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Individual N-Glycans Added at Intervals along the Stalk of the Nipah Virus G Protein Prevent Fusion but Do Not Block the Interaction with the Homologous F Protein

Qiyun Zhu,[a]* Scott B. Biering,[b,e] Anne M. Mirza,[a] Brittany A. Grasseschi,[a] Paul J. Mahon,[a] Benhur Lee,[c] Hector C. Aguilar,[b,e] Ronald M. Iorio[a,d]

Department of Microbiology and Physiological Systems[a] and Program in Immunology and Virology,[d] University of Massachusetts Medical School, Worcester, Massachusetts, USA; Paul G. Allen School for Global Animal Health[b] and Department of Veterinary Microbiology and Pathology,[e] College of Veterinary Medicine, Washington State University, Pullman, Washington, USA[b]; Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine at University of California—Los Angeles, Los Angeles, California, USA[c]

The promotion of membrane fusion by most paramyxoviruses requires an interaction between the viral attachment and fusion (F) proteins to enable receptor binding by the former to trigger the activation of the latter for fusion. Numerous studies demonstrate that the F-interactive sites on the Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) and measles virus (MV) hemagglutinin (H) proteins reside entirely within the stalk regions of those proteins. Indeed, stalk residues of NDV HN and MV H that likely mediate the F interaction have been identified. However, despite extensive efforts, the F-interactive site(s) on the Nipah virus (NiV) G attachment glycoprotein has not been identified. In this study, we have introduced individual N-linked glycosylation sites at several positions spaced at intervals along the stalk of the NiV G protein. Five of the seven introduced sites are utilized as established by a retardation of electrophoretic mobility. Despite surface expression, ephrinB2 binding, and oligomerization comparable to those of the wild-type protein, four of the five added N-glycans completely eliminate the ability of the G protein to complement the homologous F protein in the promotion of fusion. The most membrane-proximal added N-glycan reduces fusion by 80%. However, unlike similar NDV HN and MV H mutants, the NiV G glycosylation stalk mutants retain the ability to bind F, indicating that the fusion deficiency of these mutants is not due to prevention of the G-F interaction. These findings suggest that the G-F interaction is not mediated entirely by the stalk domain of G and may be more complex than that of HN/H-F.

The *Paramyxoviridae* are a family of enveloped, negative-stranded RNA viruses that includes several important human and animal pathogens, such as measles virus (MV), mumps virus, Newcastle disease virus (NDV), human parainfluenza virus types 1 to 4 (hPIV1 to hPIV4), Sendai virus, parainfluenza virus 5 (PIV5), respiratory syncytial virus, and the emerging henipaviruses, Nipah virus (NiV) and Hendra virus (HeV) (1). The last two viruses are unique among paramyxoviruses in being able to cause 40 to 75% mortality rates in humans, mainly from encephalitis (2–5). Both animal-to-human transmission and human-to-human transmission of NiV have been reported (6).

Paramyxoviruses enter and spread between cells by virus-cell and cell-cell fusion, respectively. The paramyxovirus fusion (F) protein has multiple canonical structural and functional features characteristic of class I fusion proteins (1). As is the case for many viruses in this class, receptor binding is the trigger for fusion. For most of these viruses, with HIV-1 the best-characterized example, receptor binding and membrane fusion are mediated by a single glycoprotein (7). However, in the paramyxoviruses, receptor binding and fusion promotion are contributed by separate glycoproteins, necessitating a mechanism to link the two events. This is accomplished by a virus-specific interaction between the attachment and F proteins (reviewed in references 1 and 8–12).

Paramyxoviruses can be divided into two groups according to the type of receptor recognized by their attachment proteins. Viruses that have a hemagglutinin-neuraminidase (HN) attachment protein, such as NDV and Sendai virus, bind to sialic acid-containing proteins and lipids on the cell surface and possess neuraminidase (NA) activity (1). The attachment proteins of other viruses in the family, including MV and the henipaviruses, recognize distinct protein receptors. The henipavirus attachment glycoprotein (G) recognizes ephrinB2 and -B3 as receptors (13–16), exhibits neither hemagglutinating nor NA activity, and shares little amino acid homology with other paramyxovirus attachment proteins (17, 18).

A great deal of evidence indicates the existence of a dichotomy in the relationship between receptor binding and glycoprotein complex formation for paramyxoviruses depending on the type of receptor recognized by the attachment protein (9). For HN-containing viruses, the interaction of HN and F is thought to be triggered at the cell surface by receptor binding. However, for MV and the henipaviruses, which recognize specific protein receptors, it is thought that the respective hemagglutinin (H) and G complexes with F are preformed and are dissociated upon receptor binding (8–12).

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Address correspondence to Ronald M. Iorio, ronald.iorio@umassmed.edu.
* Present address: Qiyun Zhu, Department of Avian Diseases, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, People's Republic of China.
S.B.B. and A.M.M. contributed equally to this article.
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The paramyxovirus attachment protein ectodomain consists of a stalk supporting a terminal globular head, in which resides the receptor binding site. Evidence gathered from several studies clearly demonstrates that the G/H/HN stalk domain is important for F triggering. Studies of chimeras with stalks and heads from different HN proteins have established that the stalk of HN completely determines specificity for the homologous F protein (19–22). Indeed, we have identified NDV HN stalk residues 89, 90, and 94 as part of the F-interactive domain in that protein (23). This is supported by the recent demonstration that they are located at the surface of the four-helix bundle in the NDV HN stalk (24). Analogous F-interactive residues have also been identified in the stalk of the MV H protein (25, 26).

A number of studies establish that the stalk of the henipavirus G protein is critical for fusion. Mutation of the conserved isoleucines (27) or cysteines (28) in the stalk of G abolishes fusion without a decrease in F-interactive capability. Removal of the only N-glycan in the stalk domain of NiV G at residue 159 also abolished fusion without a significant effect on G oligomerization or the G-F interaction (29). Similarly, a deletion of NiV G stalk residues 146 to 182 also abolishes fusion without decreasing the interaction with F (28). However, despite considerable effort, neither the exact role of the stalk of the G protein in fusion promotion nor the F-interactive site(s) on the henipavirus G glycoprotein has yet been identified.

One of the strategies instrumental in probing the role of the paramyxovirus attachment protein stalk in mediating the interaction with F was the determination of the effect of the addition of supernumerary N-linked glycans at various positions along the stalk on fusion and, where possible, on the interaction with the homologous F protein (26, 30, 31). Indeed, it has been shown that loss of the ability of NDV HN and MV H N-glycan stalk mutants to trigger fusion correlates with a loss of the ability of each protein to interact with its homologous F glycoprotein at the cell surface (26, 31).

We have now applied this approach to begin to understand the contribution of the NiV G stalk in mediating the interaction with F. Individual potential N-linked glycosylation sites have been introduced at intervals along the stalk of the G glycoprotein spanning the domain defined by residues 75 to 133. We were able to rule out a role for more C-terminal stalk residues by virtue of the retention of F-interactive capability by the Δ146-182 deletion mutant (28). The five most membrane distal of the seven added sites are utilized and, despite efficient surface expression, soluble ephrinB2 binding, and oligomer formation, four of the five added N-glycans completely eliminate fusion and the fifth reduces it by 80%. However, all of these fusion-deficient NiV G glycosylation mutants retain the ability to interact with F in a coimmunoprecipitation assay. Thus, unlike for analogous NDV HN and MV H mutants, we were unable to eliminate the G-F interaction by the addition of N-linked glycans to the G stalk. These findings indicate that the G-F interaction is most likely not determined entirely by the stalk domain of G and may be more complex than that of HN/H-F. We go on to show that the properties of a G-HN attachment protein chimera are consistent with this conclusion.

MATERIALS AND METHODS

Cells. Vero and PK13 (ephrinB2- and -B3-deficient) cells were obtained from the American Type Culture Collection (Manassas, VA). Vero and BHK-21F cells (gift of Rebecca Dutch) were maintained in Dulbecco's modified Eagle medium (DMEM) with high glucose, supplemented with 5% fetal calf serum, 20 mM L-glutamine, 4 U/ml of penicillin, and 4 μg/ml of streptomycin. PK13 cells were maintained in the same medium except for the use of 10% fetal calf serum and 1 mM sodium pyruvate. 293T cells (gift of Abraham Brass) were maintained in high-glucose DMEM supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 20 mM L-glutamine, 4 U/ml of penicillin, and 4 μg/ml of streptomycin.

Recombinant plasmids and site-directed mutagenesis. The preparation of pCAGGS expression vectors for the NiV G and F proteins has been described previously (32). Mutations were introduced into the NiV G gene in pBluescript SK(+) (Stratagene Cloning Systems, La Jolla, CA) using the QuikChange site-directed mutagenesis kit (Stratagene), and subsequently, the mutated genes were transferred into pCAGGS by blunt-end ligation. The presence of the desired mutation was confirmed by sequencing.

