Nipah virus entry can occur by macropinocytosis

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A R T I C L E   I N F O

Article history:
Received 12 August 2009
Returned to author for revision 27 August 2009
Accepted 14 September 2009
Available online 24 October 2009

Keywords:
Nipah virus
Entry
Macropinocytosis
Antivirals

A B S T R A C T

Nipah virus (NiV) is a zoonotic biosafety level 4 paramyxovirus that emerged recently in Asia with high mortality in man. NiV is a member, with Hendra virus (HeV), of the Henipavirus genus in the Paramyxoviridae family. Although NiV entry, like that of other paramyxoviruses, is believed to occur via pH-independent fusion with the host cell’s plasma membrane we present evidence that entry can occur by an endocytic pathway. The NiV receptor ephrinB2 has receptor kinase activity and we find that ephrinB2’s cytoplasmic domain is required for entry but is dispensable for post-entry viral spread. The mutation of a single tyrosine residue (Y304F) in ephrinB2’s cytoplasmic tail abrogates NiV entry. Moreover, our results show that NiV entry is inhibited by constructs and drugs specific for the endocytic pathway of macropinocytosis. Our findings could potentially permit the rapid development of novel low-cost antiviral treatments not only for NiV but also HeV.

Introduction

The zoonotic biosafety level 4 (BSL-4) pathogens Nipah virus (NiV) and Hendra virus (HeV) are the sole members of the Henipavirus genus in the Paramyxoviridae family. NiV first emerged 10 years ago in Malaysia from its reservoir the Pteropus fruit bat, infecting successively pigs and humans. When the virus first emerged, NiV-induced encephalitis was responsible for a mortality rate of 40% but latterly annual NiV outbreaks in Bangladesh have occurred in which no intermediate host has been identified and the mortality rate has almost doubled. At present, no antiviral treatments or prophylaxis are available to combat NiV infection. Gaining a better understanding of NiV entry into the host cell is crucial if antivirals inhibiting this process and hence infection are to be identified.

The entry of enveloped viruses into the host cell is believed to occur either by pH-independent fusion of the virion envelope with the plasma membrane or by endocytosis. In the latter case, the acidic pH of the endosome is thought to trigger conformational changes in the fusion protein leading to fusion of the virion and endosomal membranes (Earp et al., 2004). NiV entry has been assumed to occur by the former mechanism, mediated by the concerted action of the viral glycoproteins following receptor attachment. NiV uses both ephrinB2 and ephrinB3 – ligands for members of the EphB class of receptor tyrosine kinases (RTKs) – as receptors for entry (Bonaparte et al., 2005; Negrete et al., 2005, 2006). Importantly, ephrinB2 and ephrinB3 also possess RTK activity. Ephs and ephrins are plasma membrane-bound proteins and consistent with NiV’s tropism, ephrinB2 is expressed on vascular endothelial cells and neurons whereas ephrinB3 expression is limited to cells in particular areas of the CNS such as the corpus callosum and the spinal cord.

Although the entry of paramyxoviruses such as NiV has been considered to occur exclusively by fusion at the plasma membrane (Lamb and Kolakofsky, 2001), two recent studies (Cantin et al., 2007; Kolokoltsov et al., 2007) suggest that endocytosis can also play a role in the entry of two paramyxoviruses: caveolae-dependent endocytosis in the case of Newcastle disease virus (NDV) and clathrin-dependent endocytosis for respiratory syncytial virus (RSV). Recently, vaccinia virus, the prototype of the family Poxviridae, and human adenovirus of the family Adenoviridae have been shown to use macropinocytosis for entry (Amstutz et al., 2008; Mercer and Helenius, 2008). Constitutive macropinocytosis is an active endocytic pathway occurring in macrophages and immature dendritic cells, which serves to take up fluid and exogenous antigens from the extracellular milieu but is also implicated in cell migration (for a review see (Conner and Schmid, 2002; Klasse et al., 1998). In many other cell types macropinocytosis can be induced transiently by interaction of receptors with specific ligands. For example, binding of epidermal growth factor (EGF) to its receptor EGFR induces rapid internalization of both proteins and intracellular sorting to the lysosomes by a kinase-dependent pathway (Kornilova et al., 1996). Moreover, macropinocytosis occurs in regions of the plasma membrane where the formation of lamellipodia and filopodia is taking place (for a review, see Klasse et al., 1998). A recent review on viral entry by macropinocytosis (Mercer and Helenius, 2009) discusses the molecular machinery involved in this endocytic pathway and the criteria by which it can be characterized. Here, we show that both in Vero cells and CHO-K1 cells expressing human ephrinB2, entry by the paramyxovirus NiV occurs not by fusion at the...
plasma membrane but by an endocytic mechanism that fit criteria defining macropinocytosis.

Results

ephrinB2 induces filopodia formation and is endocytosed by NiV-G

The original aim of this study was to investigate whether ephrinB2 is downregulated from the cell surface upon infection. We had previously mapped the ephrinB2 binding site on the NiV-G globular head (Guillaume et al., 2006) and endeavored to map the NiV-G binding site on ephrinB2 by using receptor downregulation as a handle. We anticipated that ephrinB2 could be downregulated by NiV-G because members of the Morbivirus genus, the only paramyxovirus genus other than the Henipaviruses to use cellular proteins as receptors rather than sialic acid, internalize their receptors upon infection. A major problem in studying ephrinB2 downregulation is the lack of an adequate α-human-ephrinB2 antibody, probably explained by the very high level of conservation between human and murine ephrinB2. To overcome this difficulty, we used a recombinant system tagging human ephrinB2 with eGFP by subcloning the ephrinB2 gene into the peGFP-N1 expression vector that we used to transfect (ephrinB2-negative) CHO-K1 cells. In contrast to the expression of eGFP (Fig. 1A), we found that the expression of ephrinB2-eGFP induced the formation of multiple extended ramified filopodia (Fig. 1B). This finding is in agreement with a previous report that expression of murine ephrinB2 in B16 melanoma cells induces the formation of filopodia thereby enhancing cell migration (Meyer et al., 2005).

Using confocal microscopy analysis, we found that when ephrinB2 tagged with eGFP (green) was co-expressed with mRFP (red) in CHO-K1 cells, the ephrinB2 was present in the plasma membrane, in particular at the level of the numerous filopodia, but there was no colocalization with mRFP whose expression was cytoplasmic (Fig. 2A, top). However, when ephrinB2.eGFP was co-expressed with NiV-G, mRFP, the two proteins were endocytosed, colocalizing in intracellular vesicles and the number of filopodia appeared to be reduced (Fig. 2A, middle). Expression of mRFP-tagged NiV-G was verified by flow cytometry and its fusion promotion capacity by co-expression with NiV-F (Figs. S1A and B). Furthermore, soluble murine EphB4-Fc was used to demonstrate that its surface expression was equivalent to that of non-tagged ephrinB2 (Fig. S3). As our previous results had shown that residue E333 of NiV-G is crucial for the interaction with ephrinB2 (Guillaume et al., 2006), we also co-expressed the NiV-G, E333Q.mRFP mutant with ephrinB2.eGFP. In this case, smaller vesicles with much lower levels of colocalization were observed and the number of filopodia appeared to be unaffected (Fig. 2A bottom).

