminal ~25% of CDV H, was not recognized by the swine anti-NiV G serum. This indicates that only the N-terminal 25% of NiV G has linear epitopes, while the remainder of the protein does not. Furthermore, since the swine anti-NiV G serum has virus-neutralizing activity, this also indicates that epitopes in the globular head of NiV G are largely conformational in nature rather than linear. The neutralization of NiV by the swine anti-NiV G serum could also be directed against NiV F, but the lack of recognition of NiV F by Western blot using this antiserum would seem to

argue against this possibility, although staining by immunofluorescence assay (IFA) or FACS would clarify this issue. The addition of NiV M to cells co-expressing NiV F or CDV H and one of the chimeric glycoproteins did not result in cell-cell fusion. The MeV matrix protein has fusion inhibitory activity (Cathomen *et al.*, 1998a; Reuter *et al.*, 2006), but NiV M remains completely uncharacterized in this regard. An attempt was made to determine whether NiV M co-expression in a fusion assay with the NiV and CDV glycoproteins could force an interaction between the heterologous glycoproteins. In all experiments, none of the chimeric glycoproteins resulted in fusion when co-expressed with either NiV F or CDV F and NiV M. NiV M, therefore, does not seem to be able to force the chimeric glycoproteins to induce cell-cell fusion when expressed with either NiV F or CDV F. However, this does not preclude the possibility that, in the presence of NiV M, the chimeric glycoproteins and either NiV F or CDV F are able to interact. When cell surface IFA staining was performed, among the chimeric glycoproteins having the N-terminus of CDV H (H_{145}/G_{458}, H_{340}/G_{265}, and H_{495}/G_{105}), only H_{145}/G_{458} was expressed on the cell surface, which might provide another explanation for the lack of fusogenic activity by some of the chimeric glycoproteins.

Further modifications of NiV G were attempted in the form of truncated, secreted glycoproteins. Three different variants were constructed (sNiV G 483,

sNiV G 258, and sNiV G 98) with varying truncations of the N- and C-terminal domains, but they were not well-expressed and none could be detected in the culture supernatant. Secreted henipavirus glycoproteins have been used successfully by other groups to competitively inhibit NiV and HeV infection (Bossart *et al.*, 2005), as well as to immunize animals against NiV and HeV in a lethal animal model (Mungall *et al.*, 2006).

Fusion promotion by NiV G

Co-expression of NiV F and NiV G is clearly required for the induction of fusion. Neither NiV F nor NiV G was able to induce cell-cell fusion when singly expressed in cells. There is also a type-specific interaction required for NiV glycoprotein-mediated cell-cell fusion, since reports have shown that HeV G is able to promote NiV F-mediated fusion, but CDV F is not (Bossart *et al.*, 2002). The chimeric glycoproteins are also unable to promote fusion when co-expressed with either NiV F or CDV F. Of the chimeric glycoproteins bearing the N-terminus of CDV H, only H_{145}/G_{458} was detected on the cell surface. This indicates that transport to the cell surface is a barrier that contributes to the lack of fusion promotion. This would also imply that the chimeric glycoproteins are deficient in their interaction with NiV F and CDV F. The results with the chimeric glycoproteins would also seem to indicate that another portion of the

stalk, the transmembrane domain, or the cytoplasmic tail is required for efficient fusion promotion activity. However, to date no study has reported the involvement of the cytoplasmic tail of paramyxovirus attachment glycoproteins in fusion specificity and promotion.

Receptor binding by modified NiV G proteins

Full-length chimeric NiV G glycoproteins were constructed with the corresponding domains from CDV H. All of these chimeras are membrane anchored glycoproteins, but are hypothesized to have altered functions depending on which domains have been swapped.

The receptor binding sites for MeV H and CDV H are located in the C-terminal 35% of the protein (Li & Qi, 2002; Lecouturier *et al.*, 1996; Massé *et al.*, 2002; Vongpunsawad *et al.*, 2004; Patterson *et al.*, 1999; Hu *et al.*, 2004; Santiago *et al.*, 2002; Massé *et al.*, 2004; Hadac *et al.*, 2004). Given the relatively close genetic relationship between the henipaviruses and morbilliviruses, it was hypothesized that the receptor binding for NiV G would be located somewhere in the same region. Because of the modifications made to the chimeric glycoproteins, their structures could be perturbed to the point that they are non-functional. This possibility cannot be excluded at present since fusion complementation studies with NiV F and CDV F demonstrated that the chimeric glycoproteins are unable

