Inhibition of henipavirus infection by Nipah virus attachment glycoprotein occurs without cell-surface downregulation of ephrin-B2 or ephrin-B3

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Nipah virus (NiV) and Hendra virus (HeV) are newly identified members of the family Paramyxoviridae and have been classified in the new genus Henipavirus based on unique genetic characteristics distinct from other paramyxoviruses. Transgenic cell lines were generated that expressed either the attachment protein (G) or the fusion protein (F) of NiV. Functional expression of NiV F and G was verified by complementation with the corresponding glycoprotein, which resulted in the development of syncytia. When exposed to NiV and HeV, expression of NiV G in Crandall feline kidney cells resulted in a qualitative inhibition of both cytopathic effect (CPE) and cell death by both viruses. RT-PCR analysis of surviving exposed cells showed a complete absence of viral positive-sense mRNA and genomic negative-sense viral RNA. Cells expressing NiV G were also unable to fuse with cells co-expressing NiV F and G in a fluorescent fusion inhibition assay. Cell-surface staining for the cellular receptors for NiV and HeV (ephrin-B2 and ephrin-B3) indicated that they were located on the surface of cells, regardless of NiV G expression or infection by NiV. These results indicated that viral interference can be established for henipaviruses and requires only the expression of the attachment protein, G. Furthermore, it was found that this interference probably occurs at the level of virus entry, as fusion was not observed in cells expressing NiV G. Finally, expression of NiV G by either transient transfection or NiV infection did not alter the cell-surface levels of the two known viral receptors.

INTRODUCTION

Nipah virus (NiV) and Hendra virus (HeV) are newly emergent members of the family Paramyxoviridae and have been classified in the newly created genus Henipavirus (Murray et al., 1995; Chua et al., 2000; Mayo, 2002). HeV was first described in 1995 in connection with an outbreak of severe pneumonia among stabled horses and also caused the deaths of two horse handlers in northern Australia (Rogers et al., 1996). NiV was subsequently identified in 1999 as the causative agent of an outbreak of severe encephalitis in peninsular Malaysia and Singapore (Philbey et al., 1998; Chua et al., 1999; Paton et al., 1999). A total of 265 human cases was documented, of which 105 patients succumbed to the disease. Recently, NiV has been identified as the causative agent of several smaller outbreaks of severe encephalitis in Bangladesh (Anonymous, 2003; Butler, 2004; Hsu et al., 2004) and India (Chadha et al., 2006). Under experimental conditions, NiV is able to infect cats, pigs, guinea pigs and hamsters (Middleton et al., 2002), whilst HeV infects horses, cats and bats (Williamson et al., 1998, 2000, 2001). In a natural setting, fruit bats from the genus Pteropus appear to be the reservoir species for NiV and HeV (Halpin et al., 2000). In the Malaysian outbreak, NiV was transmitted to pigs via an uncharacterized route, resulting in a high risk of NiV exposure for both pig farmers and abattoir workers from infected pigs (Chew et al., 2000; Parashar et al., 2000). In the more recent outbreaks in Bangladesh, NiV infection appears to have been acquired from sources other than pigs, involving fruit bats or another unidentified intermediary, and person-to-person transmission was also a prominent feature of this outbreak (Hsu et al., 2004), in contrast to the Malaysian outbreak in 1998–1999 (Mounts et al., 2001). NiV and HeV have two major surface glycoproteins, a typical feature of members of the family Paramyxoviridae. The attachment glycoprotein (G) is responsible for
attachment to the host-cell receptors, two of which have been identified as ephrin-B2 and ephrin-B3 (Bonaparte et al., 2005; Negrete et al., 2005, 2006). The fusion glycoprotein (F) mediates fusion between the viral and host-cell membranes. In other related paramyxoviruses, close interaction between these two glycoproteins generally is required for fusion to occur (Sakai & Shibuta, 1989; Ebata et al., 1991; Morrison et al., 1991; Wild et al., 1991; Horvath et al., 1992; Hu et al., 1992; Tanabayashi et al., 1992; Cattaneo & Rose, 1993; Bousse et al., 1994; Heminway et al., 1994; Nishio et al., 1994; Bagai & Lamb, 1995; Bar-Lev Stern et al., 1995), although this requirement is not absolute for some species (Kahn et al., 1999; Mizuguchi et al., 1999; Seth & Shaila, 2001). NiV and HeV glycoproteins presumably share a certain number of functional domains with other paramyxovirus glycoproteins, and previous studies have demonstrated that heterologous NiV and HeV glycoproteins are able to complement each other functionally in the induction of membrane fusion and that neither glycoprotein on its own is fusogenic (Bossart et al., 2001, 2002; Tamin et al., 2002). However, when any of the NiV or HeV glycoproteins are co-expressed with other complementary paramyxovirus glycoproteins, such as those of Measles virus (MeV), no fusion results (Bossart et al., 2002). The lack of fusogenic activity for any of the henipavirus glycoproteins on their own, coupled with the lack of compatibility with other paramyxovirus glycoproteins, indicates that there are specific domains within the NiV and HeV glycoproteins that interact and allow the glycoproteins to carry out fusion between the host cell and viral membranes.