As these results appear to show that ephrinB2 is endocytosed from the cell surface following contact with NiV-G, we began to consider the possibility that the whole virus could enter the host cell by the same mechanism. Thus, it was important to determine whether intracellular vesicles were also formed when ephrinB2 and NiV-G were expressed on opposing cells. For this, cocultures of CHO cells with CHO cells expressing either eGFP or ephrinB2.eGFP, even when in close proximity, there was no transfer of NiV-G to eGFP-expressing CHO cells (Fig. 2B, top). In contrast, in cocultures of NiV-G- and ephrinB2-expressing CHO-K1 cells both proteins were found to colocalize in large vesicles in ephrinB2-expressing cells as well as in NiV-G-expressing cells (Fig. 2B, bottom).

In this experiment, NiV-G was stained intracellularly using an anti-NiV-G monoclonal antibody (5A7) and a secondary goat-α-mouse antibody conjugated with Alexa568 (red), but identical results were obtained with NiV-G.mRFP (not shown).

The intracellular vesicles containing ephrinB2 and NiV-G traffic to the lysosomes

Time-lapse microscopy of CHO cells expressing ephrinB2-eGFP and NiV-G shows trafficking of incoming vesicles towards the center of the cell (Video S1). As a particular type of endocytic vesicle – the macropinosome – has been shown to traffic and fuse with lysosomes (Racocin and Swanson, 1993), we decided to investigate whether this fate befell intracellular vesicles containing ephrinB2 and NiV-G. To determine this, CHO-K1 cells co-expressing ephrinB2.eGFP and NiV-G tagged with eCFP were stained with Lysotracker Red, a dye specific for the lysosomal compartment. The majority of vesicles containing both ephrinB2-eGFP (green) and NiV-G.eCFP (cyan-blue) co-stained with Lysotracker (red) (Fig. 3). The enlargement (Fig. 3, right) shows typical lysosomes (indicated by arrows) in proximity with giant intracellular vesicles containing ephrinB2 and NiV-G. Expression of eCFP-tagged NiV-G was verified by flow cytometry and its fusion promotion capacity by co-expression with NiV-F (Figs. S1A and B). Lamp1 is a membrane protein marker of late endosomes/lysosomes and similar results were obtained with an anti-Lamp1 antibody/Alexa568 staining (Fig. S6).

Taken together, our results strongly suggest that the intracellular vesicles containing ephrinB2 and NiV-G are endocytic – possibly macropinocytic – and traffic to the lysosomes.

VLPs expressing NiV-G or NiV-G/F/M enter cells by the same mechanism as NiV-G

We next wanted to investigate whether the interaction between NiV-G and ephrinB2 would allow whole virus to enter by this endocytic mechanism but were unable to make confocal microscopy studies with live NiV outside of the BSL-4 laboratory. To overcome this difficulty, we generated virus-like particles (VLPs) expressing NiV-G, either alone or in combination with NiV-F and NiV-M. It has been shown that expression of individual NiV proteins (for example NiV-G) from plasmids can drive the formation of VLPs that are released into

![Fig. 1. ephrinB2 induces filopodia formation. CHO cells were transfected with either 1 μg of peGFP-N1 (A) or 1 μg of peGFP-N1-ephrinB2 (B) and observed by conventional fluorescent microscopy.](image-url)
culture supernatants (Patch et al., 2007). Furthermore, the co-expression of NiV-G and NiV-F together with NiV-M leads to the production of VLPs that have the size and characteristics of authentic virus (Patch et al., 2007). It was particularly important to include NiV-F to study if VLPs containing the two NiV glycoproteins would fuse with the plasma membrane rather than enter endocytically. Thus, a western blot was made to confirm the presence of NiV-G and NiV-F in the latter VLPs (Fig. S4). We used the Patch protocol (Patch et al., 2007) as a basis to produce the VLPs which, labeled with octadecyl rhodamine B (R18), a lipophilic red fluorescent membrane dye, were added to cells expressing either eGFP or ephrinB2-eGFP (Fig. 4). CHO-K1 cells expressing eGFP did not bind NiV-G-expressing VLPs (Fig. 4A), whereas binding as well as internalization of VLPs was observed with ephrinB2-expressing cells (Figs. 4B and C). VLPs were found in association with filopodia and in large intracellular vesicles where they colocalized with ephrinB2-eGFP (Fig. 4B). In later stages of the uptake process, ephrinB2-eGFP and NiV-G-containing VLPs colocalized entirely in large intracellular vesicles (Fig. 4C). Similar results were obtained with VLPs produced by the co-expression of NiV-G, NiV-F and NiV-M, with no evidence of R18 coloration of the plasma membrane (Fig. 4D). These results led us to hypothesize that NiV can enter cells via an endocytic mechanism.

Mutation of ephrinB2 residues responsible for interaction with EphB4 abrogates NiV entry

Interestingly, ephrinB2 and its cognate receptor EphB4 are also co-endocytosed into intracellular vesicles following their interaction on opposing plasma membranes (Marston et al., 2003). We confirmed this by tagging mEphB4 with mRFP and showing that it is co-endocytosed with ephrinB2 in the same manner as NiV-G (Fig. 2). This result led us to hypothesize that NiV could gain entry into the host cell by NiV-G mimicking EphB4’s interaction with ephrinB2. To test this, we investigated the effect on NiV entry of mutating residues in the solvent-exposed G–H loop of ephrinB2 shown in a co-crystallization study (Chrencik et al., 2006) to be responsible for the interaction with EphB4. To evaluate this, we used NiV cell–cell fusion as a read-out for NiV entry. Cell–cell fusion (syncytia formation) is the hallmark of NiV infection and is dependent upon viral entry. If the virus enters, by whatever mechanism, viral replication ensues and de novo synthesized NiV glycoproteins G and F accumulate in the infected cell’s plasma membrane inducing syncytia formation by fusion with surrounding cells. Quantification was made by expressing the number of nuclei present in syncytia as a percentage of the total number of nuclei, as previously described (Guillaume et al., 2006). To ensure that the mutations do not have an inhibitory effect on the viral fusion process per se, we also tested their effect on cell–cell fusion induced by the viral glycoproteins G and F expressed from plasmids. The expression and localization of all ephrinB2 mutants was verified by flow cytometry and conventional microscopy (Figs. S2A and B).

