Characterization of African bat henipavirus GH-M74a glycoproteins

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In recent years, novel henipavirus-related sequences have been identified in bats in Africa. To evaluate the potential of African bat henipaviruses to spread in non-bat mammalian cells, we compared the biological functions of the surface glycoproteins G and F of the prototype African henipavirus GH-M74a with those of the glycoproteins of Nipah virus (NiV), a well-characterized pathogenic member of the henipavirus genus. Glycoproteins are central determinants for virus tropism, as efficient binding of henipavirus G proteins to cellular ephrin receptors and functional expression of fusion-competent F proteins are indispensable prerequisites for virus entry and cell-to-cell spread. In this study, we analysed the ability of the GH-M74a G and F proteins to cause cell-to-cell fusion in mammalian cell types readily permissive to NiV or Hendra virus infections. Except for limited syncytium formation in a bat cell line derived from Hypsignathus monstrosus, HypNi/1.1 cells, we did not observe any fusion. The highly restricted fusion activity was predominantly due to the F protein. Whilst GH-M74a G protein was found to interact with the main henipavirus receptor ephrin-B2 and induced syncytia upon co-expression with heterotypic NiV F protein, GH-M74a F protein did not cause evident fusion in the presence of heterotypic NiV G protein. Pulse–chase and surface biotinylation analyses revealed delayed F cleavage kinetics with a reduced expression of cleaved and fusion-active GH-M74a F protein on the cell surface. Thus, the F protein of GH-M74a showed a functional defect that is most likely caused by impaired trafficking leading to less efficient proteolytic activation and surface expression.

INTRODUCTION

Hendra virus (HeV) and Nipah virus (NiV) define the genus Henipavirus within the family Paramyxoviridae. Pteropid bats serve as natural virus reservoirs from which the viruses are transmitted sporadically via faeces, urine or saliva (Halpin et al., 2011; Wang et al., 2000). In bats, henipaviruses do not cause any clinical disease, but they can overcome the species barrier without adaptation causing severe infections in humans, pigs and horses. Due to their zoonotic potential and their high pathogenicity, NiV and HeV are classified as Biosafety Level 4 (BSL-4) pathogens.

HeV was discovered in 1994 after causing horse and human diseases in Queensland, Australia. Further outbreaks in horses have been reported regularly in Australia. Human HeV infections are rare, and direct bat-to-human or human-to-human transmissions have not yet been described (Marsh et al., 2010). NiV first emerged from Malaysian fruit bats in 1998 and caused infections in pigs, which transmitted the virus to humans (Chua et al., 1999). Since 2001, smaller outbreaks have occurred regularly in India and Bangladesh (Luby et al., 2009b). Here, NiV was directly transmitted from pteropid bats to humans, most often by contaminated palm sap (Khan et al., 2010). Human-to-human transmissions were also documented (Luby et al., 2009a). Clinically, henipavirus infections in horses and pigs usually present as acute respiratory and/or neurological diseases. In humans, HeV infections are mostly characterized by an influenza-like illness, which can progress to severe pneumonia and death. In contrast, NiV infection mainly causes multifocal encephalitis in humans with high mortality rates (reviewed by Escaffre et al., 2013; Maisner et al., 2009). Recently, a third member of the genus Henipavirus, Cedar virus (CedPV), has been isolated from pteropid bats in Australia. Unlike NiV and HeV, CedPV did not cause clinical disease in experimental animal infections (Marsh et al., 2012).

As with other paramyxoviruses, henipaviruses possess two surface glycoproteins. Glycoprotein G is a type II membrane protein and is responsible for binding to cellular ephrin-B2/B3 receptors (Bonaparte et al., 2005; Negrete
et al., 2005, 2006). Glycoprotein F, a type I membrane protein, is responsible for fusion between viral and cellular membranes during virus entry, and fusion with adjacent cells, resulting in the formation of multinucleated syncytia (Bossart et al., 2002). To fulfill their important functions in fusion processes, henipavirus F proteins, which are synthesized in host cells as the inactive precursor F0, must be activated proteolytically by cellular proteases into fusion-active F1/2 forms. This activation requires clathrin-mediated F protein endocytosis, cleavage by host cell-derived endosomal cathepsin L or B, and subsequent recycling to the cell surface (Diederich et al., 2005, 2012; Pager et al., 2006; Popa et al., 2012). Thus, endocytosis, recycling and cleavability of henipavirus F proteins together with functional receptor-binding capacity of the viral G proteins critically determine the ability of henipaviruses to enter and spread in cells. Knowledge about the functional characteristics of henipavirus glycoproteins might help to evaluate their potential to support virus replication in new host species.