Construction of the 188G-HN124 chimeric attachment protein gene. A chimeric attachment protein having an N-terminal segment composed of 188 NiV G-derived residues and a C-terminal segment beginning at NDV HN residue 124 (chimera 188G-HN124) was constructed in pBluescript SK(+), facilitated by the introduction of HindIII sites at the desired positions in both NDV HN and NiV G. The sequence was subsequently corrected such that the transition from G residue 188 to HN residue 124 was seamless in the chimera.

Transfections and quantitation of cell surface expression. For most experiments, cells were seeded in six-well plates at 2×10^5/well 1 day prior to transfection. Wild-type (wt) and mutant proteins were expressed using the Lipofectamine 2000 transfection reagent (Invitrogen Corp., Carlsbad, CA) and 1 μg of each DNA per well according to protocols provided by the company. All assays were performed at 48 h posttransfection except staining for fusion by the chimera, which was also performed at 24 h. For fusion staining at 24 h posttransfection, Vero and BHK-21F cells were seeded at 3×10^5/well. For fusion staining at 24 h and 48 h posttransfection, 293T cells were seeded at 4×10^5/well and at 3×10^5/well, respectively, using plates that were pretreated with 0.1 mM polylysine, rinsed with water, and allowed to dry before plating.

Cell surface expression of wt and mutated G proteins in Vero cells was quantified by flow cytometry (performed by the University of Massachusetts Medical School Flow Cytometry Core Laboratory), using a mixture of conformation-dependent G-specific monoclonal antibodies (MAbs) (33). A mixture of conformational HN-specific MAbs (34–37) was used for the G-HN chimera. Secondary antibodies (Alexa Fluor) were obtained from Invitrogen (Eugene, OR) or KPL (Gaithersburg, MD).

EphrinB2 binding assay. The ability of HN-G chimeras to bind ephrinB2 was determined by a modification of the procedure described by Negrete et al. (15). PK13 cells were transfected as described above. The medium was removed, and the monolayers were incubated for 1 h at room temperature with 2 μg of soluble ephrinB2-human Fc protein (ephrinB2-Fc) (R&D Systems, Minneapolis, MN). Binding of ephrinB2 was quantified by flow cytometry.

Receptor binding enhancement (RBE) assay. The effect of receptor binding on the recognition of the mutated G proteins by Mab45 was determined by preincubating a monolayer of PK13 cells expressing the chimeras with and without 10 nM soluble ephrinB2 and then quantifying antibody binding by flow cytometry as described in reference 33.

Hemadsorption (HAd) and NA assays. The receptor binding activity of the chimera was assayed by its ability to adsorb guinea pig erythrocytes (Bio-Link Laboratories, Liverpool, NY) (23). The NA activity of the chimera was determined with 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUN) as the substrate, as described by Tappert et al. (38).

Staining for fusion. For pictures of fusion, transfected monolayers were fixed with methanol and stained with Giemsa stain either 24 h or 48 h posttransfection (Sigma Chemical Co., St. Louis, MO).

Content-mixing assay for fusion. The ability of the mutated G proteins or the 188G-HN124 chimera to complement NiV F in the promo-

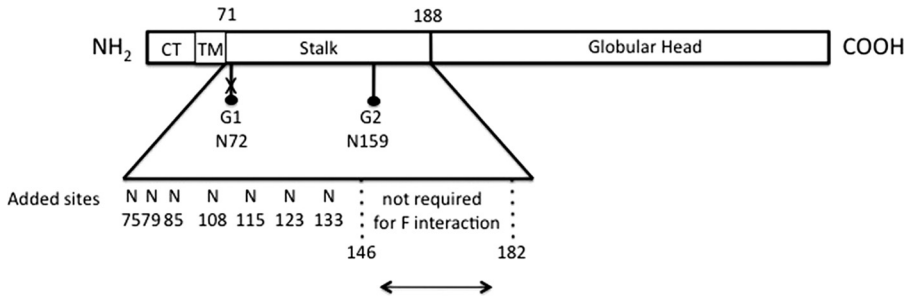

FIG 1 Schematic of N-linked glycosylation sites added, as well as those already present, in the stalk of the NiV G protein. Residues 146 to 182 can be eliminated from taking part in the interaction with F based on the findings of Maar et al. (28). The G1 site at position 72 is not used, while the G2 site at position 159 is used. CT, cytoplasmic tail; TM, transmembrane.

tion of cell-cell fusion was quantified using a modification of a content-mixing assay (32), which measures β-galactosidase activity in target cells following fusion induced by the glycoprotein-expressing effector cells. Effector Vero cells were transfected with 1 μg each of wt or mutant DNA and the NiV F DNA, as well as 1 μg of pCAGT7 DNA (39). The following day, another set of Vero cell monolayers (target) was infected with wt vaccinia virus (multiplicity of infection [MOI] of 1) and transfected with 1 μg of pG1NT7β-gal (40). Five hours later, the cells were trypsinized, and equal numbers of the two cell populations were combined and incubated overnight. The next day, the extent of fusion was quantified colorimetrically.

Immunoprecipitation. At 44 h posttransfection, transfected Vero cell monolayers were starved for 1 h at 37°C in medium lacking cysteine and methionine, radiolabeled for 3 h with 1 ml of medium containing 100 μCi of Express protein labeling mix ([^{35}S]Cys-Met) (PerkinElmer, Boston, MA) and chased for 90 min with medium (41). Cells were lysed and proteins were immunoprecipitated as described previously (32), using a G-specific polyclonal serum. Peptide-*N*-glycosidase F (PNGase F) digestion was performed as described previously (31). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence or absence of β-mercaptoethanol (BME). Rainbow markers were obtained from GE Healthcare Biosciences Corp. (Piscataway, NJ).

Coimmunoprecipitation of NiV G and F. The NiV F and wt or mutant G proteins were pseudotyped onto a reporter vesicular stomatitis virus (VSV) expressing the *Renilla* Luc gene as described previously (42, 43). NiV/VSV-rLuc virions containing wt NiV F and wt or mutant G were lysed and subjected to immunoprecipitation, as previously described (43, 44), using a mixture of conformational G-specific antibodies. Coimmunoprecipitated proteins were analyzed by Western blotting and quantified using a Li-cor Odyssey fluorimager (Li-cor Biosciences, Lincoln, NE).

RESULTS

Introduction of potential N-linked glycosyation sites in the NiV G stalk. To probe the role of the stalk of the NiV G protein in mediating the interaction with the homotypic F protein, we introduced individual potential N-linked glycosylation sites at intervals along the stalk (Fig. 1). The stalk of NiV G spans a total of 118 residues, encompassing residues 71 to 188. However, the F-interactive competence of the NiV G deletion mutant lacking stalk residues 146 to 182 (28) enabled us to focus on the remaining, N-terminal region.

N-linked carbohydrates are covalently attached to asparagines on nascent polypeptides at the motif N-X-T/S, where X is any amino acid except aspartic acid or proline. We have introduced potential N-linked glycosylation sites at convenient intervals in the segment spanning residues 75 to 133, using the mutagenesis strategy shown in Table 1 and naming the mutant according to the position that would potentially be glycosylated. This results in the potential for the addition of an N-glycan at residue 75, 79, 85, 108, 115, 123, or 133, with only the N85 mutant requiring more than a single point mutation. As it was subsequently demonstrated that the site at residue 75 is not utilized, we also attempted to add a site at the nearby residue 79 via an A81S mutation (mutant N79).

Cell surface expression of the mutated proteins and usage of the added glycosylation sites. Prior to functional analysis of the mutants, expression at the surface of Vero cells was quantified by flow cytometry (Fig. 2). While two of the more N-terminal glycosylation mutants, N75 and N85, exhibited reduced cell surface expression levels of 57 and 63% of the wt, respectively, mutants N79, N108, N115, N123, and N133 were all expressed at the surface at levels comparable to that of wt G, ranging from 83 to 115% of the wt (Fig. 2). This verifies that the potential N-linked glycosylation mutants are expressed and likely properly folded.

To determine whether the potential glycosylation sites are actually utilized, the mutants were expressed at the surface of Vero cells, radiolabeled, and immunoprecipitated with a G-specific polyclonal serum followed by SDS-PAGE under reducing conditions (Fig. 3A). All of the mutated G proteins, except the two most N-terminal ones, N75 and N79, migrated at a lower rate in the gel than did wt G, suggesting that the potential N-linked glycosylation site in each of these mutants was, indeed, utilized. However, the added sites in mutants N75 and N79 were apparently not glycosylated, as their migration rates were indistinguishable from that of wt G (Fig. 3A).

