Role of N-Linked Glycosylation of the Hendra Virus Fusion Protein

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The Hendra virus fusion (F) protein contains five potential sites for N-linked glycosylation in the ectodomain. Examination of F protein mutants with single asparagine-to-alanine modifications indicated that two sites in the F2 subunit (N67 and N99) and two sites in the F1 subunit (N414 and N464) normally undergo N-linked glycosylation. While N-linked modification at N414 is critical for protein folding and transport, F proteins lacking carbohydrates at N67, N99, or N464 remained fusogenically active. As N464 lies within heptad repeat B, these results contrast with those seen for several paramyxovirus F proteins.

Hendra virus, a newly emerged paramyxovirus in the Henipavirus genus, has two glycoproteins: the fusion (F) protein, which promotes membrane fusion (3), and the attachment protein, G. The Hendra virus F protein shows characteristics of type I viral fusion proteins (Fig. 1), including heptad repeats (HR) critical for promotion of membrane fusion (reviewed in reference 5) and posttranslational cleavage of the precursor form (F0) to the fusogenic heterodimer, F1 + F2 (13). The Hendra virus F ectodomain contains five N-X-S/T motifs for the potential addition of N-linked carbohydrates, two in F1 and three in F2. However, lectin binding analysis found no evidence that the F1 subunit contained N-linked carbohydrates (9). This is surprising for several reasons. First, the frequency of N-linked site usage is approximately 90% (7). Second, N-linked glycosylation in the transmembrane-proximal heptad repeat (HRB) is important for the folding or membrane fusion activity of several paramyxovirus F proteins (8, 18), and one potential N-linked site (N464) in the Hendra virus F1 subunit is N-link glycosylated. The mobility difference in the F2 subunits between N67A and N99A is likely due to variation in branching of the glycan. Both F1 and F2 in HRB mutant N464A show faster mobility than in the wt protein (Fig. 2A, lane 6), suggesting that N464 is also N-link glycosylated. Finally, no change in mobility is seen for N485A (Fig. 2A, lane 7), confirming that this site is not glycosylated. N-glycosidase F treatment (Calbiochem), performed as described previously (14), followed by analysis on a 15% polyacrylamide gel showed shifts in all three forms of the wt Hendra virus F protein, confirming that N-linked carbohydrates are present on the F0, F1, and F2 subunits (Fig. 2B). The subunit mobilities after N-glycosidase F treatment are similar in wt and mutant proteins, consistent with mobility changes resulting from differences in N-linked carbohydrates.

To further explore the cleavage-deficient phenotypes, two additional mutations were created. The Hendra virus F mutant S66A shows mobility and proteolytic cleavage similar to that of the wt protein (Fig. 2A, lanes 8 and 9), suggesting that asparagine 64 is not N-linked modified but is required for proper folding and processing. Hendra virus F mutant T416A displays mobility and lack of proteolytic cleavage similar to those of Hendra virus F mutant N414A (Fig. 2A, lanes 5 and 10), indicating that glycosylation at N414 is required for proper processing.

As the removal of N-linked glycans from viral fusion proteins can alter fusogenic activity (8, 18), syncytium formation in BHK-21F cells was examined (Fig. 3A). While no syncytia were observed when the wt or mutant Hendra virus F proteins were expressed alone (data not shown and reference 3), coexpression of wt Hendra virus F protein or the mutants S66A, N67A, N99A, or N464A with the Hendra virus G protein produced syncytium formation (Fig. 3A). The N64A, N414A, and T416A mutants did not form syncytia when coexpressed with the Hendra virus G protein (Fig. 3A), consistent with the cleavage deficiencies observed (Fig. 2).

To more accurately quantitate fusion, a luciferase reporter gene assay was performed (Fig. 3B). No fusion was detected when the Hendra virus F or G proteins were expressed alone or when the folding-defective mutants N64A, N414A, or T416A were coexpressed with Hendra virus G. In the presence...
of Hendra virus G, S66A resulted in no statistically significant change in fusion compared to the wt protein. Removal of carbohydrates in F2 (N67A and N99A) resulted in F proteins that were fusogenic, though the extent of fusion showed a statistically significant decrease. Surprisingly, removal of the carbohydrate in HRB (N464A) resulted in increased fusion (150% of that of the wt on average).