Whilst HeV and NiV outbreaks have only been reported in limited areas so far, henipavirus cross-reacting antibodies have been found in over 20 Pteropus and Eidolon species distributed over Australia, Papua New Guinea, Malaysia, Bangladesh, Cambodia, Thailand, Indonesia, India, China, Madagascar and Ghana (Chong et al., 2009). In addition to the serological evidence, henipavirus-like RNA has been identified in specimens from African Eidolon, Epopomorphus, Hypsignathus, Myonycteris and Rousettus species (Drexler et al., 2009, 2012). Although live virus could not be isolated from bat specimens, these findings clearly suggest a widespread existence of henipavirus-related viruses with a currently unknown potential to replicate in livestock or humans. To evaluate the ability of African bat henipaviruses to enter and spread in cells that can be productively infected by the pathogenic henipaviruses NiV and HeV, we cloned the G and F genes from RNA isolated in 2009 from the spleen of a bat (Eidolon helvum) in Ghana, referred to as GH-M74a (Drexler et al., 2012). In order to characterize the functional properties of the glycoproteins, the G and F proteins were analyzed in comparison with functional NiV glycoproteins in terms of fusion activity, receptor binding, F cleavage and surface expression.

**RESULTS**

**Co-expression of GH-M74a G and F glycoproteins does not cause cell-to-cell fusion in henipavirus-permissive cell types**

Recently, we reported that GH-M74a glycoproteins can cause some limited syncytium formation in HypNi/1.1 cells, a bat kidney cell line derived from Hypsignathus monstrosus (Krüger et al., 2013). Although this suggests a principal fusion competence, cell-to-cell fusion was not seen following GH-M74a glycoprotein co-expression in Vero cells, a monkey kidney cell line generally used to propagate henipaviruses. This result is also presented in Fig. 1(a). When we analysed syncytium formation in Vero and HypNi/1.1 cells that were co-transfected with the GH-M74a glycoproteins, only small syncytia were found in HypNi/1.1 cells. To ensure that defective syncytium formation in Vero cells was not due to the lack of GH-M74a G and F co-expression in the same cell, co-immunostaining was performed. For this, cells were fixed and permeabilized, and then incubated with mouse mAbs directed against the haemagglutinin (HA) epitope of the G protein. The GH-M74a F protein was labelled by rabbit antibodies specific for the FLAG epitope. Primary antibodies were visualized with Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 568-conjugated anti-rabbit antibodies, respectively. As shown in Fig. 1(b), HypNi/1.1 and Vero
cells readily co-expressed both GH-M74a glycoproteins. However, Vero cells did not fuse with neighbouring cells.

To determine whether the defects in fusion were restricted to Vero cells or are a more general characteristic of the GH-M74a glycoproteins, we assayed their functional activity in a larger set of cell types from different mammalian species that are known to be susceptible to pathogenic henipavirus infection. As a control, cells were co-transfected with the NiV glycoproteins, or were infected with NiV. At 24–48 h after transfection or infection, the cells were fixed and stained with Giemsa staining solution. The results for Vero, canine kidney epithelial cells (MDCK), human alveolar basal epithelial cells (A549), porcine aortic endothelial cells (PAEC-EB2), baby hamster kidney (BHK) fibroblasts and bat kidney cells isolated from *E. helvum* (EidNi/41.3) are shown in Fig. 2. In all cell types, NiV infection and co-expression of HA-tagged NiV G and F proteins caused cell-to-cell fusion (Fig. 2, right panels), whilst expression of GH-M74a G in combination with GH-M74a F protein did not induce syncytium formation within 48 h. This clearly demonstrated that the GH-M74a glycoproteins have no detectable fusion activity in cells that are readily fused by G and F proteins of pathogenic NiV.