To confirm that the lower migration rate of the putative glycosylation mutants was, in fact, due to the addition of an N-glycan, the immunoprecipitated proteins were treated with PNGase F, which cleaves the N-glycan linkage between the asparagine side group and the carbohydrate (45). After digestion with the enzyme,

TABLE 1 Introduction of additional potential N-linked glycosylation sites in the stalk of the NiV G protein[a]

Mutant name	Mutation(s)	Motif of glycosylation site
N75	R75N	75-NST-77
N79	A81S	79-NQS-81
N85	D85N + L87S	85-NAS-87
N108	K108N	108-NVS-110
N115	S115N	115-NSS-117
N123	G125S	123-NIS-125
N133	Q133N	133-NST-135

[a] Point mutations introduced in the stalk of NiV G to add potential N-linked glycosylation sites.

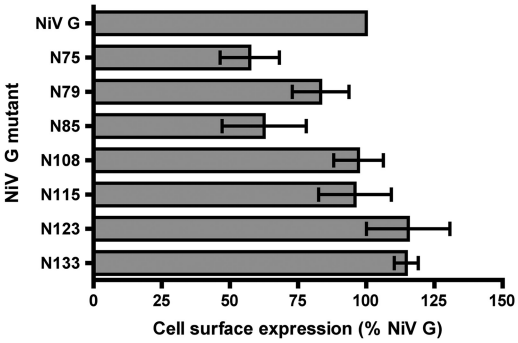

FIG 2 Cell surface expression of the mutated G protein carrying potential N-linked glycosylation sites. Expression at the surface of Vero cells was quantified by flow cytometry using a polyclonal antiserum specific for NiV G. Data are corrected for background obtained with vector alone and normalized to the value obtained with NiV G, which is set at 100%. Averages ± standard deviations are shown for three independent experiments, each performed in duplicate.

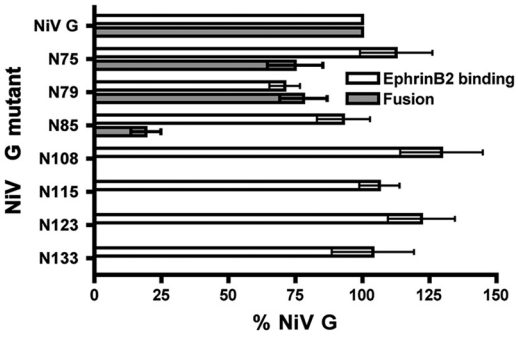

FIG 4 Addition of an N-linked glycan at any of several positions along the stalk of the NiV G glycoprotein severely compromises or eliminates fusion while retaining significant receptor binding activity. To evaluate the ability of the mutated G protein to bind NiV receptors, the wt and mutated proteins were expressed at the surface of PK13 cells, and at 48 h posttransfection, the monolayers were incubated at room temperature with 2 μg of ephrinB2-Fc. After washing, binding was detected by flow cytometry and corrected for background obtained with vector alone. Data are expressed relative to the binding obtained with NiV G, which is set at 100%. Averages ± standard deviations are shown for three independent experiments, each performed in duplicate. To quantify the ability of the mutated G proteins to complement NiV F in the promotion of fusion, Vero cells coexpressing NiV F and either a mutated or wt G were mixed with target cells overnight at 37°C. The extent of fusion was then quantified in the content-mixing assay, with data obtained with cells expressing the vector and NiV F as background. Data are expressed relative to that obtained with wt NiV G and F, which is set at 100%. Averages ± standard deviations are shown for three independent experiments; $n = 5$ for each experiment.

all the slower-migrating mutants comigrated with wt G treated in the same way, confirming that the lower migration rate of these mutants was due to a difference in N-linked glycosylation (Fig. 3B). Thus, we have successfully added N-glycans at positions 85, 108, 115, 123, and 133 along the stalk of NiV G.

Some of the added N-glycans compromise fusion promotion without affecting receptor-binding activity. The effects of the added N-glycans on the ability of NiV G to complement F in the promotion of fusion were examined by a content-mixing assay following coexpression with NiV F in Vero cells (Fig. 4). Four of the mutants, N108, N115, N123, and N133, were unable to promote a detectable level of fusion. Actually, of the five overglycosylated mutants, only N85 promoted detectable fusion, at approximately 20% of the wt level (Fig. 4). This is analogous to previous findings with NDV and PIV5 HN, which showed that addition of an N-glycan at any of several positions along the stalks of those proteins eliminated fusion-promoting activity (30, 31). Not surprisingly, mutants N75 and N79, in which the added glycosylation site was not utilized, promoted fusion quite efficiently, at 75 and 78% of the wt level, respectively (Fig. 4). This suggests that the introduced point mutations themselves, R75N and A81S, affect fusion only minimally.

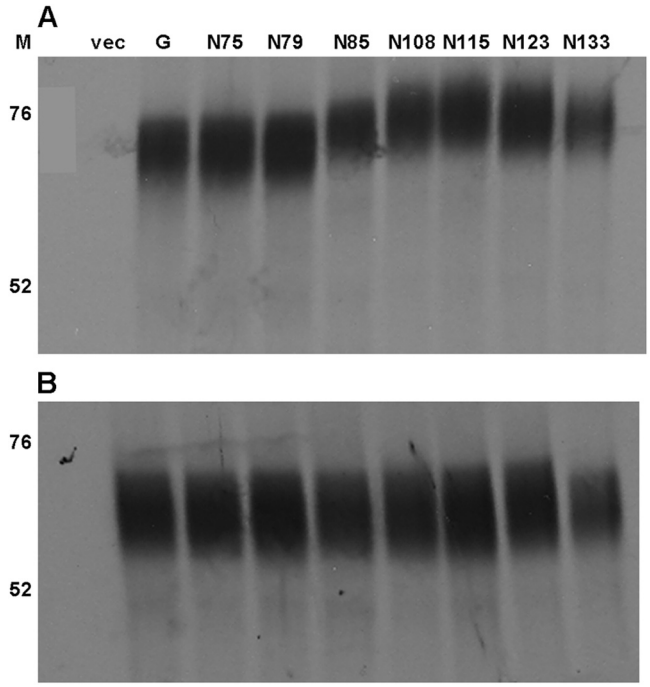

FIG 3 Usage of the potential N-linked glycosylation sites. The wt or mutated G proteins were expressed in Vero cells, radiolabeled, and chased to the surface. The G proteins were immunoprecipitated using a polyclonal serum specific for the G protein, and the immunoprecipitates were divided into two equal aliquots and either left untreated (A) or treated with 200 mU of PNGase F (B) prior to electrophoresis under reducing conditions. The numbers in the lanes marked "M" indicate the migration rates of markers in kilodaltons.

To be certain that the overglycosylated mutants were truly nonfusogenic, we compared their fusion-promoting activities to that of the wt G protein at a later time posttransfection. As shown in Fig. 5, at 48 h posttransfection, the wt G-promoted fusion is so extensive that the monolayer is almost destroyed. Similar results are obtained with the unglycosylated N75 and N79 mutants. Small syncytia are seen with the N85 mutant, consistent with the low level of fusion detected in the content-mixing assay (Fig. 4). However, even at this late time point, no syncytia are visible for either the N108, N115, N123, or N133 mutant.

Since the initial event in the fusion-triggering cascade in NiV G is thought to be binding to receptors, the possibility exists that the fusion deficiency of the glycosylation mutants could be the result of an effect on this function of G. To examine this possibility, we expressed the mutated proteins in PK13 cells, which are devoid of NiV receptors, and quantified the ability of the proteins expressed at the cell surface to bind soluble ephrinB2. As shown in Fig. 4, all