Expression of glycoproteins from some retroviruses such as Avian leukaosis virus or Murine leukaemia virus has been shown to render otherwise susceptible cells refractive to infection (Czub et al., 1995; Hunt et al., 1999; Ponferrada et al., 2003). This phenomenon is known as viral receptor interference. Due to interactions between the viral attachment glycoprotein and the endogenous host-cell receptor, functional receptor molecules appear to be neither on the cell surface nor available for virus attachment (Delwart & Panganiban, 1989; Welstead et al., 2004). Receptor interference has been characterized for gammaherpesviruses (Geraghty et al., 2000) and a number of retroviruses (Czub et al., 1995; Potash & Volsky, 1998; Lyu et al., 1999; Ponferrada et al., 2003), as well as for several other virus families (Karpf et al., 1997; Marschall et al., 1997) but among paramyxoviruses has only been investigated in some detail for Human parainfluenza virus 3 (Horga et al., 2000), Mumps virus (Hishiyama et al., 1996) and MeV (Schneider-Schaullies et al., 1995a, b; Welstead et al., 2004). Recently, it has also been demonstrated that a soluble HeV G molecule consisting of the ectodomain of the protein is able to bind to the cell surface and competitively inhibit NiV and HeV infection (Bossart et al., 2005). As viral interference may be a useful mechanism to combat viral infections, possibly including those with NiV and HeV, we generated cell lines expressing the viral proteins G and F. After exposure to henipaviruses, transgenic cells expressing the NiV G attachment glycoprotein were resistant to infection with NiV and HeV. Our results demonstrated that a viral interference system can be established for these members of the family Paramyxoviridae and that expression of the attachment glycoprotein did not result in cell-surface downregulation of the viral receptors.

METHODS

Cells, viruses and infections. Human embryonic kidney (293T) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) with 10 % fetal bovine serum (FBS; Wisent) at 37 °C and 5 % CO2. Crandall feline kidney cells (CRFK; ATCC) were maintained in minimal essential medium (MEM; Gibco) with 10 % FBS at 37 °C and 5 % CO2. NiV and HeV were obtained from the Centers for Disease Control and Prevention (Atlanta, USA); passage 3 of both viruses was used for all experiments. All infections were performed under Biosafety Level 4 (BSL4) conditions as follows: virus stock (106 TCID50 ml−1) was diluted in serum-free OptiMEM (Gibco), applied to CRFK monolayers and incubated for 1 h at 37 °C and 5 % CO2. Following virus adsorption, monolayers were flooded with MEM containing 2 % FBS and cells were incubated at 37 °C and 5 % CO2 until a CPE was observed. All experiments described here were repeated in triplicate.