**GH-M74a G protein binds to henipavirus receptor ephrin-B2**

Binding of the G protein to ephrin receptors on neighbouring cells is the initial step required for cell-to-cell fusion. To analyse whether GH-M74a G could bind to the main henipavirus receptor, ephrin-B2, the G protein was expressed in ephrin-negative HeLa cells (Thiel *et al.*, 2008). Cells were fixed with 2% paraformaldehyde and were incubated with ephrin-B2–Fc, a soluble form of the ephrin-B2 ectodomain conjugated with the Fc portion of human IgG1. To ensure the specificity of the labelling, a double staining with rabbit antibodies directed against the HA tag in the G ectodomain was performed. Bound ephrin-B2–Fc was detected with rhodamine-conjugated anti-human IgG, and HA-specific antibodies were detected by incubation with Alexa Fluor 488-conjugated anti-rabbit IgG. Fig. 3 shows a complete co-localization of both labels, confirming that both NiV G (Fig. 3a) and GH-M74a G (Fig. 3b) were

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![Fig. 2. Fusion activity of GH-M74a and NiV glycoproteins in henipavirus-permissive cell lines. Different cell lines were either co-transfected with the indicated NiV or GH-M74a G and F genes, or were infected with NiV for 24–48 h. To visualize syncytium formation, the cells were fixed with ethanol and stained with Giemsa staining solution. Transfected cells were analysed with an EVOS XL Core microscope. Images of NiV-infected cells were recorded within the BSL-4 facility with a Zeiss Axiovert 40C. Magnification, ×400.](http://vir.sgmjournals.org)
expressed on the cell surface and that both bound to soluble ephrin-B2 receptors.

To support this finding, interaction of G and ephrin-B2 was analysed by a co-precipitation assay. For this, HeLa cells were transfected with the glycoprotein genes. At 24 h post-transfection (p.t.), cells were lysed and half of the lysate was used for immunoprecipitation of G proteins with anti-HA rabbit antibodies as a control for total G protein expression. The other half of the lysate was incubated with ephrin-B2–Fc and precipitated with protein A–Sepharose beads. Precipitates were analysed by Western blotting using HA-specific mAbs. As shown in Fig. 3(c), both G proteins could be precipitated by soluble ephrin-B2–Fc (EB2), almost as efficiently as by anti-HA antibodies (HA). In contrast, NiV F was only isolated with the anti-HA antibody demonstrating that precipitation of the GH-M74a- and NiV G proteins by ephrin-B2 was specific.

**GH-M74a G protein induces syncytium formation upon co-expression with heterotypic NiV F protein**

As surface-expressed GH-M74a G was found to bind ephrin-B2, we hypothesized that the impaired fusion activity is mainly caused by the GH-M74a F protein. To evaluate this idea, heterotypic fusion assays with functional F and G proteins of NiV were performed. For this, Vero cells were co-transfected with the different homo- and heterotypic glycoprotein genes and cell monolayers were fixed at 24 h p.t. To visualize glycoprotein-expressing cells and to detect all – even very small – syncytia, immunofluorescence analysis was performed to stain transfected cells and DAPI was used to label the cell nuclei. As expected, co-expression of the two NiV glycoproteins caused the formation of huge syncytia (Fig. 4a), whilst homotypic co-expression of GH-M74a glycoproteins did not lead to any obvious cell-to-cell fusion (Fig. 4b). Interestingly, when we expressed GH-M74a G together with heterotypic NiV F, syncytium formation was detectable (Fig. 4d). Although the size of these syncytia was clearly smaller (four to eight nuclei per syncytium) than those caused by homotypic NiV glycoproteins (>10 nuclei per syncytium), this showed that the G protein of GH-M74a not only bound to ephrin-B2 but also had a fusion-helper function and could support henipavirus F-mediated fusion. In contrast, the GH-M74a F protein did not induce fusion in the