If NiV-G mimics EphB4, mutations in the G–H loop should abrogate the interaction ephrinB2/NiV-G blocking in turn NiV entry and NiV-induced cell–cell fusion. We found that this was indeed the case. Two single mutations, F120A and W125A, reduced cell–cell fusion (not shown) whereas the quadruple mutation of F120, N123, W125 and
Fig. 4. NIV-G-containing VLPs are internalized into large vesicles in ephrinB2-expressing cells. Virus-like particles (VLPs) produced from CHO cells expressing NIV-G were labeled with the fluorescent membrane dye R18 and added to CHO-K1 cells expressing either eGFP or ephrinB2-eGFP for 1 h at 37 °C. After extensive washing, cells were fixed and analyzed by confocal microscopy to determine the localization of the VLPs. (A) NIV-G-expressing VLPs do not localize in eGFP-expressing CHO cells. (B) NIV-G-expressing VLPs bind to the filopodia of ephrinB2-eGFP-expressing cells and (C) are internalized. (D) The same experiment performed with VLPs containing NIV-M, NIV-F and NIV-G.
L127 to alanine resulted in an abrogation of fusion (Figs. 5A and B). These results demonstrate that the same domain of the G–H loop of ephrinB2 is responsible for the interaction with both EphB4 and NIV-G.

Endocytic entry by NIV does not require low pH

We next used the lysosomotropic drug chloroquine (CQ), which raises the pH of late endosomes and lysosomes, to investigate whether the potential endocytic entry by NIV is dependent (as is the case for the vast majority of viruses that enter by receptor-mediated endocytosis) on the acidic conditions found in late endosomes and lysosomes. We found that 100 μM CQ reduced the number of NIV plaques in the plaque assay by 100% but importantly cell–cell fusion induced by transient co-expression of the NIV glycoproteins was also completely abrogated (not shown). Interestingly, further investigation revealed that NIV glycoproteins were present in the plasma membrane of the infected cells (Fig. S5) treated with CQ. This suggests that low pH is not required to allow NIV to exit from the endosomal vesicle and replicate but that cleavage of the NIV–F, catalyzed by the low pH requiring protease cathepsin L (Pager et al., 2006), is inhibited under these conditions. Our results confirm and extend the results of a recent study (Porotto et al., 2009) which identified CQ as an inhibitor of henipavirus replication via a high-throughput screening assay using recombinant vesicular stomatitis virus (VSV) pseudotyped with HeV-G and NIV-F.

Interaction of NIV-G with ephrinB2 induces phosphorylation of tyrosine residues in ephrinB2’s cytoplasmic tail

The cytoplasmic tail of ephrinB2 mediates reverse signaling via protein–protein interactions with intracellular proteins (Cowan and Henkemeier, 2001; Lu et al., 2001). Upon interaction with Ephs, ephrins become phosphorylated at cytoplasmic tyrosine residues within the 22 residue region between residues 301 and 322 which allows the recruitment of the adaptor protein Grb4 that transduces the signal to downstream signaling cascades regulating cytoskeleton dynamics (Cowan and Henkemeier, 2001; Holland et al., 1996). We thus investigated whether the contact of NIV-G with ephrinB2 induces the phosphorylation of tyrosine residues in ephrinB2’s cytoplasmic tail. We did this by co-expressing NIV-G, mRFP and ephrinB2-eGFP in CHO-K1 cells and then staining with an α-phosphotyrosine antibody. The quantification trace shows that phosphorylation of ephrinB2 occurs where NiV-G (red) and ephrinB2 (green) colocalize whereas cells not transfected with NIV-G phosphorysine was scattered in small punctuations (Fig. 5C). These results suggest the possibility that ephrinB2 cytoplasmic domain tyrosines are phosphorylated following contact with NIV-G.

A single tyrosine mutation (Y304F) in the ephrinB2 cytoplasmic region virtually abrogates NIV entry

We next investigated whether phosphorylation of ephrinB2 following interaction with NIV-G was relevant to entry of the whole virus. To investigate whether tyrosine residues present in the ephrinB2 cytoplasmic tail play a role in NIV entry, we first truncated ephrinB2’s cytoplasmic tail at serine268. By using cell–cell fusion as a read-out for NIV entry, we found that this truncation drastically reduced NIV entry, and hence fusion. However, this truncation did not affect cell–cell fusion induced by transient co-expression of the NIV glycoproteins (Figs. 5A and B).

Next, all six tyrosine residues present in the cytoplasmic tail were mutated individually to phenylalanine (which lacks the hydroxyl group on the phenyl ring that allows tyrosine to be phosphorylated). Mutation of the two tyrosine residues present at the C-terminus, Y330 and Y331, had no effect (Figs. 5A and B) and a similar result was obtained mutating Y311, Y316 and Y252 (not shown) but the Y304F mutation caused a 98% reduction in NiV-induced cell–cell fusion without having any negative effect on cell–cell fusion induced by transient co-expression of the NIV glycoproteins (Figs. 5A and B). Furthermore, we found that the number of NIV-G/ephrinB2 vesicles staining positive for phosphotyrosine was more than 90% reduced in CHO cells expressing the Y304F mutant of ephrinB2 rather than the wt protein (Fig. 5D). The quantification graph confirms that strong localization of ephrinB2 (green) and α-phosphotyrosine (blue) only occurs where NIV-G (red) fluorescence increases (Fig. 5C Graph). Interestingly, the Y304F mutant still localizes with NIV-G but cannot be phosphorylated (phosphotyrosine signal absent) or internalized into large vesicles (Figs. 5C and D). These results strongly suggest that phosphorylation of Y304 plays an important role in NIV entry.

If NIV entry is dependent upon phosphorylation of tyrosines in the ephrinB2 cytoplasmic tail, this raises the possibility of blocking NIV entry by inhibiting tyrosine kinase activity. We thus examined the effect of the broad-spectrum tyrosine kinase inhibitor genistein using a plaque assay. We found that 50 μM of genistein resulted in a statistically significant reduction (22%; p < 0.05) in the number of NIV plaques (Fig. 6A) but had little effect on cell–cell fusion induced by transfection of plasmids expressing NIV-G and NIV-F (Fig. 6B). Although the pleiotropic and toxic nature of this broad-spectrum inhibitor has to be taken into account, these results imply that tyrosine kinase activity plays a role in NIV entry.

Taking our results together with the Klein group’s study of EphB-ephrinB bi-directional endocytosis (Zimmer et al., 2003) led us to hypothesize that the endocytic pathway used by NIV for entry could be macropinocytosis. We thus tested the effect of a number of known inhibitors of viral entry by macropinocytosis (Mercer and Helenius, 2009) on the entry of NIV.