Generation of NiV F and G expression plasmids. The NiV F and G genes (Genbank accession no. AF212302) were amplified by PCR from viral cDNA, cloned into pBK-CMV (Stratagec) using the NheI and HindIII (New England Biolabs) sites downstream of the multiple cloning site and sequenced. The retroviral vectors pczCFG5 IEGZ and pHITBE were kindly provided by D. Lindemann (Würzburg, Germany) (Lindemann et al., 2001), pczCFG5 IEGZ and pHITBE both have multiple cloning sites immediately upstream of an internal ribosome entry site (IRES) derived from either Encephalomyocarditis virus for pczCFG5 IEGZ or immunoglobulin heavy chain-binding protein (BIP) for pHITBE. Green fluorescent protein (GFP) genes are located immediately downstream of the IRES sites, so cells expressing the NiV glycoproteins from these vectors also express GFP. For ligation into pczCFG5 IEGZ, both NiV genes were excised from pBK-CMV by digestion with NheI and HindIII, followed by Klenow (Gibco) fill-in of the overhanging ends. pczCFG5 IEGZ was digested with SwaI and ligated to the filled-in NiV genes. For ligation into pHITBE, both NiV genes in pBK-CMV were digested with NheI, followed by Klenow fill-in and digestion with HindIII, pHITBE was digested with EcoRI, followed by Klenow fill-in and digestion with HindIII and ligation to the filled-in NiV genes. pczCFG5 IEGZ was digested with SwaI (New England Biolabs) and the filled-in NiV genes were ligated into pczCFG5 IEGZ. Non-fluorescent versions of pHITBE-NiV F and pHITBE-NiV G were generated by excising the GFP gene. Briefly, pHITBE-NiV F and pHITBE-NiV G were digested with AgeI and NotI, which flank the GFP gene. After gel extraction of the plasmids, the resulting overhanging ends were filled in with Klenow fragment and religated to give the plasmids pHITAGFP-NiV F and pHITAGFP-NiV G.

Transfection. 293T cells at 80–90 % confluence were transfected in six-well dishes using serum-free OptiMEM (Gibco) and Lipofectamine 2000 (Invitrogen). Briefly, plasmid DNA (4 μg) and Lipofectamine 2000 were mixed separately in OptiMEM and incubated at room temperature for 5 min. Following incubation, an equal volume of OptiMEM: Lipofectamine 2000 was added to the OptiMEM:DNA mixture. The transfection mixtures were mixed gently and then incubated at room temperature for 15 min to allow DNA: Lipofectamine 2000 complexes to form. Following incubation,
an equal volume of OptiMEM was added and mixed, and 1 ml was pipetted onto monolayers of 293T cells. Transfections were incubated overnight at 37 °C and 5% CO₂.

Production of retroviral particles and generation of transgenic cell lines. Retroviral particles for transductions were produced by adaptation of a previously described method (Soneoka et al., 1995). The pczCFG5 IEGZ vector contains a replication-deficient retroviral genome with a Psi packaging sequence from Moloney murine leukemia virus. Plasmids (4 µg each of pczCFG5 IEGZ, pczCFG5-NiV F or pczCFG5-NiV G) were co-transfected with separate plasmids encoding vesicular stomatitis virus glycoprotein (VSV G) and murine retroviral gag–pol open reading frames as described above. After transfection, the newly transcribed plasmids 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 were harvested and stored at −70 °C until further use. Target CRFK cell monolayers were transduced with undiluted retroviral particles as described above. Monolayers were transduced twice at 16–24 h 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 400 µg Zeocin (Invitrogen) ml⁻¹ for 2 weeks. Transgene expression was assessed by FACS analysis for GFP expression in transgenic cells.