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**Fig. 3.** Binding of G proteins to ephrin-B2. (a, b) HA-tagged GH-M74a G (a) and NiV G (b) were transiently expressed in ephrin-negative HeLa cells. At 24 h post-transfection (p.t.), cells were fixed with paraformaldehyde and incubated with soluble ephrin-B2–Fc (EB2) in combination with rabbit anti-HA antibodies (HA) followed by incubation with rhodamine-labelled anti-human IgG (red) and Alexa Fluor 488-conjugated anti-rabbit IgG (green). Nuclei were counterstained with DAPI. (c) HeLa cells expressing GH-M74a G, NiV G or NiV F were lysed at 24 h p.t. Equal amounts of the lysates were immunoprecipitated with rabbit polyclonal HA-specific antibodies to evaluate total glycoprotein expression (HA), or were co-precipitated with recombinant ephrin-B2–Fc (EB2). Precipitates were separated by 10% SDS-PAGE under reducing conditions and analysed by Western blotting with anti-HA mAbs, biotinylated anti-mouse IgG and streptavidin–HRP.

**Fig. 4.** Heterotypic fusion assay. At 24 h p.t., Vero cells co-expressing the indicated NiV and GH-M74a glycoproteins were fixed with methanol/acetonitrile. The G and F proteins were immunostained with a combination of anti-HA, anti-FLAG and Alexa Fluor 488-labelled secondary antibodies. Nuclei were counterstained with DAPI. Magnification ×630.
presence of heterotypic NiV G (Fig. 4c). Similar observations were made in MDCK cells (data not shown), supporting the idea that a defect in the GH-M74a F protein rather than in the G protein is the main reason for defective fusion activity.

**Kinetics of GH-M74a F proteolytic activation is reduced**

As F cleavage is an essential prerequisite for fusion activity, we compared the cleavage efficiencies and kinetics using a pulse–chase experiment. For this, MDCK cells were labelled metabolically at 24 h after transfection with [35S]cysteine and [3H]methionine for 45 min. After pulse labelling, the medium was removed and cells were incubated further for 0–4 h in chase medium. The F proteins were then immunoprecipitated and separated by SDS-PAGE under reducing conditions. As would be predicted from the sequence (112 aa longer, with three additional potential N-glycosylation sites; Fig. 5a), precursor GH-M74a F0 and F1 cleavage products migrated more slowly than F0 and F1 of NiV. Fig. 5(b) shows that NiV F and GH-M74a F proteins were labelled with similar efficiencies and that the total protein amounts did not decrease within 4 h. This indicated that neither protein synthesis nor the degradation rate of the two F proteins differed markedly. Some cleavage of both precursor NiV and GH-M74a F0 was already observed by 45 min after pulse labelling (0 h chase). However, the amount of NiV F1 increased with longer chase times by up to 35.6% (4 h chase), whilst only 4.3% of the total GH-M74a F protein was found to be cleaved (Fig. 5b). This demonstrated that GH-M74a F is cleaved much less efficiently. The same result was found in Vero cells (data not shown). To determine whether steady-state expression levels or cleavage rates were enhanced in HypNi/1.1 cells, MDCK and HypNi/1.1 cells were transfected with GH-M74 F and subjected to Western blot analysis at 24 h p.t. As shown in Fig. 5(c), F protein expression and cleavage was very similar in both cell types, indicating that the selective syncytium formation observed in HypNi/1.1 cells was not caused by increased expression of cleaved F proteins.

The GH-M74a F cleavage site (GNAR ↓ FAG) is very similar to that of the other henipaviruses and fulfills the principal sequence requirements for proteolytic processing by cathepsin L and B. As henipavirus F protein activation depends on F transport to the plasma membrane followed by endocytosis via clathrin-coated pits, before cathepsin-mediated cleavage within acidic endosomal vesicles can occur (Diederich et al., 2005, 2012; Pager et al., 2006; Popa et al., 2012), inefficient cleavage of GH-M74a F might be the consequence of a different intracellular trafficking through the endosomal-recycling compartment. In support of this idea, we found only partial co-localization of GH-M74a F and NiV F protein (Fig. 6). Although there was substantial co-staining of the heterotypic F proteins in perinuclear regions probably representing Golgi areas, co-localization in more peripheral dot-like structures assumed to represent vesicles of the endo-lysosomal compartment was not very pronounced.