Dominant-negative mutants of Rac1 and Cdc42 reduce cell–cell fusion induced by NIV but the RhoA dominant-negative mutant has no effect

The Rho subfamily small GTPases Rac1 and Cdc42, whose expression induces the production of lamellipodia and filopodia respectively, have been implicated in the control of macropinocytosis (Garrett et al., 2000; West et al., 2000). In a preliminary investigation, we tested the effect of Toxin B (from Clostridium difficile) a monoglycosyltransferase known to inhibit Rho subfamily GTPases (Aktories and Barbieri, 2005) on NIV entry. We found that 80 mM Toxin B reduced the number of NIV plaques by 50% (Fig. 6A) but had little effect on cell–cell fusion induced by transient co-expression of the NIV glycoproteins (Fig. 6B). However, for the reason that Toxin B probably has a pluripotent effect on cells, we targeted Rac1 and Cdc42 directly. According to a recent review on viral entry by macropinocytosis (Mercer and Helenius, 2009), infection by macropinocytosis depends upon Rac1 and Cdc42 but not RhoA. We found this to be the case. Expression of the dominant-negative mutant of RhoA had no effect on NIV-induced cell–cell fusion but expression of the dominant-negative mutants of Rac1 or Cdc42 led to a reduction of by 46% and 53%, respectively (Fig. 6C). In contrast, the Rac1 and Cdc42 mutants had no or minimal effect on cell–cell fusion induced by transient co-expression of the NIV glycoproteins (Fig. 6C). These results give strong support to the hypothesis that NIV entry occurs via macropinocytosis.

PI(3)K inhibitors reduce NIV entry

As Rac activation by receptor tyrosine kinases initiates the phosphatidylinositol-3-kinase (PI(3)K) signaling pathway which modulates the closure of macropinosomes, the PI(3)K inhibitors wortmannin and LY294002 should have an effect on viral entry if this occurs by macropinocytosis (Mercer and Helenius, 2009). We found that this was indeed the case: wortmannin and LY294002 reduced the number of NIV plaques in our plaque assay by 65% and 48%, respectively.

PI(3)K inhibitors reduce NIV entry
respectively (Fig. 6A). Moreover, we found that the small molecule blebbistatin, a specific inhibitor of myosin II (required to effect the closure of macropinosomes) reduced NIV plaque numbers by 54% (Fig. 6A).

Latrunculin A strongly inhibits NIV entry

Macropinosome formation is an actin-based process. Thus, viral entry and infection via macropinocytosis should be sensitive to inhibitors of actin dynamics such as the compound Latrunculin A (Mercer and Helenius, 2009). We found that Latrunculin A, which inhibits the polymerization of actin, reduced the number of NIV plaques in our plaque assay by 83% (Fig. 6A). This result provides critical evidence that NIV entry occurs via macropinocytosis.

Amiloride abrogates NIV entry

Finally, we tested the effect of amiloride a specific inhibitor of macropinocytosis which acts by blocking the Na+/H+ exchange required for macropinocytosis to occur (West et al., 1989). Fluid uptake by cells via macropinocytosis can be monitored by the use of the fluid phase marker 70-kDa dextran (Mercer and Helenius, 2008). We hypothesized that if the NIV-G/ephrinB2 interaction induces macropinocytosis, the transient expression of NIV-G in cells permissive for NIV should result in an uptake of 70-kDa dextran that would be sensitive to ethyl isopropyl amiloride (EIPA). We found that this was indeed the case: expression of NIV-G in Vero cells resulted in a steady increase in the uptake of 70-kDa FITC-dextran over 60 min as measured by flow cytometry and treatment with 100 μM EIPA reduced this uptake by more than 35% (Fig. 6D). Moreover, we found that 100 μM EIPA reduced NIV plaque numbers in a plaque assay by 100% (Fig. 6A) but had minimal effects on cell–cell fusion induced by the transient co-expression of the NIV glycoproteins (Fig. 6B). When we investigated the effect of lower EIPA concentrations in the plaque assay, we found that the number of plaques (pfu) formed was inversely proportional to the EIPA concentration (Fig. 6E).

Discussion

The major conclusion from this study is that NIV can enter the host cell by receptor-mediated endocytosis and that the particular endocytic pathway used is macropinocytosis. To our knowledge, this is the first demonstration of paramyxovirus entry by macropinocytosis—the vast majority are assumed to enter the host cell by fusion (at neutral pH) at the plasma membrane. Moreover, our results obtained with the lysosomotropic drug chloroquine suggest that the endocytic pathway

Fig. 6. NIV entry is dependent on macropinocytosis. Vero cells were either (A) infected with NIV or (B) co-transfected with phCMV-NIV-G and phCMV-NIV-F in the presence or absence of 100 μM S-(N-ethyl-N-isopropyl) amiloride (EIPA), 80 μM Toxin B (ToxB), 50 μM genistein, 100 μM LY294002, 50 μM Blebbistatin (Blebb), 50 nM Wortmannin (Wort) and 2 μM Latrunculin A (LatA). (A) Virus entry was evaluated by plaque assay after 72 h p.i. and (B) fusion activity by cell–cell fusion assay 48 h p.i. (C) Dominant-negative mutants N17Rac1-eGFP, N17Cdc42-eGFP or N19RhoA were expressed in Vero cells 24 h before infection by NIV or co-transfected with NIV-G and NIV-F. Syncytia formation was quantified as described above 24 h p.t. or 48 h p.i., respectively. Experiments were performed in triplicate and data are represented as the standard deviation of the mean. (D) FITC-Dextran uptake by NIV-G-expressing Vero cells is inhibited by EIPA in a fluid uptake assay. Vero cells, expressing either NIV-G, treated with PMA (positive control) or left untreated, were incubated with FITC-Dextran for the indicated time points. Uptake of FITC-Dextran is indicated by the mean fluorescence intensity (MFI). One representative experiment out of three is shown. (E) EIPA dose effect on virus entry was evaluated by plaque assay after 72 h p.i.

Fig. 5. NIV entry requires tyrosine 304 in the ephrinB2 cytoplasmic domain. (A) CHO cells transfected to express ephrinB2-eGFP (non-mutated), ephrinB2Δ268-eGFP with four G→C-H loop residues mutated (F120, N123, W125, L127/AAAA), ephrinB2 lacking the cytoplasmic tail (Δ268) or with cytoplasmic tail tyrosine residues mutated to phenylalanine (Y304F, YY330/1FF). Cells were either infected with NIV (200 PFU/well) 24 h p.t. (upper panel) or co-transfected with phCMV-NIV-G and phCMV-NIV-F (lower panel). Cells were examined 48 h p.i. or p.t. for syncytia formation. (B) Cell-to-cell fusion in Fig. 4A was quantitatively analyzed and is expressed as the ratio of nuclei in syncytia to total nuclei in three randomly selected images. Experiments were performed in triplicate and data are represented as the standard deviation of the mean. (C) ephrinB2 is phosphorylated upon interaction with NIV-G. Upper panels: CHO cells expressing ephrinB2-eGFP with or without NIV-G-mRFP were fixed, permeabilized, stained with an α-phosphotyrosine antibody and a secondary antibody, goat-α-mouse-Alexa647 and then analyzed by confocal microscopy. Lower panels: the same experiment made with ephrinB2Δ268Y304F. Scale bar: 15 μm. (D) Effect of ephrinB2 Y304F mutation on colocalization of ephrinB2 and anti-phosphotyrosine, with and without NIV-G, in intracellular vesicles. The number of ephrinB2 Δ/anti-phosphotyrosine−vesicles per cell was counted. For each set, 10 cells were analyzed and data are represented as the standard deviation of the mean.
used by NIV does not depend upon low pH, an entry mechanism that few viruses are known to utilize. Our results suggest that NIV achieves endocytic entry by virtue of its attachment protein NIV-G mimicking the interaction of EphB4 with its ligand ephrinB2. Importantly, both EphB4 and ephrinB2 have receptor tyrosine kinase (RTK) activity and are implicated in numerous cellular processes important in development. Notably, they are expressed respectively on venous and arterial endothelial cells. During vasculogenesis, their interaction ensures that repulsion rather than adhesion occurs when a venous cell encounters an arterial cell. Repulsion is achieved by bi-directional trans-endocytosis of the intact EphB4-ephrinB2 complex that is concomitant with depolymerization of the actin skeleton and retraction of filopodia and lamellipodia. This mechanism strongly resembles receptor-mediated macropinocytosis (Wilkinson, 2003).

