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.

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The promotion of membrane fusion by most paramyxoviruses requires an interaction between the viral attachment (HN, H, G) 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) HN and measles virus (MV) H proteins reside entirely within the stalk region of those proteins. Indeed, stalk residues of NDV HN and MV H that likely mediate the F-interaction have been identified. However, despite extensive effort, the F-interactive site(s) on the Nipah virus (NiV) G attachment glycoprotein has not been identified. Here, 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 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 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.
INTRODUCTION

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 1-4, Sendai virus, parainfluenza virus 5, respiratory syncytial virus and the emerging henipaviruses, nipah (NiV) and hendra (HeV) (1). The latter two viruses are unique among paramyxoviruses in being able to cause 40-75% mortality rates in humans, mainly from encephalitis (2-5). Both animal-to-human and human-to-human transmission of NiV has 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 1, 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 neuraminidase (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 H/G-F complex fusion is pre-formed and
is dissociated upon receptor binding (8-12).

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 146-182 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 is yet 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 75-133. 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 co-immunoprecipitation 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 American Type Culture Collection (Manassa, VA). Vero and BHK-21F (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 penicillin and 4 \( \mu g/ml \) 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 penicillin and 4 \( \mu g/ml \) 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 QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) 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
146  subsequently corrected such that the transition from G residue 188 to HN residue 124 is
147  seamless in the chimera.
148
149  Transfections and quantitation of cell surface expression. For most
150  experiments, cells were seeded in six-well plates at 2x10^5 cells/well one day prior to
151  transfection. Wt and mutant proteins were expressed using Lipofectamine 2000
152  transfection reagent (Invitrogen Corp, Carlsbad CA) and 1 μg of each DNA per well
153  according to protocols provided by the company. All assays were performed at 48h
154  post-transfection except staining for fusion by the chimera, which was also performed at
155  24h. For fusion staining at 24h post-transfection, Vero and BHK-21F cells were seeded
156  at 3x10^5 cells/well. For 24h and 48h post-transfection fusion staining, 293T cells were
157  seeded at 4x10^5 cells/well and at 3x10^5 cells/well, respectively, using plates that were
158  pre-treated with 0.1 mM polylysine, rinsed with water and allowed to dry before plating.
159
160  Cell surface expression of wt and mutated G proteins in Vero cells was quantified by
161  flow cytometry (performed by the University of Massachusetts Medical School Flow
162  Cytometry Core Laboratory), using a mixture of conformation-dependent G-specific
163  Mabs (33). A mixture of conformational HN-specific Mabs (34-37) was used for the G-
164  HN chimera. Secondary antibodies (Alexa Fluor) were obtained from Invitrogen
165  (Eugene, OR) or KPL Laboratories (Gaithersburg, MD).
166
167  EphrinB2 binding assay. The ability of HN-G chimeras to bind ephrinB2 was
168  determined by a modification of the procedure described by Negrete et al. (15). PK13
169  cells were transfected as above. The medium was removed and the monolayers were
170  incubated for 1h at room temperature with 2 μg of soluble ephrinB2-human Fc protein
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 pre-incubating a monolayer of PK13 cells expressing the chimeras with and without 10 nM soluble ephrinB2 and then quantification of antibody binding by flow cytometry as in (33).

**Hemadsorption (HAd) and neuraminidase (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). NA activity of the chimera was determined with 2-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUN) as 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 24h or 48h post-transfection (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 promotion 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 (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 44h post-transfection, transfected Vero cell monolayers were starved for 1h at 37°C in medium lacking cysteine and methionine, radiolabeled for 3h with 1 ml of medium containing 100 μCi of Express Protein Labeling Mix (35S-cysteine-methionine) (Perkin-Elmer, 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. 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).

**Co-immunoprecipitation 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. Co-immunoprecipitated proteins were analyzed by Western blot and quantified using a Li-cor Odyssey fluorimager (Li-cor Biosciences, Lincoln, NE).
RESULTS

Introduction of potential N-linked glycosylation 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 71-188. However, the F-interactive competence of the NiV G deletion mutant lacking stalk residues 146-182 (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 75-133, 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, exhibit reduced cell surface expression
levels of 57 and 63% of wt, respectively, mutants N79, N108, N115, N123 and N133 were all expressed at the surface at levels comparable to wt G, ranging from 83 to 115% of 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, migrate at a slower rate in the gel relative to wt G, suggesting that the potential N-linked glycosylation site in each of these mutants is, indeed, utilized. However, the added sites in mutants N75 and N79 are apparently not glycosylated, as their migration rates are indistinguishable from that of wt G (Fig. 3A).

To confirm that the slower migration rate of the putative glycosylation mutants is, 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, all the slower-migrating mutants comigrate with wt G treated in the same way, confirming that the slower migration rate of these mutants is 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 was examined by a content-mixing assay
following co-expression 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 promotes detectable fusion, approximately 20% of wt (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 is not utilized, promote fusion quite efficiently, 75 and 78% of wt, respectively (Fig. 4). This suggests that the introduced point mutations themselves, R75N and A81S, affect fusion only minimally.