to promote fusion. Using monoclonal antibodies and point mutations in the globular head of NiV G, Guillaume and colleagues have implicated the residues Trp504, Glu505, Arg557, Gln530, Thr531, Ala532, and Glu533 in binding of ephrin-B2, with mutation of Glu533 having the strongest effect on fusion (Guillaume *et al.*, 2006). These residues are located in several surface exposed loops on the globular head of NiV G. The residues in this region of NiV G correspond very closely to the Asp505, Asp 507, Tyr529, Asp530, Arg533, and Tyr553 residues of MeV H which are critical for SLAM-dependent fusion (Massé *et al.*, 2004; Vongpunsawad *et al.*, 2004). Further studies of this region of NiV G and HeV G have revealed that Val507 (as found in NiV G) is critical for binding of ephrin-B3, whereas Ser507 at the same position (as found in HeV G) binds ephrin-B3 very inefficiently (Negrete *et al.*, 2007). The effect of these differences between NiV G and HeV G and the relationship to pathogenesis remains to be determined. Bishop and colleagues have recently reported several new clusters of residues in HeV G which they identify as being responsible for binding to ephrin-B2 and ephrin-B3. These include Asp257, Asp260, Gly439, Lys443, Gly449, Lys465, and Asp468 (Bishop *et al.*, 2007). The involvement of these residues in receptor is somewhat controversial, since it has been noted that they are not predicted to be exposed on the surface of the HeV G globular head

(Negrete *et al.*, 2007). It is possible, perhaps, that these residues are also involved in F-G interaction or fusion promotion.

Antibodies directed against NiV G

The Western blots of the sNiV G constructs and the chimeric glycoproteins using the swine anti-NiV G serum reveal some interesting structural features of NiV G. The swine 30 anti-NiV G serum has neutralizing activity against NiV in a plaque reduction neutralization assay, yet it is only able to recognize the N-terminal 25% of NiV G by Western blot. Only the chimeras G_{145}/H_{463}, G_{338}/H_{268}, and G_{498}/H_{113} were detected by Western blot, indicating that the only linear epitopes recognized by this anti-NiV serum are in that region. However, since the serum has NiV neutralizing activity, it should also recognize conformational epitopes, which are likely located in the globular head of NiV G.

Despite having 75% of the C-terminus of the NiV G, H_{145}/G_{458} is not recognized by the swine 30 anti-NiV G serum, indicating that the missing NiV G sequence is crucial for binding of linear epitopes. Not surprisingly then, the two remaining chimeric glycoproteins bearing the N-terminus of CDV H, H_{340}/G_{268} and H_{495}/G_{105}, are also not recognized by the swine 30 anti-NiV G serum. The attachment glycoproteins of paramyxoviruses all seem to share a large degree of structural homology. The HN proteins of NDV, HPIV-3, and SV5 have all been

crystallized and have similar features in their 3D structures (Crennell *et al.*, 2000; Lawrence *et al.*, 2004; Yuan *et al.*, 2005). The globular ectodomain of the paramyxovirus HN protein is of six β-sheets each composed of 4 anti-parallel β-strands arranged radially around the central axis of the protein, referred to as a β-propeller. Homology modelling of MeV H and CDV H has indicated that the morbillivirus H proteins share this same structure (Langedijk *et al.*, 1997; Massé *et al.*, 2004). Guillaume and colleagues performed the same type of modelling with NiV G, and though it required more iterations to obtain what they considered a reasonable structural model, they determined that NiV G also fold into this same β-propeller structure (Guillaume *et al.*, 2006). By contrast, the stalk and membrane-proximal domains of paramyxovirus attachment proteins have very little tertiary structure, although they likely have α-helical and β-sheet motifs. The Western blot results for both the secreted NiV G proteins and the chimeric glycoproteins support this structural model. Neutralizing antibodies are able to recognize conformational epitopes in the globular head of NiV G, while linear epitopes are directed against the membrane-proximal and stalk regions. This indicates that the portions of NiV G that are not recognized by the anti-serum in Western blots contain a high degree of secondary and tertiary (and perhaps quaternary) structure. Conversely, since they contain all of the linear epitopes in NiV G, the membrane-proximal and stalk regions likely contain

much lower amounts of tertiary structure, although they likely have elements of secondary structure (i.e. α-helices and β-sheets).