Recent publications have already hinted that endocytosis is involved in Henipavirus replication although the mechanism by which this is achieved and indeed its significance, have remained obscure. The first indication was the finding that the protease Cathepsin L – which localizes to low pH late endosomes and lysosomes – is responsible for the proteolytic processing of both the HeV-F protein (Pager and Dutch, 2005) and NIV-F (Himanen et al., 2001). Another study using transient expression of HeV glycoproteins in Vero cells found that expression of dominant-negative mutants of GTPases Rac1 and Cdc42 had little effect on cell–cell fusion induced by plasmids expressing NIV-G and NIV-F (Schowalter et al., 2006), but these authors did not investigate the effect of these mutants on cell–cell fusion induced by the virus. Finally, a recent publication (Diederich et al., 2008) reported that NIV can be endocytosed but the authors – who did not test any inhibitors of receptor-mediated macropinocytosis and did not demonstrate NIV-G-induced ephrinB2 downregulation – concluded that endocytosis is ‘definitely not required for the NIV entry process’. We believe that this conclusion is emphatically contradicted by our results.

In effect, our preliminary findings show that when ephrinB2 and NIV-G are expressed on opposing cells, both proteins are trans-endocytosed into intracellular vesicles which are then transported towards the lysosomes. Not only does this demonstrate, for the first time, that the NIV receptor is internalized from the cell surface upon interaction with NIV-G but also suggests that NIV-G can potentially mimic EphB4’s interaction with ephrinB2 and thereby allow NIV to enter by the same endocytic mechanism. Hitherto, the question of ephrinB2 downregulation by NIV-G has been controversial. A recent report (Sawatsky et al., 2007) concluded that both the expression of NIV-G and NIV infection had no effect on the cell-surface expression of ephrinB2. We found this result surprising because the closely related Morbilliviruses internalize their receptors upon infection. Viruses of the Morbillivirus genus and the Henipavirus genus differ from the other paramyxoviruses in that they use cellular proteins as receptors rather than sialic acid and for the Morbillivirus measles virus (MV) it has been shown that its two known receptors, CD46 and SLAM (CD150), are downregulated from the cell surface upon infection (Naniche et al., 1993; Tanaka et al., 2002). Moreover, ephrinB2 downregulation could potentially be responsible for the vasculitis that characterizes NIV pathogenesis, in particular encephalitis, as vascular smooth muscle mural cells require ephrinB2 for their association with small diameter blood vessels (Foo et al., 2006).

Our finding that expression of ephrinB2 provokes the formation of extensive polarized filopodia was an initial indication that macro-pinocytosis could be involved in NIV entry. Filopodia are protrusions of the plasma membrane important not only for cell movement and tentative encounters with other cells but also – in specialized cell types such as macrophages and immature dendritic cells – for uptake of fluid and the entrapment of exogenous antigen (for a review see Conner and Schmid, 2002; Klasse et al., 1998; West et al., 1989). If NIV-G mimics EphB4, the same residues of ephrinB2 should be responsible for the interaction with both of these proteins. We have demonstrated that this is indeed the case: mutating ephrinB2 residues in the solvent-exposed G–H loop that have been shown to be responsible for interaction with EphB4 in co-crystallization studies (Kolokoltsov et al., 2007) abrogated syncytia formation by NIV. A recent co-crystallization study of ephrinB2 and NIV-G (Bowden et al., 2008) confirms that this stretch of ephrinB2’s G–H loop is the binding site for NIV-G. Significantly, by tagging EphB4 with mRFP, we were able to show that this protein is co-endocytosed with ephrinB2 in the same manner as for NIV-G confirming the results of the Klein group (Zimmer et al., 2003). Because Klein’s group did not find any colocalization with markers of the clathrin and caveolae pathways, they speculated that the endocytic mechanism responsible for ephrinB–EphB endocytosis was either phagocytosis or macropinocytosis, especially as they found that the process was dependent on the GTPase Rac1 (Zimmer et al., 2003). Taking this into consideration together with our results, we decided to test the effect of a number of inhibitors known to disrupt viral entry by macropinocytosis (Mercer and Helenius, 2009) on NIV entry. Viral entry by macropinocytosis is dependent both on a multi-branched signaling cascade and cytoskeleton dynamics (Mercer and Helenius, 2009). The results obtained with the Rac1, Cdc42 and RhoA dominant-negative mutants, wortmannin, LY294002, blebbistatin and in particular Latrunculin A and the amiloride derivative EIPA provide compelling evidence that the receptor-mediated endocytic pathway that NIV uses for entry is macropinocytosis.

Although EIPA abrogated NIV infection, 100% the effect of amiloride is no longer considered to be the definitive test for macropinocytosis (Mercer and Helenius, 2009). However, as macro-pinocytosis, unlike fusion at the plasma membrane, requires actin polymerization to allow the necessary changes in cytoskeleton dynamics our finding that Latrunculin A gives an 83% inhibition of NIV infection provides very strong support that macropinocytosis is involved. It is interesting to note that inhibitors such as genistein and Toxin B, which are toxic and have pluriptotent effects in the cell, show relatively lower inhibition rates (22% and 50%, respectively) whereas the more specific inhibitors give higher inhibition rates. Obtaining less than 100% inhibition with macropinocytosis inhibitors could be interpreted as meaning that NIV does not enter exclusively by macropinocytosis into the cell lines we have used. We advance two major reasons why we believe that this is not the case. Firstly, as macropinocytosis is induced via a multi-branched signaling cascade (Mercer and Helenius, 2009) redundancy could provide an explanation as to why inhibition with certain drugs and dominant-negative mutants was not total. For example, Rac1 and Cdc42 dominant-negative mutants gave reductions in NIV infection by 46% and 53%, respectively. However, although Cdc42 is regarded to be responsible for filopodia formation (Garrett et al., 2000), filopodia are still present on cells which do not express Cdc42 (Czuchra et al., 2005). Secondly, the use of macropinocytosis for entry by NIV is supported by our finding that the cytoplasmic domain of ephrinB2 is required for entry—but significantly, not for viral spread (syncytia production).