RT-PCR of NiV- and HeV-exposed transgenic cells. Wild-type (wt) CRFK, CRFK-pcz, CRFK-NiV F and CRFK-NiV G cells were seeded into two 24-well dishes. Cells were exposed to HeV and NiV as described above at dilutions ranging from 5 × 10⁻¹ to 5 TCID₅₀ per well. At 5 days post-exposure, surviving cells were trypsinized and RNA was extracted using an RNasy mini kit (Qiagen). Extracted total cellular RNA was first subjected to first-stand cDNA synthesis using a SensiScript reverse transcriptase kit (Qiagen) and a reverse transcriptase primer. The resulting cDNA was amplified using a Master Mix PCR kit (Qiagen) and primers that were designed to target HeV and NiV positive-sense mRNA from the N, M and G genes and negative-sense genomic viral RNA (vRNA) at the N/M, M/F and F/G gene junctions. RNA extracts from all CRFK cell lines and CRFK-derived cells were verified with an internal mRNA control using primers for feline glyceraldehyde-3-phosphate dehydrogenase mRNA (GFAPDH; GenBank accession no. AB038241): GFAPDH fwd (5′-TTCACCCGACAGTCAGGGACAGTGAACGAGA-3′) and GFAPDH rev (5′-GGTTGGAGGAGGCGTGCAATCT-3′). Amplification of mRNA in all samples gave the expected 294 bp RT-PCR product.

Fluorescent fusion inhibition assay. 293T cells were seeded into six-well dishes. When the cells were approximately 80–90% confluent, they were transfected with 4 µg each of pczCFG5 IEGZ, pczCFG5-NiV F, pczCFG5-NiV G or pHITAGFP-NiV F + pHITAGFP-NiV G. After 8 h, 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 pHITAGFP-NiV F + pHITAGFP-NiV G-transfected cells in a fresh 12-well dish. The cells were incubated overnight, fixed with 3.7% PBS-buffered formaldehyde and examined for green fluorescent syncytia the next day.

Production of polyclonal NiV antisera. Two female guinea pigs (Hartley, 500 g; Charles River Laboratories) were inoculated intraperitoneally with 10⁷ p.f.u. live NiV per guinea pig. The guinea pigs were boosted intraperitoneally with a further 10⁷ p.f.u. per guinea pig at 14 days post-inoculation and were bled at 28 days post-inoculation. Guinea pigs were anesthetized prior to inoculation, bleeding or exsanguination by intramuscular administration of xylazine (5 mg kg⁻¹) and ketamine (40 mg kg⁻¹). The generation of swine antisera against NiV G has been described elsewhere (Weingartl et al., 2005, 2006). All animal work was performed under BSL4 conditions and according to Canadian Council of Animal Care guidelines.

Western blots. For Western blots, cells were lysed in 1 × SDS gel loading buffer [50 mM Tris/HCl (pH 7.5), 1% SDS, 8.75% glycerol and 0.125% bромophenol blue] with 4% 2-mercaptoethanol. Lysates were boiled for 5 min before being run on 10% resolving SDS-polyacrylamide gels. Protein gels were transferred to PVDF membranes (Amersham) using a Mini-PROTEAN 3 Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) overnight at 30 V in transfer buffer [25 mM Tris/HCl (pH 8.3), 192 mM glycine and 20% methanol]. Membranes were blocked for at least 1 h at room temperature in blocking buffer (5% skimmed milk, 0.1% Tween 20 in PBS). Membranes were washed three times for 5 min in PBS/0.1% Tween 20 (PBS-T) and then probed with a guinea pig anti-NiV immune serum as the primary antibody; primary antibodies were diluted 1:800–1:1000 in blocking buffer containing 1% normal rabbit serum (Sigma). Primary antibody incubation was performed at room temperature for 1–2 h on a rocker. Membranes were washed three times for 10 min each in PBS-T. A rabbit anti-guinea pig or goat anti-swine horseradish peroxidase-conjugated antibody (Sigma) was used as the secondary antibody at a dilution of 1:10000 in blocking buffer. Secondary antibody incubation was performed at room temperature for 1 h, followed by washing with PBS-T three times for 10 min each. Blots were developed using the ECL Plus kit (Amersham) and exposed to Hyperfilm (Amersham) to visualize the bands.