**Surface expression of GH-M74a F protein is reduced compared with that of NiV F**

Regarding the complex trafficking pathway needed to get fusion-active F proteins exposed on the cell surface, we wanted to determine whether GH-M74a F differed from NiV F not only in terms of cleavage kinetics and subcellular distribution but also in its surface expression. To analyse the amount of cleaved and surface-expressed F proteins in Vero and MDCK cells, two cultures were transfected with NiV F and GH-M74a F. To determine the total amount of F protein, one of the cultures was lysed at 24 h p.t. The F proteins were immunoprecipitated with polyclonal HA and FLAG-specific antibodies, and subjected to Western blot analysis. The second culture was labelled with non-membrane-permeating sulfo-N-hydroxysuccinimidobiotin. After surface biotinylation, the cells were lysed and the F proteins were immunoprecipitated, separated by SDS-PAGE and blotted onto nitrocellulose. To selectively detect surface-expressed F proteins, the blots were probed with streptavidin–HRP. In agreement with the comparable synthesis rates observed in the pulse–chase analysis (Fig. 5b), Western blot analysis demonstrated that the total amounts of NiV and GH-M74a F proteins expressed in the cells were similar (Fig. 7a). However, the amount of surface-biotinylated F proteins differed substantially. Compared with NiV F, surface expression of the precursor F0 and F1/F2 cleavage products was reduced in GH-M74a F-expressing MDCK and Vero cells. We thus concluded that the functional inability of the GH-M74a F protein to cause fusion in standard henipavirus-permissive cells was mainly due to insufficient expression of cleaved and fusion-active F protein on the cell surface.

**DISCUSSION**

The existence of henipavirus-related viruses in African bats raises questions about the potential of these viruses to replicate in bat and non-bat mammalian cell types. Hayman et al. (2011) reported that 5% of 97 pig sera sampled in Ghana contained cross-reactive antibodies to henipaviruses. This might suggest that pigs have already been exposed to henipaviruses in this part of Africa. However, it is completely unclear whether these African bat henipaviruses can infect pigs. Evaluation of the pathogenic potential of African henipaviruses is hampered mainly by the fact that isolation of infectious virus from bats in the field or after experimental infections is rarely successful (Halpin et al., 2011). Due to the unavailability of replication-competent African bat viruses, functional characterization of cloned genes is a key tool to evaluate the potential of newly identified viruses to enter and spread within different cell types. Our studies on the functional properties of the glycoproteins of the prototype African
Henipavirus GH-M74a revealed substantial differences in these central determinants for productive replication. In contrast to what has been shown for NiV, HeV and CedPV, we did not detect cell-to-cell fusion in standard mammalian cell types readily permissive to henipavirus infections. Whilst GH-M74a G protein was found to interact with ephrin-B2 receptors and induced syncytium formation upon co-expression with heterotypic NiV F

**Fig. 5.** Cleavage kinetics of GH-M74a and NiV F proteins. (a) Schematic diagram of NiV F and GH-M74a F proteins. The two F protein subunits, F₁ and F₂, are indicated. Arrowheads indicate the locations of the potential N-glycosylation sites. Numbers indicate the amino acid positions. FP, fusion peptide; LD, luminal domain; TMD, transmembrane domain; CD, cytoplasmic domain. (b) Transfected MDCK cells were radiolabelled with [³⁵S]cysteine and [³⁵S]methionine for 45 min and then incubated in chase medium for the indicated times. After lysis, the F proteins were immunoprecipitated and separated by 15% SDS-PAGE, followed by autoradiography using a Biomager. The amount of F₁ protein (% F₁) as a percentage of total F protein (F₀ plus F₁) was calculated to yield the percentage cleavage. (c) To analyse steady-state expression and F cleavage, MDCK and HypNi/1.1 cells were transfected with GH-M74a F and lysed at 24 h p.t. The F proteins were immunoprecipitated and detected by Western blotting using anti-FLAG mAbs, biotinylated anti-mouse IgG and streptavidin–HRP.
protein, GH-M74a F did not cause evident fusion in the presence of heterotypic NiV G protein. Reduced F cleavage kinetics, only partial co-localization with NiV F and reduced expression of cleaved and fusion-active GH-M74a F protein on the cell surface support the idea that the functional defect is basically caused by impaired trafficking, leading to an insufficient expression of fusion-competent F proteins. It remains to be elucidated whether this is the consequence of a defective transport of newly synthesized F proteins via the secretory pathway to the cell surface, or rather is the result of mistargeting within the endosomal-recycling compartment after uptake of surface-expressed GH-M74a F proteins.