Macropinocytosis is induced by a receptor tyrosine kinase (RTK)-dependent signaling cascade (Mercer and Helenius, 2008) and importantly the NIV receptor ephrinB2 has RTK activity. If NIV entry is dependent upon macropinocytosis the phosphorylation of ephrinB2 upon interaction with NIV would thus appear to be a necessity. Our results show that the interaction of NIV-G with ephrinB2 indeed induces the phosphorylation of the latter and that this involves a single tyrosine (Y304) in ephrinB2’s cytoplasmic domain. Significantly, mutation of this single tyrosine to phenylalanine abrogates NIV entry. However, in this respect it has recently been reported that ephrinB2’s cytoplasmic tail is not required for NIV entry (Thiel et al., 2008). If true, this would deal a near-fatal blow to our hypothesis of NIV entry by macropinocytosis but close examination of a western blot in this article reveals that there is a potential problem with their cytoplasmic tail-deleted ephrinB2. The western blot appears to show
that the untruncated ephrinB2 has a molecular weight (MW) of around 45 kDa whereas that of the truncated ephrinB2 appears to be close to 40 kDa. However, the MW of untruncated ephrinB2 is in fact 37 kDa (Masood et al., 2009). We suggest that this size difference could well be the basis for the difference between our results and those of the Maisner group, pertaining to the role of ephrinB2’s cytoplasmic tail in NiV entry.

A recent review on virus entry by macropinocytosis (Mercer and Helenius, 2009) poses the rhetorical question of whether there are different types of macropinocytosis and if so how do they differ? According to this review, viruses that enter by macropinocytosis cause cytoskeletal rearrangements to occur in the host cell (with the notable exception of dendritic cells) that lead to ruffling and blebbing at the plasma membrane. These changes in actin polymerization are induced by signaling from receptor tyrosine kinases (RTKs) which are indirectly activated by the virus binding to a plasma membrane protein. When the small filopodia or lamellipodia which constitute the ruffles melt back into the plasma membrane they engulf the virus to enclose it together with extracellular fluid in a macropinosome. We suggest that NiV entry by macropinocytosis represents a variation of this because the NiV receptor itself is an RTK and induces the formation of filopodia constitutively. Our interpretation of our results is that rather than inducing filopodia formation, the interaction with NiV-G with ephrinB2 triggers a signaling pathway that results in filopodia formation being turned off so that they retract and in doing so engulf the NiV particle (Fig. 7).

An obvious strategic advantage of macropinocytosis over plasma membrane fusion for viral entry is that – as for other endocytic mechanisms – no trace of the virus is left at the plasma membrane to attract the attention of the host immune response. However, further study will be required to investigate whether our results obtained with Vero and CHO-K1 cells can be extrapolated to other cell types. In particular it will be interesting to determine whether NiV entry occurs in cells such as macrophages and dendritic cells which operate continuous constitutive macropinocytosis.

Interestingly, our results suggest that the endocytic mechanism of entry used by NiV does not require low pH. But then why does fusion not occur at the plasma membrane? We suggest two possible explanations for this enigma. First, that in the case of NiV, endocytosis of the virus could occur more rapidly than plasma membrane fusion. The second possible explanation that we have considered is that NiV entry and NiV spread (cell–cell fusion) require different conformations of NiV-G. We are currently investigating both of these possibilities.

It is becoming clear that the mere docking of a virus to its cellular receptor is often insufficient for entry. Viruses have evolved to make use of the cellular machinery to enter the host cell and a prime means to do this is via signalization. That virus attachment to receptors triggers essential intracellular signals has only recently been appreciated (Marsh and Helenius, 2006). Interestingly, it has been reported that phosphorylation of Y304 of ephrinB2 confers high-affinity binding to the SH2 domain of the SH2/SH3 adaptor protein Grb4 which transduces signals via its SH3 domains (Su et al., 2004). As Grb4 transduces signals to downstream effectors of cytoskeleton dynamics Rac1 and Cdc42, this provides a plausible explanation of how NiV-G-induced ephrinB2 phosphorylation...
induces macropinocytosis. This is summarized in our schematic model for NiV entry shown in Fig. 7.

It is conceivable that our results can be generalized for those viruses whose receptor (i) is downregulated upon infection, (ii) is a signaling protein and (iii) induces the formation of filopodia. Human adenovirus subverts CtBP1-controlled macropinocytosis to gain entry (Amstutz et al., 2008), but although the actual mechanism by which macropinocytosis is subverted was not deduced by these authors, interestingly, this adenovirus uses CD46 as a receptor. CD46 is also a receptor for MV. That MV has the capacity to enter cells by macropinocytosis has been reported (Crimeen-Irwin et al., 2003) but this process was suggested to function to remove fusion-incompetent virus. Interestingly, we have discovered that CD46 – as well as MV's other cellular receptor SLAM (CD150) – induces the formation of filopodia extremely similar to those induced by ephrinB2 (Kweder and Buckland, unpublished results). CD46 is used as a receptor by other viruses such as human herpes virus 6 and certain bacteria such as Neisseria spp (Liszewski et al., 2005). Moreover, for Neisseria gonorrhoeae, cell entry has been shown to occur by macropinocytosis (Zenni et al., 2000) and that CD46 is phosphorylated at tyrosine residue 354 upon infection (Lee et al., 2002). Furthermore, a cellular protein to which oncolytic MV has been retargeted is EGFR (Nakamura et al., 2005). This choice was perhaps serendipitous as it appears to have been made, not because of EGFR's capacity to be macropinocytosed (following EGF binding) but for EGFR's frequent overexpression on human cancer cells.

In conclusion, the data we have accumulated provides convincing evidence that NiV entry occurs by macropinocytosis in the cell lines we have studied. Set against the present lack of NiV vaccines and the unlikelihood of their future development due to commercial considerations, the discovery that NiV can enter by this endocytic mechanism gives important new hope that a low-cost alternative antiviral strategy can potentially be developed against this deadly virus. In this regard, amiloride and CQ are particularly attractive candidates as they have both been long utilized in human medicine for treating hypertension and malaria, respectively. We are currently conducting trials in our P4 laboratory to evaluate the capacity of these drugs to block NiV infection in our animal model, the hamster.

Materials and methods

Cells

Chinese hamster ovary (CHO-K1) cells (# ACC110 obtained from DSMZ) were maintained in F12 medium containing 10% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 U/ml Fungizone and 10 mM HEPES. VeroE6 cells (African green monkey kidney fibroblast) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 U/ml Fungizone and 10 mM HEPES. All cell culture reagents were obtained from Invitrogen. Typically, 5 × 10^5 CHO or 2.5–5 × 10^5 Vero cells were seeded per well of a 6-well culture plate prior to transfection or infection.