To be certain that the overglycosylated mutants were truly non-fusogenic, we compared their fusion-promoting activity to that of the wt G protein at a later time post-transfection. As shown in Fig. 5, at 48h post-transfection, 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 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 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 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 non-reducing conditions. The NiV G protein migrates in the gel predominantly as a mixture of disulfide-linked dimers and tetramers with a lesser 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 the wt G protein, although the N85 and N133 mutants appear to exhibit 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-deficiency of
both NDV HN (31) and MV H (26) stalk N-glycan mutants correlates 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 co-immunoprecipitated NiV Fα and F₁ 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 is immunoprecipitated at an efficiency comparable to the wt protein (G-IP, Fig. 7). Also, Mab-mediated immunoprecipitation of the wt F protein from the respective virions is comparable whether they are co-expressed with wt G, the unglycosylated mutant or the glycosylated mutants (F-IP). Interestingly, all the G proteins retained the ability to interact with F (F-co-IP). This includes all mutants, including N85, N108, N115, N123 and N133, that exhibited severely compromised or undetectable fusion. Indeed, only the N85 mutant seems to exhibit a discernible slight reduction in the level of co-IP. The lack of F in the F-only co-IP (last lane) indicates that the co-IP 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 receptor binding-enhanced (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 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 extent 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 two-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 wt G), retained the Mab45 RBE (177% of 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 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 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 of time. This finding also raised the possibility that NiV G residues 183-188 might somehow be critical for fusion triggering (46).
To address these findings, we have 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 5 mM zanamivir treatment (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 is 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 post-transfection. Thus, the
extent of syncytium formation induced by the chimera and by wt G were compared at 24h and 48h post-transfection by visualization of stained monolayers. Consistent with the content-mixing data, syncytia were not visible at 24h post-transfection in Vero cell monolayers co-expressing 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 48h post-transfection, 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) is far more robust. Indeed, the fusion in NiV G-F-expressing Vero cells after 48 hours is so extensive that the monolayer is almost completely destroyed (Fig. 10A). Similar results were obtained in BHK-21F cells (data not shown). We also obtained weak fusion at 48h post-transfection 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 co-expressing 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 24h (Fig. 10B). In these cells, NDV HN and F gave weaker, but clearly visible, syncytium formation at 24h and much more robust fusion after 48h (Fig. 10B). However, no syncytium formation is visible in 293T cell monolayers co-expressing the 188G-HN124 chimera and NiV F, even at 48h post-
transfection. They are indistinguishable from cells expressing only NiV F. Thus, in our hands, we are unable to demonstrate the triggering of NiV F by the 188G-HN124 chimera in three different mammalian cell lines.
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 of specific domains in the interaction between the paramyxovirus attachment and fusion proteins.

Here, 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 71-188, 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 146-182, 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 pre-existing N-linked glycosylation sites in the NiV G stalk. One just outside the membrane at position 72 is not utilized (29). A 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 wt NiV G with the lone exception of N85 (62.6% of wt) and all exhibited receptor binding activity comparable to 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 of 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 co-immunoprecipitation 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

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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 146-182 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 is 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 same chimera capable of triggering NiV F-mediated fusion of
erthrocytes (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 of time. However, we were unable to repeat this finding; chimera 188G-HN124 does 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 post-transfection. However, at this 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/B3 and NDV HN sialic acid binding sites co-localize 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/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, as well as both of the unglycosylated N75 and N79 mutants, all exhibit
Mab45 RBE of nearly two-fold. 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 position exhibits Mab45 RBE. Indeed, in some cases,
most notably 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 or converts to a post-receptor-bound conformation.

In summary, we have shown that, similar to 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.
ACKNOWLEDGEMENTS

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

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

Point mutations introduced in the stalk of NiV G to add potential N-linked glycosylation sites.
FIGURE LEGENDS

**Fig. 1.** Schematic of N-linked glycosylation sites added, as well as those already present, in the stalk of the NiV G protein. Residues 146-182 can be eliminated from taking part in the interaction with F based on the findings of Maar et al. (27). The G1 site at position 72 is not used, while the G2 site at position 159 is used. CT, cytoplasmic tail; TM, transmembrane.

**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 ± S.D. are shown for three independent experiments, each performed in duplicate.

**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.
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 post-transfection 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 ± S.D. 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 co-expressing 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 vector and NDV F as background. Data are expressed relative to that obtained with wt NiV G and F, which is set at 100%. Averages ± S.D. are shown for three independent experiments where n=5 for each experiment.

Fig. 5. Syncytium formation in monolayers co-expressing 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: vector control; wt G; N75; N79; N85; N108; N115; N123; and, N133. At 48 hours post-transfection, the monolayers are fixed with methanol and stained with Giemsa.
Fig. 6. Each of the glycosylation mutants oligomerizes similar 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. Tet, di and mono indicate tetramers, dimers and monomers, respectively.

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 glycoproteins (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, basically as previously described (43, 44). Co-immunoprecipitated 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.

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 1h. 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 receptor, which is set at 100%. Averages ± S.D. are shown for three independent experiments each performed in duplicate.

**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 performed as above except that, in some cases, upon mixing of the two cell populations, 5 mM zanamivir was also added and left on the cells overnight until the assay was performed. The data are expressed relative to that of wt NiV G and F. Averages ± S.D. are shown for a minimum of six determinations.

**Fig. 10.** The ability of the 188G-HN124 chimera to trigger NiV-F-mediated fusion of Vero and 293T cells. The extent of syncytium formation at 24h and 48h in Vero cells (A) and 293T cells (B) after transfection with NiV F plus either vector, wt G, chimera 188G-HN124 or NDV HN or with wt NDV HN and F. The monolayers are fixed with methanol and stained with Giemsa stain at the indicated times post-transfection.
Figure 4

NiV G
N75
N79
N85
N108
N115
N123
N133

% NiV G

EphrinB2 binding
Fusion
Figure 9

% wt G+F Fusion

NiV G chimera -zana chimera +zana

BHK-21F Vero