It has been shown that henipavirus glycoproteins of NiV and HeV are capable of highly efficient heterotypic functional activity with each other but not with envelope glycoproteins of the morbilliviruses measles virus and canine distemper virus (Bossart et al., 2002). As heterotypic fusion activity is obviously restricted to viruses of the same genus, our finding that GH-M74a G can induce syncytium formation upon co-expression with NiV F strengthens the classification into the genus Henipavirus, which is so far based mainly on sequence similarities in the L gene (Drexler et al., 2012).

Although lack of fusion activity in most cell types might suggest that the GH-M74a F protein is fusion incompetent, syncytium formation in HypNi/1.1 cells indicates that expressed F proteins are principally biologically active. Thus, F cleavage results in the release of a functional fusion peptide and generates a functional protein that can undergo the conformational changes triggered by F–G interaction required for any subsequent fusion event (Aguilar et al., 2010; Smith et al., 2009). However, compared with the F proteins of other henipaviruses, there is a significant restriction in either the F cleavage within the endosomal-recycling compartment and/or trafficking to the cell surface. In most cases, the resulting low-level surface expression of fusion-active GH-M74a F proteins does not allow cell-to-cell fusion in otherwise henipavirus-permissive cell types. There is, however, at least one exception. HypNi/1.1 cells allow a certain extent of fusion, although expression levels and F cleavage rates are not increased compared with cells that do not support fusion. It remains to be elucidated which conditions are provided in HypNi/1.1 cells that are not given in other cell types, such as extraordinary receptor expression levels or membrane lipid compositions that facilitate fusion pore formation, thereby supporting viral glycoprotein-mediated cell-to-cell spread.

The fact that the GH-M74a genome has been isolated from *E. helvum* clearly indicates that GH-M74a can replicate in...
these bats. Thus, it must be assumed that there are cells in infected bats that produce sufficient amounts of cleaved and fusion-active F proteins to spread the infection to other cells and new bat hosts. As we did not see fusion activity upon co-expression of the GH-M74a glycoproteins in the Eidolon kidney cell line EidNi/41.3, it must be assumed that conditions either supporting expression of increased amounts of fusion-active GH-M74a glycoproteins, or facilitating cell-to-cell fusion by low amounts of functional G–F complexes are not generally provided in bat cells but are restricted to certain cell types or special tissues. Future studies are needed to identify such cell types that support fusion by the GH-M74a glycoproteins: cell types with HypNi/1.1-like characteristics.

In summary, we have provided evidence that the glycoproteins of the novel African henipavirus GH-M74a have a reduced biological activity in most mammalian cell types compared with G and F proteins of the highly pathogenic henipavirus NiV. The reported characteristics of the GH-M74 glycoproteins may explain why to date, no infectious henipaviruses have been isolated in Africa.

**METHODS**

**Cell culture.** Vero76, A549, BHK, PAEC-EB2 (Thiel et al., 2008), HypNi/1.1 (Hoffmann et al., 2013), EidNi/41.3 (Biesold et al., 2013) and MDCK cells were cultivated in Dulbecco’s modified Eagle’s medium (Gibco) or Eagle’s minimal essential medium (Gibco), with 10% FCS (Life Technologies), 100 U penicillin ml⁻¹, and 0.1 mg streptomycin ml⁻¹ and 4 mM l-glutamine (Gibco).