Production of mutants and fusion proteins

Site-directed mutagenesis was performed as previously described (Guillaume et al., 2006). The NiV-G coding sequence was amplified by PCR (FastStart; Roche) and inserted into the pcEGFP-N1 (enhanced cyan fluorescent protein) plasmid (Clontech) between the BsrGI and NotI restriction sites or between the NotI restriction sites of the NotI-modified pcDNA3-mRFP (monomeric red fluorescent protein) plasmid. NotI-modified pcDNA3-mRFP is a pcDNA3-mRFP (Addgene) plasmid with an additional NotI site 8 bases before the EcoRI restriction site. The murine EphB4 (a gift from Ralf H. Adams, CRUK London) was cloned between the HindIII and BamHI sites of pcDNA3-mRFP. The plasmids pcMV-NiV-G, pcMV-NiV-F E533Q and pcMV-NiV-F have been previously described (Guillaume et al., 2006). The human ephrinB2 DNA sequence was cloned from the commercially available expression plasmid pcMV6-XL4-ehnB2 (Origene) and inserted into the pEGFP-N1 (enhanced green fluorescent protein) plasmid (Clontech), between the BglII and SalI sites. The human CD46 DNA sequence was cloned from plasmid pgt.CD46 (Masse et al., 2004) and inserted into pEGFP-N1 (Clontech), between the BglII and SalI sites. The mutated plasmids were amplified and purified as described previously (Masse et al., 2004). Mutations were introduced using QuickChange kit (Qagen, France) according to the manufacturer's instructions. All mutations were verified by DNA sequencing. Primer sequences are available on request.

Transfections

Cells were transfected in 1 ml OptiMEM medium containing plasmid DNA and 1% Lipofectamine (both Invitrogen). For single and double transfections, 1 μg of each plasmid was used (unless indicated otherwise). For triple transfections, 1 μg of pcMV-NiV-G, 0.5 μg of pcMV-NiV-F and 0.25 μg of pEGFP-N1-ephrinB2 or its mutants were transfected. After 5–7 h incubation at 37 °C, the cells were washed and fresh medium added.

Infections

As NiV is a BSL class 4 agent, infections were performed in the Jean Mérieux BSL-4 laboratory, Lyon, France. NiV (isolate UMMC2, GenBank accession number AY029768.1) isolated from the cerebro-spinal fluid of a patient was a generous gift from Kwang Bing Chua (University of Malaya, Kuala Lumpur, Malaysia). Virus was grown and titrated in Vero cells, and a stock was made on the seventh passage on Vero cells following virus isolation. Vero cells or transfected CHO cells were infected with 200 PFU NiV per well for 45 min at 37 °C using a virus stock diluted in 1 ml of non-supplemented DMEM. Infection media were then removed and fresh medium added. Images were taken 48 h later.

Microscopy analysis

Transfected cells, in D-PBS supplemented with MgCl2 and CaCl2, were observed using a Zeiss Axiovert 200M microscope. Confocal microscopy analyses were performed with either a Leica SP5 or a Zeiss Axiovert100M LSM510 microscope. Time-lapse analyses were performed using a Zeiss Time-lapse Axiovert 100M microscope at 37 °C and 5% CO2. Cells were analyzed after the indicated time points p.t. or p.i. Microscopy data were analyzed using ImageJ freeware and Leica LAS AF lite software.

Cellular and immuno-staining

Cells cultivated on LabTek slides (or on coverslips) were washed with PBS supplemented with CaCl2 and MgCl2. To detect NiV-G, cells were fixed with 4% PFA, permeabilized with PBS–0.25% Triton X-100 and immuno-stained using a monoclonal mouse-α-NiV-G antibody (clone 5A7, generated in our laboratory by V. Guillaume) diluted 1:250 and a secondary goat-α-mouse antibody conjugated with Alexa568 (diluted 1:500, Molecular Probes). To localize ephrinB2 on CHO-K1 cells (Fig. S3), soluble mEphB4-Fc was stained with goat anti-human antibody conjugated with Alexa 568 (diluted 1:500; Molecular Probes). For lysosome staining, cells were treated with 50 nM Lysotracker Red DND 99 (Molecular Probes) for 20 min at 37 °C, washed twice before fixation with cold 4% PFA for 10 min at room temperature and mounting in Faramount Mounting Medium (Dako).
Alternatively, cells were first fixed, permeabilized with 0.25% PBS–Triton X-100 and then stained with 1:200 diluted polyclonal murine anti-lamp1 antibody (Santa Cruz, CA; a kind gift from D. Muriaux) followed by 1:500 diluted goat-α-mouse antibody conjugated with Alexa568 (Molecular Probes). Tyrosine phosphorylation was detected in PFA-fixed cells using a mouse-α-phosphotyrosine antibody (clone P66, Sigma) diluted 1:1000 in PBS supplemented with 1% BSA and 0.33% saponin and a goat-α-mouse antibody conjugated with Alexa680 (diluted 1:600, Molecular Probes) in BSA–saponin buffer. Samples were mounted prior to analysis. In some experiments, mounting medium was supplemented with 0.5% DRAQ5 DNA dye (Biotostatus Limited) or Prolong antifade reagent containing DAPI (Invitrogen).

Flow cytometry

To determine the expression levels of ephrinB2-eGFP and its mutants as well as NiV-G and its fluorescently labeled or mutated versions, CHO-K1 or Vero cells were transfected as described above and analyzed by flow cytometry after 48 or 24 h p.t., respectively. Briefly, cells were washed with PBS, detached using trypsin and washed in 1 ml FACS buffer (0.5% bovine serum albumin and 0.02% sodium azide in D-PBS). Cells expressing NiV-G or one of its derivatives were fixed for 10 min in 4% PFA (in PBS), washed and immunostained using 5A7 monoclonal mouse-α-NiV-G antibody diluted 1:200 in FACS buffer followed by a goat-α-mouse antibody conjugated with fluorescein isothiocyanate (FITC, Dako) diluted 1:100 in FACS buffer for 30 min at 4 °C. Cells were analyzed using a FACSCalibur 3C cytometer (BectonDickinson) and CellQuest-Pro Software.

Co-culture assays

First, cells were separately cultured and transfected as described. Then cells were detached with trypsin and re-seeded for co-culture at a ratio of 40:60 (ephrinB2-expressing cells: NiG-expressing cells) on a cover slide for 24 h. Cells were fixed, immuno-stained for NiV-G and mounted as described above for subsequent analysis by confocal microscopy.