Cell-surface staining using fluorescence activated cell sorting (FACS). In order to assess the effect of NiV glycoprotein expression and NiV infection on cell-surface ephrin-B2 and ephrin-B3, cell-surface staining was performed and stained cells were analysed by FACS on a FACSCalibur flow cytometer (BD). For infected cells, a 75 cm² flask of 293T cells was infected with approximately 10⁵ TCID₅₀ ml⁻¹ of NiV and incubated overnight at 37 °C. Approximately 18–20 h after infection, cells were removed from the flask with Versene (Invitrogen), pelleted by centrifugation at 500 g for 10 min, resuspended in 5 ml Mg²⁺/Ca²⁺-free PBS ( Gibco) and fixed in an equal volume of PBS-buffered 4% paraformaldehyde (PFA) overnight at 4 °C. The following day, infected cells were pelleted by centrifugation (10 min, 500 g), resuspended in 5 ml Mg²⁺/Ca²⁺-free PBS, diluted with an equal volume of PBS-buffered 4% PFA and removed from BSL4 containment. Rabbit polyclonal antibodies against human ephrin-B2 and human ephrin-B3 were purchased from Genex Biosciences, diluted in Mg²⁺/Ca²⁺-free PBS to a concentration of 1 mg ml⁻¹ and then used as described below. Polyclonal goat anti-rabbit R-phycocerythrin conjugate was purchased from Jackson ImmunoResearch. Mock and transfected cells were removed from 75 cm² flasks using 10 ml Versene per flask 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 min at 500 g, the supernatant was discarded and the cells were resuspended in 5 ml fresh Mg²⁺/Ca²⁺-free PBS. For FACS staining, 2.5 × 10⁶ cells per tube were blocked with 10 µl human gammaglobulin for 10 min at room temperature. Primary antibody diluted in Mg²⁺/Ca²⁺-free PBS (100 µl per tube) was added to cells, mixed and incubated at 4 °C for 30 min. Cells were washed twice with Mg²⁺/Ca²⁺-free PBS (1 ml per tube) with centrifugation for 5 min at 500 g between each wash. Secondary antibody diluted in Mg²⁺/Ca²⁺-free PBS was added to cell pellets, mixed and incubated for 30 min at 4 °C, followed by two washes with Mg²⁺/Ca²⁺-free PBS (1 ml per tube). Final cell pellets were resuspended in 500 µl Mg²⁺/Ca²⁺-free PBS and fixed overnight at 4 °C with an equal volume of PBS-buffered 4% PFA. Primary antibodies were used at a dilution of 1:100; whilst secondary antibody dilutions were generally used at dilutions of 1:200 to 1:400.
RESULTS

Requirements for syncytium formation

In order to study the fusogenic behaviour of the NiV glycoproteins, transfection experiments in 293T cells were performed. 293T cells were used because of their high transfectability. Expression of NiV glycoproteins was detected by Western blots. The major forms of NiV F were the uncleaved F0 precursor and the large cleavage fragment F1 (Fig. 1); the \( \sim 19 \) kDa cleavage fragment F2 was not visible on the blot. NiV G was detected as one band of \( \sim 75–80 \) kDa (Fig. 1). We then attempted to assess the fusogenic behaviour of the glycoproteins in cell culture. When NiV F or G was transfected alone into 293T cells, there was no formation of syncytia (Fig. 2). When NiV F and G were co-transfected, extensive fusion resulted and large syncytia developed (Fig. 2). However, when NiV F and G were transfected singly into separate cell populations followed by mixing of the cell populations, no syncytia were detected (Fig. 2). These results confirmed the observations by other groups that NiV F and G are both required for fusion to occur (Bossart et al., 2002; Tamin et al., 2002). The lack of fusion in the mixed populations of singly transfected NiV F and NiV G cells indicated that there is a requirement for a specific interaction between NiV F and G in the same cell for fusion to occur.