**Virus infections.** All experiments with live NiV were performed under BSL-4 conditions at the Institute of Virology, Philipps University Marburg, Germany. The NiV strain used in this study was a human isolate and was propagated as described previously (Lamp et al., 2013; Moll et al., 2004). For NiV infection, confluent cell monolayers were infected with NiV at an m.o.i. of 0.0001–0.01. After incubation for 1 h at 37 °C, input virus was removed and the cells were cultured and monitored for cell-to-cell fusion for 24–48 h at 37 °C. To visualize syncytia, the cells were fixed with ethanol and were stained with 1:10-diluted Giemsa staining solution.

**Plasmids and transfections.** Cloning and characterization of the tagged NiV F and G proteins have been described previously (Diedrich et al., 2008, 2012; Lamp et al., 2013). cDNA fragments spanning the F and G genes of the African henipavirus GH-M74a isolated from a straw-coloured fruit bat (E. helvum) sampled in 2009 in Ghana (Drexler et al., 2012) were cloned into the pCAGGS vector (Niwa et al., 1991), using the restriction enzymes NcoI and NheI. To allow detection with commercially available antibodies, tagged versions of the GH-M74a glycoproteins were constructed. As for the NiV G protein, an HA epitope was added to the C-terminal ectodomain of the GH-M74a G protein. We further generated two GH-M74a F proteins with either a cytoplasmic HA tag or a double FLAG epitope. Comparison of the two F proteins in terms of expression levels, intracellular distribution, cleavage rates and surface expression did not reveal any difference. We decided, however, to use the FLAG-tagged GH-M74a F protein in this study because it has been reported that an HA tag but not a FLAG epitope added to the C terminus can influence the fusion activity of HeV F protein (Popa et al., 2011).

MDCK cell transfections were performed using the cationic lipid transfection reagent Lipofectamine 2000 (Invitrogen). All other cells were transfected with FuGENE HD transfection reagent (Promega).

**Fusion assays.** To compare the biological activity of the GH-M74a and NiV glycoproteins, different cell lines permissive to NiV and HeV infection were co-transfected with plasmids bearing either the genes encoding the NiV glycoproteins, or plasmids encoding GH-M74a G in combination with pCAGGS-GH-M74a-F, respectively. To monitor syncytium formation, cells were fixed with ethanol at 24–48 h p.t. and stained with 1:10-diluted Giemsa staining solution.

For heterotypic fusion assays, Vero cells were co-transfected with different combinations of the GH-M74a and NiV G and F genes. At 24 h p.t., cells were fixed and permeabilized with methanol/aceton (1:1) for 5 min on ice. To detect transfected cells and to visualize syncytia, the glycoproteins were labelled with anti-HA and anti-FLAG primary antibodies (diluted 1:250; Sigma) for 45 min on ice. Primary antibodies were then detected with Alexa Fluor 488-conjugated secondary antibodies (1:250; Invitrogen), and nuclei were counterstained with DAPI. The samples were mounted in Mowiol (Hoechst) and 10% 1,4-diazabicyclo(2,2,2)octane (Sigma) and examined using a Zeiss Axiovert 200M microscope.

**Co-immunofluorescence analysis.** To analyze the co-expression of pCAGGS-GH-M74a-G and -F, co-transfected cells were fixed with methanol/aceton (1:1) at 24 h after transfection. Mouse antibodies directed against the HA epitope (1:200) were used to detect the G protein, and rabbit antibodies directed against the FLAG epitope (1:100) were added for 45 min on ice to detect the F protein. Primary antibodies were visualized by incubation for 45 min on ice with Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 568-labelled anti-rabbit IgG antisera (1:250).

To analyse co-localization of GH-M74a F and NiV F, co-transfected Vero cells were fixed, and FLAG-tagged GH-M74a F protein was detected by FLAG-specific mouse antibodies and Alexa Fluor 568-conjugated secondary antibodies. HA-tagged NiV F proteins were stained with HA-specific rabbit antibodies and Alexa Fluor 488-labelled anti-rabbit IgG.

**Ephrin-B2 surface binding assay.** To determine the ability of GH-M74a G to bind ephrin-B2, ephrin-negative HeLa cells were transfected with pCAGGS-GH-M74a-G or NiV G. At 24 h p.t., the monolayers were fixed with 2% paraformaldehyde, and incubated for 45 min on ice with 2 μg soluble ephrin-B2-human Fc protein (ephrin-B2–Fc; R&D Systems) and rabbit anti-HA antibodies (1:200), followed by incubation with Alexa Fluor 488-conjugated anti-rabbit IgG and rhodamine-labelled anti-human IgG (1:50; JacksonImmunoResearch). Nuclei were counterstained with DAPI.