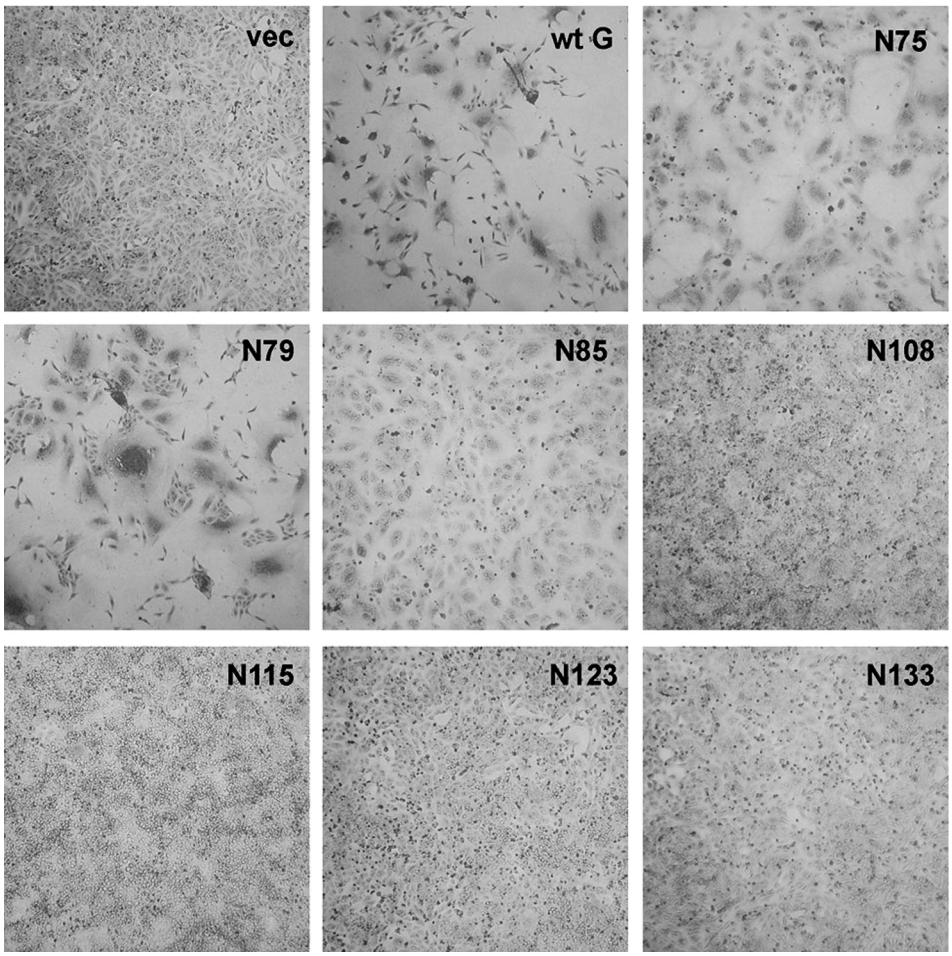

FIG 5 Syncytium formation in monolayers coexpressing wt or glycosylation site mutants of NiV G with the NiV F protein. The extent of syncytium formation is shown in monolayers expressing wt F with the following: a vector control (vec), wt G, N75, N79, N85, N108, N115, N123, and N133. At 48 h posttransfection, the monolayers were fixed with methanol and stained with Giemsa.

of the mutants retain at least 90% of the ephrinB2-binding activity of the wt G protein, with the exception of the unglycosylated N79 mutant, which still exhibits 71% of wt activity. Indeed, some mutants, e.g., N108 and N123, exhibit more than a 20% increase in binding activity relative to that of the wt protein. Thus, the fusion deficiency of the glycosylation mutants is not the result of a defect in receptor binding.

The NiV G stalk N-glycan mutants oligomerize at an efficiency comparable to that of the wt protein. The oligomeric structure of the stalk glycosylation mutants was examined by immunoprecipitation of the radiolabeled G protein that had been chased to the cell surface, using a G-specific polyclonal antiserum followed by SDS-PAGE under nonreducing conditions. The NiV G protein migrates in the gel predominantly as a mixture of disulfide-linked dimers and tetramers, with a smaller amount of monomer (Fig. 6). The intermolecular disulfide bonds responsible for these oligomers are mediated by cysteines at positions 146, 158, and 162 in the stalk region of NiV G (28).

As shown in Fig. 6, all of the mutants, including those with added N-glycans, are capable of forming both dimers and tetramers at the cell surface to an extent comparable to that of the wt G protein, although the N85 and N133 mutants appear to exhibit

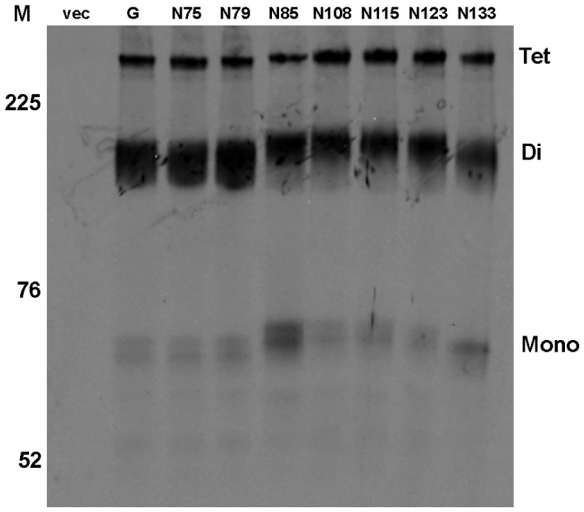

FIG 6 Each of the glycosylation mutants oligomerizes similarly to the wt G protein. The wt and mutated NiV G proteins were expressed in Vero cells, radiolabeled, and chased to the surface by incubation with medium for 90 min. Cells were lysed and the G proteins were immunoprecipitated with NiV G-specific antiserum. Proteins were resolved by SDS-PAGE in the absence of BME. The numbers in the lanes marked "M" indicate the migration rates of markers in kilodaltons. vec, vector; Tet, tetramers; Di, dimers; Mono, monomers.

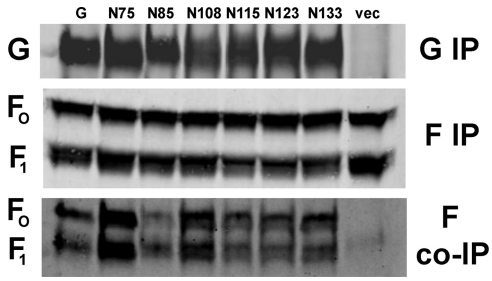

FIG 7 Each of the glycosylation mutants retains the ability to interact with the homologous F protein. wt NiV F (AU1 tagged) and wt or mutant G (and F only as a control [last lane]) were pseudotyped onto *Renilla* luciferase VSV virions as previously described (42, 43). Virions were lysed and subjected to immunoprecipitation (IP), basically as previously described (43, 44). Coimmunoprecipitated (co-IP) proteins were analyzed by Western blotting using a polyclonal G-specific antiserum to detect G and anti-AU1 serum to detect F and visualized and quantified using a Li-cor Odyssey fluorimager. Three independent experiments were conducted, and a representative experiment is shown. vec, vector.

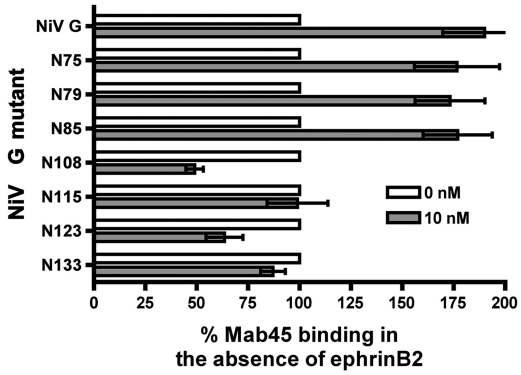

FIG 8 The fusion-null phenotype of stalk glycosylation mutants correlates with an inability to exhibit the enhanced binding of Mab45 in the presence of soluble ephrinB2 that is exhibited by the wt NiV G protein. The wt and mutated G proteins were expressed at the surface of PK13 cells and either left untreated or treated with 10 nM soluble ephrinB2 at room temperature for 1 h. Binding of Mab45 was then quantified by flow cytometry, using polyclonal G-specific antiserum. Data for each protein are expressed relative to that of the protein expressed in the absence of the receptor, which is set at 100%. Averages ± standard deviations are shown for three independent experiments, each performed in duplicate.

slightly reduced amounts of tetramers. Nonetheless, these data indicate that the fusion deficiency exhibited by the stalk N-glycan mutants is not the result of altered oligomerization. It is also noteworthy that unlike wt G and the other mutants, the N133 mutant fails to exhibit a doublet monomer band (Fig. 6). The significance of this is unclear.

The fusion-deficient G stalk glycosylation mutants retain the ability to interact with the F protein. It has previously been demonstrated that the fusion deficiencies of both NDV HN (31) and MV H (26) stalk N-glycan mutants correlate with an interference with the ability of the protein to interact with its cognate F protein. This is even true for NDV HN N-glycans added at sites in the stalk that are distant from the putative F-interactive site (23, 31).