VLP assay

VLPs were produced, following the protocol of Patch et al. (2007) by transfection of CHO-K1 cells with phCMV-NiV-G or phCMV-NiV-N′G plasmids (GFP-plasmids) for 36 h. VLP-containing cell culture supernatants were stained with 1 μg/ml R18 for 1 h on a shaker at RT and then centrifuged (10 min, 3000× g at 4 °C). Supernatants were diluted in up to 3 ml NTE buffer (100 mM NaCl; 10 mM Tris–HCl, pH 7.5; 1 mM EDTA), loaded onto a 1-ml 20% sucrose cushion and ultra-centrifuged for 2 h at 150,000× g at 4 °C (Himac CS-150GX ultra-centrifuge with an SS0AT3 rotor). Pellets were washed with NTE buffer and re-ultra-centrifuged. Pellets were finally resuspended in 200 μl NTE buffer. VLP preparations were added to CHO-K1 cells expressing either ephrinB2-eGFP or eGFP for 1 h at 37 °C. Cells were then washed extensively, fixed, mounted and analyzed by confocal microscopy. A western blot was made to confirm the presence of NiV-G and NiV-F in the VLP’s. Briefly, the purified VLPs were resuspended and reduced in Laemmli buffer containing 5% β-mercaptoethanol (Sigma), at 100 °C for 10 min. Proteins were then separated on SDS–PAGE (12% acrylamide-bisacrylamide) and transferred onto a PVDF membrane. The NiV glycoproteins were stained using a murine α-NiV serum (generated in our laboratory by V. Guillaume) as a primary antibody and then a rabbit anti-mouse antibody (Dako) conjugated with horseradish peroxidase (HRP) as secondary antibody. HRP was revealed using an ECL kit and CL-Exposure film (both Pierce).

Quantification of NiV entry

In this study, two methods have been to quantify the effect of various inhibitors and mutations on NiV entry and determine its basis: a cell–cell fusion assay and a plaque assay. Both methods were used in tests involving viral infection (under BSL-4 conditions), but for infections due to inherent problems of maintaining monolayer integrity, only the cell–cell fusion assay was used.

**Plaque assay**

The 5×10⁵ Vero cells per 6-well culture plate were seeded and confluent cell layers were infected as described above. After infection, cells were kept in medium containing 33% carboxymethyl cellulose (Interchim) supplemented with inhibitors as indicated. Three days p.i., cells were washed with D-PBS, incubated with crystal violet solution (0.2% crystal violet, 10% formol and 20% ethanol) for 15 min and subsequently rinsed with water. The number of plaques (syncytia) was counted and the percentage relative to non-treated cells (set to 100%) calculated. Experiments were performed in triplicate and the error bars represent the standard deviation between experiments. A Student’s t-test was applied to determine the statistical relevance of the results obtained with Toxin B and genestein based on the original plaque counts in three experiments.

**Quantification of cell–cell fusion**

Cell–cell fusion in transfected cells was quantified as described previously (Guillaume et al., 2006). Briefly, 24 h p.t. or 48 h p.i., respectively, images of three microscope fields were taken randomly and the proportion of nuclei in syncytia relative to the total number of nuclei was determined by counting. Experiments were performed in triplicates and error bars represent the standard deviation.

**Inhibition of macropinocytosis/viral entry**

5-(N-Ethyl-N-isopropyl) amiloride (EIPA) (100 mM in DMSO), Toxin B (10 mM in non-supplemented DMEM), Wortmannin (10 mM in DMSO), LY294002 (100 mM in DMSO), Blebbistatin (100 mM in DMSO) and genistein (10 mM in ethanol) stock solutions were prepared (all Sigma). One hour before infection (or 7 h p.t. for controls with plasmids), inhibitors were added to the culture medium as well as to the infection and post-infection media (10× diluted for long-term lethal inhibitors Latrunculin, Wortmannin LY294002 and Blebbistatin). Quantification of the inhibition of non-transfected cells was measured by a plaque assay as described below whereas for cells transfected prior to infection the cell–cell fusion assay was used. Rho GTPases Cdc42, Rac1 and RhoA were inhibited by their respective N17 dominant-negative mutants: for the infection assay, 2.5×10⁵ Vero cells were transfected with 0.4 μg of pECP-CI-N17Cdc42 or pECP-CI-N17Rac1 (gifts from P. Jurdic) or pEGP-CI-N19RhoA (a gift from Delphine Muriaux) in 6-well culture plates and infected 18 h p.t. as described above. For the fusion assay, 0.4 μg of pECP-CI-N17Cdc42 or pECP-CI-N17Rac1 or pECP-CI-N19RhoA dominant-negative mutants were co-transfected with 1 μg of phCMV-NiV-G and 1 μg of phCMV-NiV-F.

**Fluid phase uptake analysis**

Fluid phase uptake was assessed using a modified protocol previously described (Mercer and Helenius, 2008). Vero cells (5×10⁴ per well) were seeded in 24-well culture dishes and either transfected with phCMV-NiV-G or phCMV-NiV-N-G the following day or left untreated. At 20 h p.t., all cells were serum starved for 3 h at 37 °C and 5% CO₂ before adding 100 μM EIPA in serum-reduced DMEM (containing 0.1% FCS) for 1 h. Cells not receiving any EIPA treatment were left in non-supplemented starvation medium (DMEM + 0.1% FCS). For PMA
treatment (positive control), cells were incubated in medium containing 100 μM PMA for 1 h at 4 °C. PMA-treated cells were washed twice with cold D-PBS and 70-kDa FITC-conjugated Dextran (Sigma) was added to all cells for 10, 30 or 60 min, respectively. During the incubation, cells were agitated regularly. Finally, cells were washed twice with cold PBS, once with low pH buffer (0.1 M sodium acetate, 0.05 M NaCl, pH 5.5), and then prepared for subsequent flow cytometric analysis as described above.

Acknowledgments

This study was supported by the INSERM and the ANR 2005 program "Microbiologie, infections et immunités" (contract # ANR-05-MIM-017-02). C.P received a post-doctoral grant from the same ANR contract; this was followed by a research fellowship from the Deutsche Forschungsgemeinschaft. O.P. has an MRT grant from the French Ministry of Research. H.K. has a grant from the Syrian Government. M.A. and R.B. have INSERM and CNRS positions, respectively. We thank Pierre Jurdic for the plasmids expressing the dominant-negative mutants N17Rac1-eGFP and N17Cdc42-eGFP, Delphine Muriaux both for the plasmid expressing the N19RhoA-eGFP dominant-negative mutant and for the anti-Lamp1 antibody, Ralf H. Adams for a murine EphB4 plasmid and Vanessa Guillaumue for the o-NiV-G monoclonal antibody 5A7 and a murine-anti-NiV polyclonal antibody. We thank Hervé Raoul director of the INSERM Jean Mérieux P4 laboratory and the laboratory’s staff, in particular Sandra Lacote, and we thank the staff of the IFR128 PLATIM, in particular Fabienne Simian-Lerme and Claire Lioniert.

Appendix A. Supplementary data


References