Rescue of recombinant viruses

The rescue of several recombinant viruses was attempted. The only virus that was recovered successfully was rCDV eGFPH NiVFG, which has the rCDV eGFPH strain 5804P backbone and has its F and H ORFs substituted with those of NiV F and G. Despite many efforts, the rescue of the other recombinant virus clones (rCDV eGFPM, rCDV eGFPH, rCDV eGFPH NiVMFG, rNiV, and rNiV eGFPG) could not be rescued, although further optimization of these systems is required. rCDV eGFPM, rCDV eGFPH, rCDV eGFPH NiVFG, and rCDV eGFPH NiVMFG are all produced from vectors exclusively driven by the T7 RNAP promoter. Both NiV genomes (rNiV and rNiV eGFPG) were produced either from vectors driven by the T7 RNAP promoter or the CMV_{IE} promoter. While the recovery of all of these viruses from cDNA is possible, the recovery and optimization of virus rescue tends to be the most difficult part of the process. The lone virus that was recovered thus far, rCDV eGFPH NiVFG, was rescued using the proscribed accessory plasmid amounts of 1 μg each of CDV N and CDV P, 0.5 μg of CDV L, and 7 μg of rCDV eGFPH NiVFG genome plasmid. Clearly, these amounts of accessory plasmid are able to drive transcription and

replication of the rCDV eGFPH NiVFG genome. It remains unclear why these same ratios, then, have not worked for the recovery of rCDV eGFPH NiVMFG, rCDV eGFPM, and rCDV eGFPH. All other factors being equal, these viruses should have been rescued as well. The fact that they have not been rescued illustrates just how unpredictable virus rescues can be. It will be necessary to continue rescue attempts and to systematically alter the plasmid ratios until these viruses are produced.

The rescue of the rNiV and rNiV eGFPG has also proven quite challenging. Rescues were attempted for the T7 RNAP and CMV_{IE} promoter-driven system using the same plasmid amounts as for the CDV rescues. While this seemed like a good starting point, it may be that the initiation of NiV transcription requires different protein ratios than CDV. NiV minigenome experiments performed by Halpin and colleagues determined that in their assay, plasmid ratios of 1.25 μg of NiV N, 0.8 μg of NiV P, and 0.4 μg of NiV L, and 3.5 μg of minigenome were required for optimal expression of the CAT reporter gene (Halpin *et al.*, 2004). This translates into an accessory plasmid ratio of ~3:2:1 for NiV N:NiV P:NiV L when normalized to the amount of NiV L plasmid. Thus, it seems that slightly more NiV N plasmid might be required in further NiV rescue experiments.

The successful rescue of rCDV eGFPH NiVFG also demonstrates that the substitution of the glycoproteins of CDV with those of the more distantly related NiV still allows for virus rescue. The successful rescues of a variety of chimeric paramyxoviruses bearing foreign envelope proteins have been previously reported. In one report, the F and H glycoproteins of MeV were removed and replaced with either the full-length VSV G ORF or a chimeric VSV G in which the cytoplasmic tail was replaced with that of the MeV F protein (Spielhofer *et al.*, 1998). In a similar report, the SeV F and HN glycoproteins were replaced with chimeric RSV F proteins containing either the SeV F cytoplasmic tail or the cytoplasmic tail and transmembrane domain (Zimmer *et al.*, 2005). Recombinant MeVs and CDVs have also been produced in which the glycoproteins from vaccine strains are substituted into the wild-type virus backbone, and vice versa (Johnston *et al.*, 1999; Takeda *et al.*, 1999). Chimeric RPVs have also been generated using a similar strategy to the chimeric rCDV eGFPH NiVFG/MFG viruses, except the glycoproteins and the matrix protein of RPV have been substituted with those of the closely related PPRV (Das *et al.*, 2000; Mahapatra *et al.*, 2006).

Much more work of this nature has been published for the parainfluenza viruses. A number of studies have been published in which the F and HN glycoproteins of HPIV-3 have been replaced with those of HPIV-1 (Skiadopoulos

et al., 1999; Tao *et al.*, 1999; Tao *et al.*, 2001; Tao *et al.*, 1998), or BPIV-3. The reciprocal chimeric viruses in which the BPIV-3 glycoproteins are replaced by the glycoproteins of HPIV-3 have also been rescued (Haller *et al.*, 2000; Schmidt *et al.*, 2000). However, HPIV-1, HPIV-3, and BPIV-3 are classified in the *Respirovirus* genus of the subfamily *Paramyxovirinae*, so they are still quite closely related. The ectodomains of the HPIV-3 F and HN glycoproteins have been replaced with those of the HPIV-2 F and HN glycoproteins, but the HPIV-3 F and HN cytoplasmic tails and transmembrane domains were still present (Tao *et al.*, 2000). The versatility of these types of chimeric viruses has been further illustrated by the rescue of a recombinant HPIV-3 in which the nucleocapsid protein was replaced with that of BPIV-3, yielding an attenuated virus. However, there has been no published report to date in which the glycoprotein ORFs of one paramyxovirus have been replaced with those from a paramyxovirus in a different genus. The successful rescue of rCDV eGFPH NiVFG shows that this is indeed possible, although the efficiency of this rescue is debatable.