Generation of transgenic cells

In order to study the behaviour of cells expressing NiV G, transgenic CRFK cells were created using retroviral particles containing the recombinant NiV F or G gene. CRFK cells were transduced with vector alone (pczCFG5 IEGZ), NiV F (pczCFG5-NiV F) or NiV G (pczCFG5-NiV G) and were

![Fig. 1. Western blots of NiV F and G glycoproteins. 293T cells were transfected with plasmid encoding NiV F or G and lysed in SDS-PAGE gel loading buffer containing 2% SDS and 10% 2-mercaptoethanol. The samples were run on SDS-PAGE gels, transferred to PVDF membranes and probed with guinea pig anti-NiV F serum (left panel) or swine anti-NiV G serum (right panel). The precursor band of NiV F, F0, is cleaved to F1 and F2 (F2 is \( \sim 19 \) kDa and is not visible on the blot).](http://vir.sgmjournals.org)

![Fig. 2. Expression of NiV F and G. 293T cells were transfected with 4 \( \mu \)g plasmid and examined 24 h post-transfection. NiV F and NiV G cells were transfected singly with each protein. NiV F+NiV G cells were co-transfected with both glycoprotein genes. NiV F+NiV G (co-culture) cells were transfected singly with NiV F or G and then mixed at approximately 20 h post-transfection to allow cells to grow to confluence. Arrows indicate syncytia.](http://vir.sgmjournals.org)
denoted CRFK-pcz, CRFK-NiV F and CRFK-NiV G, respectively. Transgene expression in CRFK-NiV F cells was 93.08% and in CRFK-NiV G cells was 88.79% (Fig. 3b, left and right panels). These data indicated that we obtained a high level of transgene expression in the transgenic cells. In our experience, the read-through rate of the IRES between the first and second genes was > 90% and was a very reliable indicator of upstream gene expression. In order to verify functional expression of the glycoproteins, CRFK-NiV F or CRFK-NiV G cells were transduced with the complementary retroviral particle (CRFK-NiV F + NiV G particles or CRFK-NiV G + NiV F particles). Following incubation for 48 h, cells were examined for fusion. Syncytia developed containing approximately 15–20 nuclei per syncytium (Fig. 3a, bottom left and right). No syncytia developed in any of the singly transduced transgenic cells (Fig. 3a, top left and right). These data demonstrated the functional expression of NiV F and G in transgenic cells. The GFP levels in the CRFK-NiV F and CRFK-NiV G transgenic cells were verified by FACS analysis to assess transgene expression.

CRFK-NiV G cells are resistant to infection with NiV and HeV

Viral interference is a well-investigated phenomenon for retroviruses, but it has also been characterized for several other viruses, albeit to a lesser extent. We attempted to investigate whether expression of NiV F or G, or both, was able to inhibit infection with NiV and the closely related HeV. Wt CRFK cells, CRFK-pcz (vector control), CRFK-NiV F and CRFK-NiV G were seeded into rows in 24-well plates. NiV and HeV stocks were diluted from $10^6$ to $10^1$ TCID$_{50}$ ml$^{-1}$, corresponding to $5 \times 10^4$ to $5 \times 10^0$ TCID$_{50}$ per well. Virus was then applied to the transgenic cells in the 24-well dishes. The plates were examined daily for development of CPE. CRFK cells were used in this assay as they display a very clear CPE and therefore provided more clarity in this experiment than 293T cells.