To determine whether the fusion-deficient phenotypes of the NiV G stalk glycosylation mutants similarly correlate with a block in the interaction with the homologous F protein, we have determined the ability of the NiV G mutants to interact with F in VSV virions pseudotyped with wt NiV F and either wt or mutant NiV G (43). We coimmunoprecipitated NiV F_o and F_1 from viral lysates using anti-G antibodies (Fig. 7). As an additional control, we included the N75 mutant, in which the added glycosylation site is not utilized. As expected from the flow cytometric data (Fig. 2) and the SDS-PAGE analyses (Fig. 3 and 6), each of the G mutants was immunoprecipitated at an efficiency comparable to that of the wt protein (G IP [Fig. 7]). Also, the efficiencies of MAb-mediated immunoprecipitation of the wt F protein from the respective virions were comparable whether they were coexpressed with wt G, the unglycosylated mutant, or the glycosylated mutants (F IP [Fig. 7]). Interestingly, all the G proteins retained the ability to interact with F (F co-IP [Fig. 7]). This includes all mutants, including N85, N108, N115, N123, and N133, that exhibited severely compromised or undetectable fusion. Indeed, only the N85 mutant seemed to exhibit a discernible slight reduction in the level of coimmunoprecipitation. The lack of F in the F-only coimmunoprecipitation (Fig. 7, last lane) indicates that the coimmunoprecipitation of the F proteins through their interaction with G is specific. Thus, these data indicate that there is no correlation between the fusion deficiency of an overglycosylated G stalk mutant and an effect on the ability of the protein to interact with the F protein.

The fusion deficiency of the G stalk N-glycan mutants correlates with a loss of the RBE phenotype of an MAb that appears to detect a step in the triggering cascade in NiV G. Mab45 is a conformational, anti-G MAb whose binding inhibits virus entry and is enhanced upon receptor engagement (33). The receptor binding enhancement (RBE) for this MAb is predicted to reflect a required step in the fusion-triggering cascade in NiV G. Based on a deletion mutant analysis, the epitope recognized is thought to reside near the base of the globular domain quite distant from the receptor binding site (33).

To determine whether the Mab45 RBE is affected by the overglycosylation of the stalk of G, we used flow cytometry to compare the extents of antibody binding to wt or mutated G expressed at the surface of PK13 cells in the presence of 10 nM soluble ephrinB2 to that in the absence of receptor (Fig. 8). As expected, the wt G protein (190% RBE) as well as the unglycosylated N75 (177% RBE) and N79 (173% RBE) mutants exhibited a nearly 2-fold enhancement of Mab45 binding in the presence of receptor relative to that in its absence. Indeed, even the N85 mutant, which is only relatively weakly fusogenic (19% of the value for wt G), retained the Mab45 RBE (177% of the value for the wt). These values approach the 2.5-fold RBE originally reported for NiV G (33).

However, each of the overglycosylated, fusion-deficient stalk mutants failed to exhibit the Mab45 RBE. Indeed, the N-glycans at positions 108 and 123 actually result in a significant receptor-induced decrease in the binding of Mab45, with antibody binding reduced to 49 and 64%, respectively, in the presence of ephrinB2 compared to that in its absence (Fig. 8). Even the N115 and N133 glycosylation mutants exhibit no significant Mab45 RBE (99 and 87% of binding, respectively, with receptor relative to that in its absence) (Fig. 8). Thus, the fusion deficiency of all of the glycosylation mutants correlates completely with a loss of the Mab45 RBE phenotype.

A chimeric attachment protein with a complete NiV G stalk and intact NDV HN head triggers NiV F for fusion only minimally. The properties of the NiV G N-glycan stalk mutants are

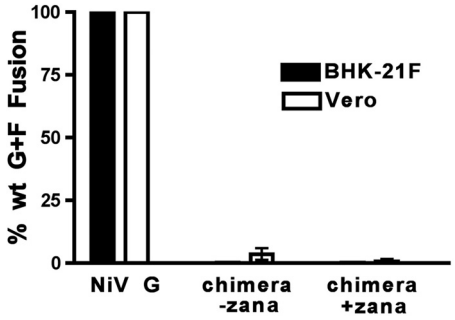

FIG 9 Chimera 188G-HN 124 does not trigger NiV F for fusion to a significant extent, as detected in a reporter gene content-mixing assay. Cells were transfected and the content-mixing assay was performed as described in the text except that in some cases, upon mixing of the two cell populations, 5 mM zanamivir (zana) was also added and left on the cells overnight until the assay was performed. The data are expressed relative to those of wt NiV G and F. Averages ± standard deviations are shown for a minimum of six determinations.

consistent with our previous characterization of chimeras having NiV G-derived stalks and NDV HN-derived heads (G-HN chimeras) (32). Whereas several chimeras having NDV HN-derived stalks and NiV G-derived heads (HN-G chimeras) are capable of efficiently complementing the NDV F protein for fusion promotion (32), the reciprocal was not true. Attachment proteins having NiV G-derived N-terminal segments of 144 and 166 amino acids and a complete, functional HN-derived head were expressed and bound receptor, but they did not trigger NiV F to a detectable extent in a content-mixing assay (32). This lack of fusion was observed in BHK-21F, Vero, and 293T cells. We concluded from this that the G triggering cascade may be more complex than that of HN (32).

Subsequently, it was reported by another group that a chimera with a 188-amino-acid G-derived N-terminal segment and a complete HN head beginning at HN residue 124 (chimera 188G-HN124, using our nomenclature) could trigger NiV F-mediated fusion of erythrocytes, but only if the NA activity of the chimera was inhibited with zanamivir (46). It was concluded that the inhibitor was required to enable the protein to stay attached to receptors for a longer period. This finding also raised the possibility that NiV G residues 183 to 188 might somehow be critical for fusion triggering (46).

To address these findings, we prepared the 188G-HN124 chimera and evaluated its functional properties. The chimera exhibits HAd activity (38% ± 9% of wt HN) commensurate with its cell surface expression (42% ± 5% of wt HN), indicating that receptor binding activity is unaffected. However, in the reporter gene content-mixing assay, it did not trigger a detectable level of NiV F-mediated fusion of BHK-21F cells either with or without treatment with 5 mM zanamivir (Fig. 9). In Vero cells, only extremely weak triggering of NiV F was detected with this assay (4% ± 2% of wt G), and it was actually decreased even further with zanamivir treatment (1% ± 1% of wt G) (Fig. 9). Thus, in our hands, the 188G-HN124 chimera was not capable of triggering NiV F-induced fusion of either BHK-21F or Vero cells to a significant extent in a quantitative content-mixing assay, even with zanamivir treatment.

The lack of an effect of zanamivir in two cell types is consistent with the demonstration that the chimera has only 2% ± 1% of the NA activity of wt NDV HN. Thus, even after correcting for somewhat reduced cell surface expression, the chimera has <10% of wt NA activity. This is somewhat expected because the NA activity of NDV HN is known to be hypersensitive to even point mutations in the stalk (23, 47, 48), which may be related to the cooperative substrate saturation kinetics exhibited by the NA of this strain of NDV (49). Given this hypersensitivity, it is not surprising that replacement of the entire stalk would modulate NA activity.

Since the same group subsequently reported that a 186G-HN124 chimera is capable of triggering NiV F for fusion about 1/3 as effectively as wt G, with no mention of the need for zanamivir treatment (50), we wondered whether the promotion of fusion by the 188G-HN124 chimera could be detected at later times posttransfection. Thus, the extents of syncytium formation induced by the chimera and by wt G were compared at 24 h and 48 h posttransfection by visualization of stained monolayers. Consistent with the content-mixing assay data, syncytia were not visible at 24 h posttransfection in Vero cell monolayers coexpressing the 188G-HN124 chimera and NiV F (Fig. 10A), while robust fusion was obtained with wt G and F, as well as with NDV HN and F, but not with NDV HN and NiV F. At 48 h posttransfection, some chimera-triggered, NiV F-induced syncytium formation was detected. However, at this time, fusion promotion by NiV G and F (as well as NDV HN and F) was far more robust. Indeed, the fusion in NiV G-F-expressing Vero cells after 48 h was so extensive that the monolayer was almost completely destroyed (Fig. 10A). Similar results were obtained in BHK-21F cells (data not shown). We also obtained weak fusion at 48 h posttransfection in both Vero and BHK-21F cells with a chimera (182G-HN124) (31) having a shorter N-terminal, G-derived segment (data not shown). Thus, the weak syncytium formation in Vero and BHK-21F monolayers coexpressing the G-HN chimera and NiV F is visible only at times when fusion promotion induced by wt NiV G and F is so extensive that the monolayer is essentially destroyed.

Finally, since 293T cells are inherently more susceptible to syncytium formation and these cells were used by the other group (50), we also tested the 188G-HN124 chimera for its ability to trigger NiV F-mediated fusion in these cells. In 293T monolayers, NiV G and F gave extremely robust fusion after only 24 h (Fig. 10B). In these cells, NDV HN and F gave weaker, but clearly visible, syncytium formation at 24 h and much more robust fusion after 48 h (Fig. 10B). However, no syncytium formation was visible in 293T cell monolayers coexpressing the 188G-HN124 chimera and NiV F, even at 48 h posttransfection. They are indistinguishable from cells expressing only NiV F. Thus, we were unable to demonstrate the triggering of NiV F by the 188G-HN124 chimera in three different mammalian cell lines.