The data generated by infection of Vero dogSLAMtag, BHK-21, and CHO-K1 cells by rCDV eGFPH NiVFG also sheds some light on the requirements for paramyxovirus assembly. Very little data has been published in which assembly of chimeric paramyxoviruses bearing foreign glycoproteins has been analyzed,

particularly with respect to the role of the M protein. A chimeric MeV in which the F and H glycoprotein genes were replaced by the single VSV G glycoprotein yielded attenuated viruses (Skiadopoulos *et al.*, 2001). When chimeric virus containing full-length VSV G was produced, the resulting viral particles were found to lack the MeV M protein. However, when a virus containing a chimeric VSV G in which the cytoplasmic tail and transmembrane domain was replaced with the homologous domains from the MeV F protein, the MeV M protein could then be detected in viral particles. Taken together with other reports in which only the ectodomains of viral glycoproteins have been exchanged with those of foreign glycoproteins (Tao *et al.*, 2000; Zimmer *et al.*, 2005), assembly of chimeric paramyxoviruses in which full-length foreign glycoproteins have been replaced with full-length heterologous glycoproteins seems to be an unlikely event. Indeed, assembly of rCDV eGFPH NiVFG seemed to be highly impaired due to its inability to produced detectable virus progeny in any of the cells that were tested (i.e. Vero dogSLAMtag, BHK-21, and CHO-K1). Further experiments using wild-type rCDV eGFPH or rCDV eGFPM and rCDV eGFPH NiVMFG would serve to clarify this matter. If a matrix protein homologous to the surface glycoproteins was included in the viral genome, it might be expected that the interaction between the homologous fusion/attachment/matrix protein complex would be more efficient and would thus generate more infectious virus in

culture supernatant. There is also an intriguing connection between the release of virus and cell surface down-regulation of ephrin-B2 and ephrin-B3. Infection of cells by NiV results in efficient virus release, while infection by rCDV eGFPH NiVFG is very inefficiently released, if at all. NiV infection does not change cell surface levels of ephrin-B2 or ephrin-B3, but rCDV eGFPH NiVFG infection clearly results in down-regulation of both receptors. It is possible that the cell surface down-regulation of ephrin-B2 and ephrin-B3 by rCDV eGFPH NiVFG impairs virus release by sequestering NiV G, which is complexed with the internalized ephrin-B2 and ephrin-B3. Normal cell surface expression of ephrin-B2 and ephrin-B3 in NiV-infected cells might aid virus release by keeping NiV G on the cell surface where NiV can bud more efficiently from the cell.

The inclusion of the PPRV M protein in a chimeric RPV virus bearing the PPRV F and H glycoproteins enhanced virus production to levels comparable to unmodified PPRV, but not to the same level as unmodified RPV (Mahapatra *et al.*, 2006). Based on these results, it is reasonable to speculate that inclusion of NiV M along with NiV F and G in the CDV viral genome backbone should produce a virus with growth characteristics that are at least comparable to wild-type virus. Viral release should therefore also be enhanced in Vero dogSLAMtag, BHK-21, and CHO-K1 cells. Curiously, this report also noted that a chimeric RPV in which only the M protein was replaced with the PPRV M

protein grew with kinetics similar to the parental RPV. It would appear that the nature of the interaction between the paramyxovirus matrix, fusion, and attachment proteins is more complex than previously thought.

The result that rCDV eGFPH NiVFG can down-regulate both ephrin-B2 and ephrin-B3 cell surface expression was quite surprising. Neither NiV G nor any of the chimeric glycoproteins were ever able to induce down-regulation of these molecules. This would imply that neither NiV G nor CDV H has an inherent internalization signal, but perhaps some component of the CDV backbone is responsible for receptor down-regulation.