**G protein and ephrin-B2 co-precipitation.** To analyse further the ability of GH-M74a G to interact with ephrin-B2, ephrin-negative HeLa cells were transfected with HA-tagged GH-M74a G, NiV G or NiV F. At 24 h p.t., cells were lysed with 1% Triton X-100 in PBS and clarified by centrifugation (13 000 g, 20 min, 4 °C). For co-precipitation of G protein with receptors, cell lysates were incubated overnight with 2 μg ephrin-B2–Fc on ice followed by precipitation with a suspension of protein A–Sepharose (Amersham). As an expression control, equal amounts of lysates were immunoprecipitated with HA-specific rabbit polyclonal antiserum (1:500). Precipitates were washed three times with lysis buffer and finally boiled in sample buffer with 2% β-mercaptoethanol. Samples were analysed by SDS-PAGE, transferred to nitrocellulose and visualized by Western blotting with mouse anti-HA tag specific mAbs (1:1000; Covance) and biotinylated anti-mouse IgG (1:2000; Amersham). Following incubation with HRP-conjugated streptavidin (1:4000; Amersham),
proteins were detected by enhanced chemiluminescence (SuperSignal West Dura; Pierce) and a ChemiDoc Imaging System (Bio-Rad).

**Pulse–chase analysis.** To monitor F cleavage, MDCK cells transiently expressing GH-M74a or NiV F proteins were incubated at 24 h.p.t. for 40 min with medium lacking cysteine and methionine, followed by incubation with medium containing [35S]cysteine and [35S]methionine (Promix; Perkin Elmer) at a final concentration of 100 Ci ml⁻¹ for 45 min (pulse). The labelling medium was then replaced by non-radioactive medium and the cells were incubated at 37 °C for 0–4 h (chase times). After labelling, the cells were washed extensively with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 0.15 M NaCl, 10 mM EDTA, 1 mM iodoacetamide, 1 mM PMSF, 50 U aprotinin ml⁻¹ and 20 mM Tris/HCl, pH 8.5), followed by centrifugation for 45 min at 13,000 g. The F proteins were then immunoprecipitated using rabbit polyclonal anti-HA or anti-FLAG antibodies. After addition of protein A–Sepharose, the immune complexes were washed with RIPA buffer and suspended in reducing sample buffer for separation by 12 % SDS-PAGE. Dried gels were subjected to autoradiography and analysed with a BAS1000 Bio-Image analyser (Fuji).

**Western blot analysis.** To determine total F expression and cleavage rates, F-expressing cells were lysed in RIPA buffer at 24 h.p.t. and F proteins were immunoprecipitated as described above. Samples were separated by 12 % SDS-PAGE under reducing conditions, blotted onto nitrocellulose and probed with HA- or FLAG-specific antibodies. After addition of protein A–Sepharose, the immune complexes were washed with RIPA buffer and suspended in reducing sample buffer for separation by 12 % SDS-PAGE. Dried gels were subjected to autoradiography and analysed with a BAS1000 Bio-Image analyser (Fuji).

**Surface biotinylation analysis.** Surface labelling with biotin was performed as described previously (Diederich et al., 2005). Briefly, MDCK and Vero cells were transfected with F-encoding plasmid DNA. At 24 h.p.t., cells were washed and incubated twice for 20 min at 4 °C with 2 mg sulfo-N-hydroxysuccinimidobiotin (Calbiochem) ml⁻¹. Following cell lysis, the F proteins were immunoprecipitated using rabbit polyclonal anti-HA or anti-FLAG antibodies. After addition of protein A–Sepharose, the immune complexes were washed with RIPA buffer and suspended in reducing sample buffer for separation by 12 % SDS-PAGE. Dried gels were subjected to autoradiography and analysed with a BAS1000 Bio-Image analyser (Fuji).

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