DISCUSSION

N-glycan shielding has been used effectively to probe the role of the stalk region of both NDV HN (31) and MV H (26) in mediating the interaction with the cognate F protein. Inhibition of PIV5 fusion by the addition of an N-glycan at any of several positions along the stalk of HN has also been reported, though the effect on HN-F complex formation could not be determined (30). We have even used this approach (31) to rule out a role for a domain in the head of NDV HN previously predicted to be involved in mediating the interaction with the F protein (51). All of these findings confirm that N-glycan addition can be used as a tool to probe the role

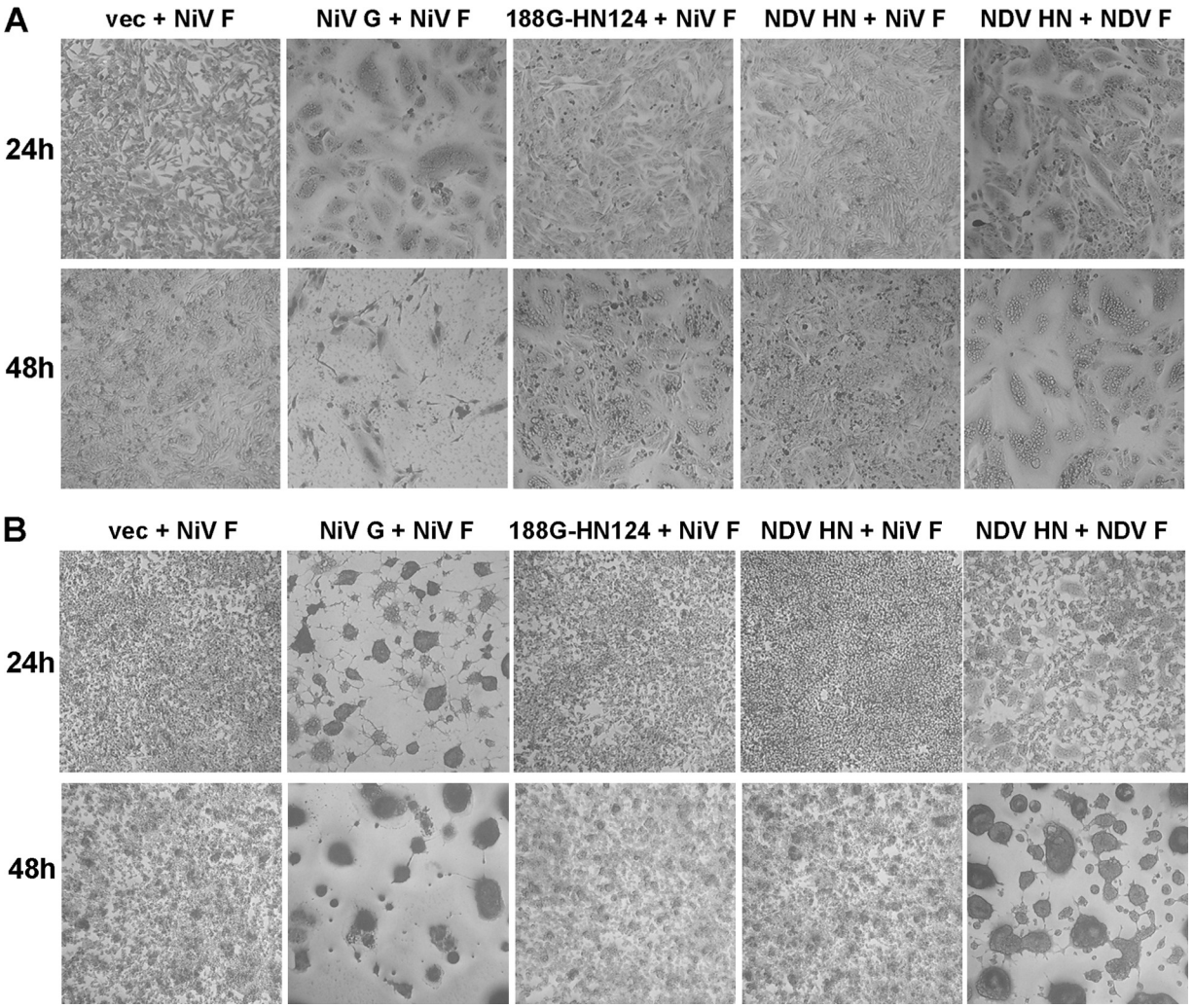

FIG 10 Ability of the 188G-HN124 chimera to trigger NiV-F-mediated fusion of Vero and 293T cells. Shown are the extents of syncytium formation at 24 h and 48 h in Vero cells (A) and 293T cells (B) after transfection with NiV F plus either a vector (vec), wt G, chimera 188G-HN124, or NDV HN or with wt NDV HN and F. The monolayers were fixed with methanol and stained with Giemsa stain at the indicated times posttransfection.

of specific domains in the interaction between the paramyxovirus attachment and fusion proteins.

In this study, we have used this approach to probe the role of the stalk of NiV G in mediating the interaction with its cognate F protein. Though the stalk spans residues 71 to 188, we could exclude a significant portion of the membrane-distal part of the stalk of G from playing a role in the F interaction based on the demonstration that a NiV G deletion mutant lacking stalk residues 146 to 182 retains the ability to interact with the homotypic F protein (28). This enabled us to focus our analysis on the membrane-proximal part of the stalk.

There are two preexisting N-linked glycosylation sites in the NiV G stalk. One, just outside the membrane at position 72, is not utilized (29). The second site, at position 159, is utilized, and its deletion severely decreases fusion (29). To try to probe the role of the base of the stalk in the F interaction, we attempted to introduce glycosylation sites very close to the membrane at positions 75 and 79. However, similar to the nearby site at position 72, neither of these sites was utilized for N-glycosylation. This may be related to the proximity of the domain to the membrane.

However, all five of the potential N-linked sites introduced at positions progressively more distal to the membrane were utilized, resulting in N-glycan addition at position 85, 108, 115, 123, or 133. Remarkably, all five overglycosylated proteins were expressed at levels comparable to that of wt NiV G with the lone exception of N85 (62.6% of the wt level), and all exhibited receptor binding activity comparable to that of the wt protein. Analogous to the studies with NDV and PIV5 HN, all five added N-glycans severely impaired or completely eliminated fusion promotion, with only the most membrane-proximal N85 mutant promoting a detectable level of fusion, approximately 20% of that of wt G.

Surprisingly, despite their defects in fusion promotion, all five overglycosylated mutants retained the ability to interact with the homologous F protein in coimmunoprecipitation assays, indicating that the loss of fusion for these mutants is not the result of a block in G-F complex formation. This is in direct contrast to the findings obtained with NDV HN in which N-glycan addition, even quite distant from the putative F-interactive residues 89, 90, and 94, blocks both fusion and HN-F complex formation (31).

Since the added N-glycans in the NiV G stalk are spaced at intervals along the stalk, this suggests that the role of the stalk of NiV G in its interaction with F is likely different from that of the stalk of HN with NDV F. NiV G also likely differs from MV H in this regard, as N-glycan addition near F-interactive residues 110, 114, and 118 in the stalk of MV H blocks H-F complex formation (26).

Whereas the stalks of NDV HN and MV H entirely mediate the interaction with F, this appears not to be the case for NiV. If a domain in the NiV G stalk did entirely mediate the F interaction, it seems reasonable to expect that at least one of the N-glycans introduced at intervals along the stalk of G would eliminate this interaction. We can speculate that the head region of G may also be involved in making contact with the F protein. However, based on the ability of the NiV G deletion mutant lacking stalk residues 146 to 182 to interact with F (28), it is unlikely that the head alone entirely mediates G-F complex formation. A deletion of 37 residues in the stalk would be expected to result in a misalignment of an F-interactive site in the globular domain of G with the complementary domain in F. If such a domain did entirely mediate the interaction with F, glycoprotein complex formation would be eliminated by the stalk deletion.

These findings are consistent with our inability to identify a chimera having a NiV G-derived stalk and NDV HN-derived head that can effectively complement NiV F for fusion and are in sharp contrast to the demonstration that the head of NDV HN can be replaced by that of hPIV3 HN (20) or even NiV G (32) and still effectively trigger NDV F for fusion. This suggests that the triggering of NiV F by the G protein may involve a secondary contribution from the globular head of G in addition to its receptor binding activity. It even seems plausible that the G-F interaction may be bidentate, involving domains in both the stalk and head of G, accounting for our inability to eliminate complex formation by interfering only with the stalk-mediated arm of the interaction.