At all doses of NiV and HeV, all control cells (wt CRFK, CRFK-pcz and CRFK-NiV F not shown) died by 5 days post-exposure. Transgenic CRFK-NiV G cells survived NiV exposure at all doses over the course of the experiment (Fig. 4), as did CRFK-NiV G cells exposed to HeV (Fig. 4). These data indicated that there was specific protection conferred by expression of the NiV G attachment glycoprotein. In contrast, the lack of protection in cells expressing NiV F also demonstrated that this is a phenomenon specific to NiV G expression and was not due to non-specific interference from NiV genes or gene products. The lack of protection in vector control cells (CRFK-pcz) also demonstrated the specificity of NiV G-conferred protection by excluding any components of the pczCFG5 IEGZ-based vector backbone. We also checked transgenic cells exposed to NiV and HeV for viral mRNA and genomic vRNA. We were not able to detect vRNA in CRFK-NiV G cells exposed to 5 TCID$_{50}$ of virus, indicating that nucleic acid from NiV and HeV could not be detected in cells that had survived 5 days of exposure to NiV or HeV (data not shown). In contrast, CRFK-NiV F and wt CRFK cells exposed to HeV and NiV were positive for all HeV and NiV mRNA and vRNA (data not shown). Mock-infected
cells did not have positive PCR results with any primer set. Total cellular RNA was tested for each sample using fGAPDH primers. The inability to detect viral nucleic acid in NiV- and HeV-exposed CRFK-NiV G cells seemed to indicate that virus was unable to enter these cells or was able to enter but was unable to replicate.

**Expression of NiV G inhibits NiV F- and G-mediated fusion**

The lack of viral nucleic acid and CPE in CRFK-NiV G cells that had been exposed to low doses of NiV and HeV indicated that NiV G was somehow restricting virus replication or entry in these cells. To elucidate further which step of the virus life cycle was inhibited, we developed a fluorescent fusion inhibition assay. 293T cells that had been transfected singly with pczCFG5-NiV G were mixed with cells that had been co-transfected with pHITΔGFP-NiV F and pHITΔGFP-NiV G (Fig. 5a, right). As controls, cells singly transfected with pczCFG5 IEGZ or pczCFG5-NiV F were also mixed in the same manner (Fig. 5a, left and centre). Approximately 16 h after mixing, pczCFG5-NiV F-transfected cells had fused with cells expressing NiV F and G and formed green fluorescent syncytia (Fig. 5b, centre), indicating that NiV F expression alone was not able to inhibit fusion induced by NiV F and NiV G co-expression. However, pczCFG5-NiV G-transfected cells did not fuse with cells expressing NiV F and G, and therefore did not form green fluorescent syncytia; they remained as single green cells (Fig. 5b, right). This indicated that expression of

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**Fig. 4.** Exposure of wt and transgenic cells to NiV and HeV. Cell monolayers were exposed to NiV as described in Methods. Cells were examined at 5 days post-exposure for CPE. Monolayers were fixed with 3.7% formaldehyde in PBS.
NiV G was sufficient to inhibit fusion induced by NiV F and G co-expression. The lack of fusion in NiV G-expressing cells suggested that NiV G had downregulated the expression of the cellular receptor on the surface of the cell, thereby rendering these cells resistant to fusion and infection. If NiV G interfered with replication steps downstream of virus attachment or entry, it would be expected that these NiV G-expressing cells would fuse in this assay; this did not occur.

Ephrin-B2 and ephrin-B3 are present on the surface of NiV-infected cells and cells transfected with NiV G

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. With the identification of ephrin-B2 (Bonaparte et al., 2005; Negrete 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. Untransfected control cells stained positive for cell-surface expression of ephrin-B2 and ephrin-B3 (Fig. 6, green line). Cells transfected with pczCFG5 IEGZ (empty vector) or pczCFG5-NiV F showed no differences in the cell-surface levels of ephrin-B2 or ephrin-B3 (not shown), indicating that expression of NiV F had no effect on cell-surface levels of ephrin-B2 or ephrin-B3 ligand. Interestingly, when 293T cells were transfected with pczCFG5-NiV G there was no difference in the cell-surface level of ephrin-B2 or ephrin-B3 (Fig. 6, blue line). There was also no cell-surface downregulation of ephrin-B2 or ephrin-B3 in cells infected with NiV (Fig. 6, brown line). Expression of NiV G, whether
by a recombinant plasmid-based system or by NiV infection, had no effect of the cell-surface levels of these two known NiV receptors.