NiV glycoproteins and virus assembly

Recombinant systems have provided a great amount of insight into the functions of paramyxovirus glycoproteins. However, the behaviour of the glycoproteins might be substantially different in the context of a viral infection. Since we did not possess a recombinant system for the production of infectious NiV, the NiV F and G glycoprotein ORFs were inserted into the recombinant CDV strain 5804P backbone. Two chimeric viruses were constructed, one with the NiV F and G ORFs substituted for the CDV F and H ORFs (designated rCDV eGFPH NiVFG), and the other with both the glycoproteins and the NiV M protein substituted for the corresponding CDV proteins (designated rCDV

eGFPH NiVMFG). Recently, we have also acquired a reverse genetics system for NiV and a recombinant NiV carrying a GFP gene (rNiV eGFPG).

Experimentally, the rescue of recombinant viruses has proven more difficult than anticipated. Fortunately, rCDV eGFPH NiVFG was rescued and its ability to infect different cells and the extent to which virus was released was assessed. Whether the infections were performed in Vero dogSLAMtag, BHK-21, or CHO-K1 cells, virus release appeared to be extremely low. When the rCDV eGFPH NiVFG stocks were grown, only one stock (Stock #3) reached a titer of 10^5 $TCID_{50}$, while the other three stocks (Stock #1, Stock #2, and Stock #4) reached titers of ~10^4 $TCID_{50}$, despite one freeze-thaw cycle. Without a freeze-thaw cycle, no virus was detected in the supernatant, indicating that rCDV eGFPH NiVFG is highly cell-associated. Viral replication was clearly occurring in infected cells, based on strong cell-cell fusion observed in monolayers infected with rCDV eGFPH NiVFG. By contrast, NiV was able to efficiently infect Vero dogSLAMtag, BHK-21, and CHO-K1 cells and infectious virus could easily be transferred to fresh Vero dogSLAMtag monolayers, indicating that there is no impediment to the release of NiV in the cell lines used for the experiment. The lack of release of rCDV eGFPH NiVFG from infected cells implies that close interaction is required between the paramyxovirus glycoproteins and the matrix protein in the promotion of virus release (Cathomen *et al.*, 1998a; Cathomen *et al.*,

1998b). This finding also implicates the cytoplasmic tails of NiV F and NiV G in virus assembly. The removal of the CDV F and H glycoproteins and their replacement with NiV F and G seem to show that interaction between the CDV M protein and the glycoproteins is lost to a significant degree. The addition of the NiV M into this virus system (rCDV eGFPH NiVMFG) should have a significant effect on virus release and viral titer. Chimeric RPVs in which the M, F, and H ORFs have been replaced with the corresponding ORFs from PPRV show growth kinetics equivalent to that of wild-type RPV, so it would be reasonable to expect that rCDV eGFPH NiVMFG would grow much better in culture (Mahapatra *et al.*, 2006).

CONCLUSIONS

Fusion by NiV F and NiV G

When co-expressed in a suitable cell line, NiV F and NiV G induce the formation of large syncytia. When singly expressed in cells, neither NiV F nor NiV G is able to induce fusion, which indicates that there is a specific interaction required for both of these glycoproteins to perform this function. Neither NiV F nor NiV G, when co-expressed with either CDV F or CDV H, were able to induce fusion in cells expressing canine SLAM. This demonstrates that glycoproteins from another genus of paramyxovirus are unable to complement the functions of the NiV glycoproteins. When the glycoproteins were singly expressed in separate cell populations and then mixed, no fusion resulted. In addition to requiring a type-specific interaction between NiV F and NiV G for fusion, there is also a requirement for the glycoproteins to be in the correct orientation.

Receptor interference by NiV G

Cellular expression of NiV G by transgenic cells resulted in protection from infection by NiV and HeV. No viral nucleic acid was detected in NiV G-

expressing cells. NiV G was also able to inhibit NiV F+NiV G-mediated fusion. The cell surface levels of the *Henipavirus* receptors, ephrin-B2 and ephrin-B3, were unchanged despite NiV G expression. NiV G expression inhibits NiV and HeV entry into cells, which results in viral receptor interference.

Chimeric CDV H-NiV G glycoproteins

Chimeric CDV H-NiV G glycoproteins were fusion deficient and also could not be co-immunoprecipitated with NiV F. They also did not affect the cell surface level of ephrin-B2 and ephrin-B3.

Chimeric viruses

rCDV eGFPH NiVFG was successfully rescued and grown into a stock with a titer of 10^5 $TCID_{50}$/mL. rCDV eGFPH NiVFG was poorly released from cells and infectivity could not be transferred without a freeze-thaw cycle. We conclude that rCDV eGFPH NiVFG is deficient in virus assembly.

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