Our findings with chimera 188G-HN124 are consistent with this conclusion but stand in contrast to the report that zanamivir treatment of glycoprotein-expressing 293T cells rendered this chimera capable of triggering NiV F-mediated fusion of erythrocytes (46). This treatment was ostensibly required to inhibit the NA activity of the chimera, enabling it to stay attached to the target membrane for an extended period. However, we were unable to repeat this finding; chimera 188G-HN124 did not promote fusion of either Vero or BHK-21F cells in a simple content-mixing assay either with or without zanamivir treatment.

The lack of an effect of zanamivir is consistent with the minimal NA activity of the chimera. It is not clear why zanamivir treatment would be required to inhibit the NA activity of the chimera, when it is not required for NDV HN-F-mediated fusion, despite the more than 10-fold-greater NA activity of wt NDV HN. We did go on to show that very weak syncytium formation could be detected in Vero cell monolayers at a later time posttransfection. However, at the same time point, monolayers expressing wt G and F had essentially been obliterated. Thus, we have established that the G-HN chimera triggers NiV F only very minimally compared to the wt G protein. Again, this is in sharp contrast to the extensive triggering of NDV F by chimeras having HN-derived heads that bind sialic acid receptors (32). We propose that this points to a secondary role for the head of G in fusion promotion in addition to its receptor binding function.

This idea is made more tenable when one considers that the NiV G ephrinB2 and -B3 and NDV HN sialic acid binding sites colocalize at the center of the β-sheet propeller in the head of each monomer (52). Apparently, an aspect of the triggering cascade in HN by its binding to sialic acid receptors must be conserved in NiV G's binding to ephrinB2 and -B3, as evidenced by the ability of HN-G chimeras to trigger robust NDV F-induced fusion. Thus, it was reasonable to expect the reciprocal switch in receptors to result in the efficient triggering of NiV F. But, we have shown that it does not, indicating that binding to NiV receptors results in aspects of the fusion-triggering cascade that are not a part of the cascade induced by binding to sialic acid receptors. In other words, the NiV fusion-triggering cascade is more complex than that of NDV. We contend that the difference in NiV F-induced fusion by wt G and the G-HN chimera is due to a deficiency in a specific, yet-unidentified contribution from the head of G in the chimeras, possibly even involving the interaction with F.

If the fusion deficiency of the NiV G stalk N-glycan mutants cannot be accounted for by an interference with the interaction with F, what, then, is responsible for the fusion-deficient phenotype of these mutants? This may be explained by the failure of all four of the fusion-null glycosylation mutants to exhibit the Mab45 RBE characteristic of the wt G protein. NiV G and both of the unglycosylated N75 and N79 mutants all exhibit nearly 2-fold Mab45 RBE. Even the poorly fusogenic N85 mutant retains the Mab45 RBE phenotype. However, none of the fusion-null mutants with N-glycans added at more membrane-distal positions exhibits Mab45 RBE. Indeed, in some cases, most notably those of mutants N108 and N123, the binding of the antibody is actually reduced in the presence of soluble receptor. This suggests that the added N-glycans in the stalk may convert the protein to a post-receptor-bound conformation even in the absence of receptor, thus accounting for their defect in triggering. In this regard, these stalk glycosylation mutants are similar to the I-to-A HeV stalk mutants described by Bishop et al. (27). Mab45 likely binds to the base of the NiV G head. Congruent with our findings, Aguilar et al. reported that a receptor-induced conformational change in NiV G depended on the presence of the stalk, suggesting a strong communication between the head and stalk of NiV G (33). Our findings confirm that the stalk can influence whether the head remains in a pre-receptor-bound conformation or converts to a post-receptor-bound conformation.

In summary, we have shown that similar to the case with other paramyxovirus attachment proteins, the addition of individual N-glycans at several positions along the stalk of NiV G prevents fusion promotion. However, in contrast to NDV HN and MV H, this fusion deficiency does not correlate with the prevention of the interaction with the homologous F protein. These data strongly suggest that the contact(s) between NiV G and F in the fusion-relevant complex are different, and possibly more complex, than those between either NDV HN or MV H and the respective homotypic F protein.