**DISCUSSION**

Our initial data confirmed previous observations on the expression and behaviour of the NiV glycoproteins in cell culture. By themselves, NiV F and NiV G are not fusogenic, but they are highly fusogenic when expressed in the same cell. The expression of NiV F and 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 G must be expressed in the same cell and that interaction between the proteins is important for function, probably at the intracellular level. A similar result has been observed in co-cultures of cells separately expressing the F and haemagglutinin–neuraminidase (HN) glycoproteins of Human parainfluenza virus 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 Human parainfluenza virus 2 were mixed together (Hu et al., 1992).

Cellular expression of the NiV G glycoprotein led to a high degree of resistance towards infection with either NiV or HeV. Several lines of evidence indicated that protection was conferred specifically by 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 wt CRFK cells. This indicated that components of the pczCFG5 IEGZ vector backbone (GFP–Zeocin resistance fusion protein) were not playing a role in resistance to NiV. Secondly, CRFK-NiV F cells died following exposure to virus, indicating that NiV F had no protective effect. The lack of protection seen in CRFK-NiV F cells also demonstrated that resistance was not due to antisense RNA interference from cellular NiV F positive-sense gene transcripts with the incoming negative-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. Thirdly, viral nucleic acid (mRNA and vRNA) was not detected in CRFK-NiV G cells that had been exposed to 5 TCID50 HeV and NiV per well. Lastly, expression of NiV G rendered cells resistant to NiV F- and G-mediated fusion. This indicated that the blockage occurred at the level of virus binding and/or entry. If NiV G had inhibited downstream steps in virus replication, we would have expected to see fusion occurring with NiV G-expressing cells, a phenomenon that clearly was not observed.

All of the above lines of evidence point towards 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. As 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 downregulate (Marshall et al., 1997; Breiner et al., 2001) or perhaps even induce degradation of their cellular receptors (Horga et al., 2000), leading to the phenomenon of receptor interference. Expression of NiV G specifically inhibited the ability of cells to fuse with other fusogenic cells that expressed both NiV F and G, which indicated a block at the level of interaction with the cellular receptor. This was supported by the lack of any viral nucleic acid in CRFK-NiV G cells that had been exposed to low doses of NiV and HeV, and the accompanying lack of CPE in these cells. We have also observed that the haemagglutinin protein of Canine distemper virus does not protect cells from NiV infection, further confirming the specificity of NiV G-mediated protection (data not shown).

In prior studies, it has been suggested 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 it can 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 indirectly indicated a common mechanism of inhibition by binding of a soluble HeV G to cells, which resulted in competitive inhibition of NiV and HeV binding and infection (Bossart et al., 2005). One intriguing aspect of our study is the result that the two known NiV receptors, ephrin-B2 and ephrin-B3, are clearly present on the surface of cells expressing NiV G. We had expected that ephrin-B2 and ephrin-B3 would exhibit decreased cell-surface expression in NiV G-expressing cells; this 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. Based on our data, we conclude that expression of NiV G and infection by NiV have no effect on the cell-surface levels of ephrin-B2 and ephrin-B3.

In our studies, the result of NiV G expression was resistance to NiV- and HeV-induced CPE. We hypothesize that this resistance is due to viral receptor interference, although neither one of the known viral receptors, ephrin-B2 and ephrin-B3, is downregulated from the cell surface. Further work will focus on identification of the receptor-binding domain(s) within the attachment protein and regions responsible for interaction with the fusion protein, which are probably encoded by separate regions of the protein.

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