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REFERENCES

1. **Lamb RA, Parks GD.** 2007. Paramyxoviridae: the viruses and their replication, p 1449–1496. *In:* Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), Fields virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
2. **Chadha MS, Comer JA, Lowe L, Rota PA, Rollin E, Bellini WJ, Ksiazek TG, Mishra AC.** 2006. Nipah virus identified as the agent responsible for an outbreak of encephalitis in Siliguri, India. Emerg. Infect. Dis. **12**:235–240.
3. **Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, Lam SK, Ksiazek TG, Rollin PE, Zaki SR, Shieh W, Goldsmith CS, Gubler DJ, Roehrig JT, Eato B, Gould AR, Olson J, Field H, Daniels P, Ling AE, Peters CJ, Anderson LJ, Mahy BW.** 2000. Nipah virus: a recently emergent deadly paramyxovirus. Science **288**:1432–1435.
4. **Luby SP, Hossain MJ, Gurley ES, Ahmed BN, Banu S, Khan SU, Homaira N, Rota PA, Rollin PE, Comer JA, Kenah E, Ksiazek TG, Rahman M.** 2009. Recurrent zoonotic transmission of Nipah virus into humans, Bangladesh, 2001–2007. Emerg. Infect. Dis. **15**:1229–1235.
5. **WHO.** 2004. Nipah virus outbreak (s) in Bangladesh, January–April 2004. Wkly. Epidemiol. Rec. **17**:168–171.
6. **Butler D.** 2004. Fatal fruit bat virus sparks epidemics in southern Asia. Nature **429**:7.
7. **White JM, Delos SE, Brecher M, Schornberg K.** 2008. Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme. Crit. Rev. Biochem. Mol. Biol. **43**:189–219.
8. **Aguilar HC, Iorio RM.** 2012. Henipavirus membrane fusion and viral entry. Curr. Top. Microbiol. Immunol. **359**:79–94.
9. **Iorio RM, Melanson VR, Mahon PJ.** 2009. Glycoprotein interactions in paramyxovirus fusion. Future Virol. **4**:335–351.
10. **Lee B, Ataman ZA. 21 April** 2011, posting date. Modes of paramyxovirus fusion: a Henipavirus perspective. Trends Microbiol. doi:10.1016/j.tim.2011.03.005.
11. **Plemper RK, Brindley MA, Iorio RM.** 2011. Structural and mechanistic studies of measles virus illuminate paramyxovirus entry. PLoS Pathog. **7**(6):e1002058. doi:10.1371/journal.ppat.1002058.
12. **Smith EC, Popa A, Chang A, Masante C, Dutch RE.** 2009. Viral entry mechanisms; the increasing diversity of paramyxovirus entry. FEBS J. **276**:7217–7227.
13. **Bonaparte MI, Dimitrov AS, Bossart KN, Crameri G, Mungall BA, Bishop KA, Choudhry V, Dimitrov DS, Wang LF, Eaton BT, Broder CC.** 2005. Ephrin-B2 ligand is a functional receptor for Hendra virus and Nipah virus. Proc. Natl. Acad. Sci. U. S. A. **102**:10652–10657.
14. **Negrete OA, Chu D, Aguilar HC, Lee B.** 2007. Single amino acid changes in the Nipah and Hendra virus attachment glycoproteins distinguish ephrinB2 from ephrinB3 usage. J. Virol. **81**:10804–10814.
15. **Negrete OA, Levroney EL, Aguilar HC, Bertoletti-Ciarlet A, Nazarian R, Tajyar S, Lee B.** 2005. EphrinB2 is the entry receptor for Nipah virus, an emergent deadly paramyxovirus. Nature **436**:401–405.
16. **Negrete OA, Wolf MC, Aguilar HC, Enterlein S, Wang W, Muhlberger E, Su SV, Bertolotti-Ciarlet A, Flick R, Lee B.** 2006. Two key residues in ephrinB3 are critical for its use as an alternative receptor for Nipah virus. PLoS Pathog. **2**:e7. doi:10.1371/journal.ppat.0020007.
17. **Harcourt BH, Tamin A, Ksiazek TG, Rollin PE, Anderson LJ, Bellini WJ, Rota PA.** 2000. Molecular characterization of Nipah virus, a newly emergent paramyxovirus. Virology **271**:334–349.
18. **Yu M, Hansson E, Langedijk JP, Eaton BT, Wang LF.** 1998. The attachment protein of Hendra virus has high structural similarity but limited primary sequence homology compared with viruses in the genus Paramyxovirus. Virology **251**:227–233.
19. **Deng R, Mirza AM, Mahon PJ, Iorio RM.** 1997. Functional chimeric HN glycoproteins derived from Newcastle disease virus and human parainfluenza virus-3. Arch. Virol. Suppl. **13**:115–130.
20. **Deng R, Wang Z, Mirza AM, Iorio RM.** 1995. Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. Virology **209**:457–469.
21. **Tanabayashi K, Compans RW.** 1996. Functional interaction of paramyxovirus glycoproteins: identification of a domain in Sendai virus HN which promotes cell fusion. J. Virol. **70**:6112–6118.
22. **Tsurudome M, Kawano M, Tabata N, Nishino M, Kusagawa S, Komada H, Matsumura H, Ito Y, Tsurodome M.** 1995. Identification of regions on the hemagglutinin-neuraminidase protein of human parainfluenza virus type 2 important for promoting cell fusion. Virology **213**:190–203.
23. **Melanson VR, Iorio RM.** 2004. Amino acid substitutions in the F-specific domain in the stalk of the Newcastle disease virus HN protein modulate fusion and interfere with its interaction with the F protein. J. Virol. **78**:13053–13061.
24. **Yuan P, Swanson KA, Leser GP, Paterson RG, Lamb RA, Jardetzky TS.** 2011. Structure of the Newcastle disease virus hemagglutinin-neuraminidase (HN) ectodomain reveals a four-helix bundle stalk. Proc. Natl. Acad. Sci. U. S. A. **108**:14920–14925.
25. **Lee JK, Prussia A, Paal T, White LK, Snyder JP, Plemper RK.** 2008. Functional interaction between paramyxovirus fusion and attachment proteins. J. Biol. Chem. **283**:16561–16572.
26. **Paal T, Brindley MA, St Clair C, Prussia A, Gaus D, Krumm SA, Snyder JP, Plemper RK.** 2009. Probing the spatial organization of measles virus fusion complexes. J. Virol. **83**:10480–10493.
27. **Bishop KA, Hickey AC, Khetawat D, Patch JR, Bossart KN, Zhu Z, Wang LF, Dimitrov DS, Broder CC.** 2008. Residues in the stalk domain of the Hendra virus G glycoprotein modulate conformational changes associated with receptor binding. J. Virol. **82**:11398–11409.
28. **Maar D, Harmon B, Chu D, Schulz B, Aguilar HC, Lee B, Negrete OA.** 2012. Cysteines in the stalk of the Nipah virus G glycoprotein are located in a distinct subdomain critical for fusion activation. J. Virol. **86**:6632–6642.
29. **Biering S, Huang A, Vu AT, Robinson LR, Bradel-Tretheway B, Choi E, Lee B, Aguilar HC.** 2012. N-glycans on the Nipah virus attachment glycoprotein modulate fusion and viral entry as they protect against antibody neutralization. J. Virol. **86**:11991–12002.
30. **Bose S, Welch BD, Kors CA, Yuan P, Jardetzky TS, Lamb RA.** 2011. Structure and mutagenesis of the parainfluenza virus 5 hemagglutinin-neuraminidase stalk domain reveals a four-helix bundle and the role of the stalk in fusion promotion. J. Virol. **85**:12855–12866.
31. **Melanson VR, Iorio RM.** 2006. Addition of N-glycans in the stalk of the Newcastle disease virus HN protein blocks its interaction with the F protein and prevents fusion. J. Virol. **80**:623–633.
32. **Mirza AM, Aguilar HC, Zhu Q, Mahon PJ, Rota PA, Lee B, Iorio RM.** 2011. Triggering of the Newcastle disease virus fusion protein by a chimeric attachment protein that binds to Nipah virus receptors. J. Biol. Chem. **286**:17851–17860.
33. **Aguilar HC, Ataman ZA, Aspericueta V, Fang AQ, Stroud M, Negrete OA, Kammerer RA, Lee B.** 2009. A novel receptor-induced activation site in the Nipah virus attachment glycoprotein (G) involved in triggering the fusion glycoprotein (F). J. Biol. Chem. **284**:1628–1635.
34. **Iorio RM, Borgman JB, Glickman RL, Bratt MA.** 1986. Genetic variation within a neutralizing domain on the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. J. Gen. Virol. **67**:1393–1403.
35. **Iorio RM, Bratt MA.** 1983. Monoclonal antibodies to Newcastle disease virus: delineation of four epitopes on the HN glycoprotein. J. Virol. **48**:440–450.
36. **Iorio RM, Glickman RL, Riel AM, Sheehan JP, Bratt MA.** 1989. Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. Virus Res. **13**:245–262.
37. **Iorio RM, Syddall RJ, Sheehan JP, Bratt MA, Glickman RL, Riel AM.** 1991. Neutralization map of the HN glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition activity. J. Virol. **65**:4999–5006.
38. **Tappert M, Smith DF, Air GM.** 2011. Fixation of oligosaccharides to a surface may increase the susceptibility to human parainfluenza virus 1, 2, or 3 hemagglutinin-neuraminidase. J. Virol. **85**:12146–12159.
39. **Okuma K, Nakamura M, Nakano S, Niho Y, Matsuura Y.** 1999. Host range of human T-cell leukemia virus type I analyzed by a cell fusion-dependent reporter gene activation assay. Virology **254**:235–244.
40. **Nussbaum O, Broder CC, Berger EA.** 1994. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia-virus-based assay quantitating cell fusion-dependent reporter gene activation. J. Virol. **68**:5411–5422.
41. **Mahon PJ, Mirza AM, Musich TA, Iorio RM.** 2008. Engineered intermonomeric disulfide bonds in the globular domain of the Newcastle disease virus hemagglutinin-neuraminidase protein: implications for the mechanism of fusion promotion. J. Virol. **82**:10386–10396.
42. **Aguilar HC, Matreyek KA, Choi DY, Filone CM, Young S, Lee B.** 2007. Polybasic KKR motif in the cytoplasmic tail of Nipah virus fusion protein

modulates membrane fusion by inside-out signaling. J. Virol. **81**:4520–4532.

43. **Aguilar HC, Matreyek KA, Filone CM, Hashimi ST, Levroney EL, Negrete OA, Bertolotti-Ciarlet A, Choi DY, McHardy I, Fulcher JA, Su SV, Wol MC, Kohatzu L, Baum LG, Lee B.** 2006. N-glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. J. Virol. **80**:4878–4889.
44. **Levroney EL, Aguilar HC, Fulcher JA, Kohatsu L, Pace KE, Pang M, Gurney KB, Baum LG, Lee B.** 2005. Novel innate immune functions for galectin-1: galectin-1 inhibits cell fusion by Nipah virus envelope glycoproeins and augments dendritic cell secetion of proinflammatory cytokines. J. Immunol. **175**:413–420.
45. **Tarentino AL, Gómez CM, Plummer TH, Jr.** 1985. Deglycosylation of asparagine-linked glycans by peptide:*N*-glycosidase F. Biochemistry **24**: 4665–4671.
46. **Porotto M, DeVito I, Palmer SG, Jurgens EM, Yee JL, Yokoyama CC, Pessi A, Moscona A.** 2011. Spring-loaded model revisited: paramyxovirus fusion requires engagement of a receptor binding protein beyond initial triggering of the fusion protein. J. Virol. **85**:12867–12880.
47. **Stone-Hulslander J, Morrison TG.** 1999. Mutational analysis of heptad repeats in the membrane-proximal region of the Newcastle disease virus HN protein. J. Virol. **73**:3630–3637.
48. **Wang Z, Iorio RM.** 1999. Amino acid substitutions in a conserved region in the stalk of the Newcastle disease virus HN glycoprotein spike impair its neuraminidase activity in the globular domain. J. Gen. Virol. **80**:749–753.
49. **Mahon PJ, Deng R, Mirza AM, Iorio RM.** 1995. Cooperative neuraminidase activity in a paramyxovirus. Virology **213**:241–244.
50. **Porotto M, Salah Z, DeVito I, Talekar A, Palmer SG, Xu R, Wilson IA, Moscona A.** 2012. The second receptor binding site of the globular head of the Newcastle disease virus hemagglutinin-neuraminidase activates the stalk of multiple paramyxovirus receptor binding proteins to trigger fusion. J. Virol. **86**:5730–5741.
51. **Gravel KA, Morrison TG.** 2003. Interacting domains of the HN and F proteins of Newcastle disease virus. J. Virol. **77**:11040–11049.
52. **Bowden TA, Crispin M, Jones EY, Stuart DI.** 2010. Shared paramyxoviral glycoprotein architecture is adapted for diverse attachment strategies. Biochem. Soc. Trans. **38**:1349–1355.