As lowered surface expression can decrease fusion activity (6), a biotinylation assay with Vero cells transiently expressing wt or mutant Hendra virus F proteins was performed according to the manufacturer’s instructions (Pierce), with the same number of cells being used for each biotinylation. The mature F1 subunit and some precursor F0 are present on the cell surface for wt Hendra virus F and the mutants N67A, N99A, and N464A (Fig. 4A, lanes 1, 3, 4 and 6), consistent with reports of uncleaved F protein in Hendra virus virions (9). Only uncleaved N64A and N414A are present on the cell surface (Fig. 4A, lanes 2 and 5), consistent with the cleavage defects previously demonstrated. Quantitation of F1 from four separate experiments using ImageQuant (Amersham) showed 40% (N67A), 70% (N99A), and 50% (N464A) reductions in surface expression. The decrease in fusion for N67A or N99A correlates with a decrease in surface expression. In contrast, higher levels of fusion are seen with N464A (Fig. 3B), even with lowered surface density, confirming the hyperfusogenic nature of this mutant.

Other viral glycoproteins undergo additional posttranslational modifications, such as O-linked glycosylation (2), acylation (17), or phosphorylation (11). To determine whether Hendra virus F has additional posttranslational modifications, protein mobility after synthesis in the presence of tunicamycin (1 μg/ml; Calbiochem), which inhibits N-linked glycosylation in the endoplasmic reticulum (16), was compared to mobility after N-glycosidase F treatment. Tunicamycin treatment gave a single band (Fig. 4B; lane 2), consistent with the requirement for N-linked glycosylation at N414 for protein processing. N-glycosidase F-treated Hendra virus F0 runs slower than F0 from the tunicamycin-treated cells (Fig. 4B, lanes 2 and 4), suggesting that additional modifications occur during transport through the secretory pathway. The SV5 F protein, which requires N-linked glycosylation for folding (1) but has no other identified modifications, had mobilities similar to those of the F0 precursor either synthesized with tunicamycin present or after treatment with N-glycosidase F (Fig. 4B, lanes 6 and 8).

Our studies of the Hendra virus F protein clearly demonstrate that both the F2 (at N67 and N99) and F1 (at N414 and N464) subunits contain N-linked carbohydrates, in contrast to lectin binding studies which did not detect N-linked modifications in the F1 subunit (9). Our findings indicate that the Hendra virus and Nipah virus F proteins utilize N-linked carbohydrates in a similar manner: the sites of addition match (10), glycosylation at residue 414 is critical for processing (Fig.
FIG. 3. Membrane fusion promoted by the Hendra virus F wt and mutant proteins. (A) Syncytium formation. Hendra virus wt and mutant F proteins were coexpressed in BHK-21F cells with the Hendra virus G attachment protein. Approximately 18 h posttransfection, photographs were taken at a magnification of ×10 using the Nikon Eclipse TS100 microscope outfitted with a Nikon Coolpix 995 digital camera. (B) Luciferase fusion assay of Hendra virus wt and mutant F proteins. pCAGGS Hendra virus F wt or the indicated mutants, along with pCAGGS Hendra virus G and a plasmid containing the luciferase gene under the control of the T7 promoter, were transfected into Vero cells. BSR cells, which stably express the T7 polymerase (4), were overlaid onto the F- and G-expressing cells, and the mixed cell populations were incubated at 37°C for 3 h. Cells were lysed and analyzed for luciferase activity on a luminometer (Molecular Devices). Samples used were averages of triplicates and are representative of five separate experiments. G, Hendra virus G protein alone; F, Hendra virus F protein alone; V, the pCAGGS vector alone.

FIG. 4. Cell surface expression and examination of possible additional modifications to the Hendra virus F protein. (A) Biotinylation assay of wt and mutant Hendra virus proteins for cell surface expression. Vero cells were transfected and metabolically labeled, and biotinylation on an identical numbers of cells was performed as described in the text. The biotinylated proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed via the STORM PhosphorImager system. (B) Comparison of mobilities of Hendra virus F and SV5 F proteins transiently expressed in Vero cells after tunicamycin treatment (T) or N-glycosidase F digestion (P). For tunicamycin samples, cells were preincubated in 1 μg/ml tunicamycin, labeled for 30 min, and chased for 3 h. N-glycosidase F digestions were performed as described previously (14). Samples were separated on a 10% acrylamide gel and analyzed using the STORM PhosphorImager system.
2A) (10), and removal of the N-linked carbohydrate in HRB (N464A) gives a significant decrease of cell surface expression (10). Many paramyxovirus F proteins contain a carbohydrate within HRB. This modification is required for SV5 F protein cleavage and cell surface expression (1) and is important for fusion promotion of the Newcastle disease virus and respiratory syncytial virus F proteins (8, 18). In contrast, the Nipah virus mutant lacking HRB glycosylation promoted efficient syncytium formation even with an 80% decrease in surface expression (10). Removal of the HRB carbohydrate from the Hendra virus F protein results in both decreased surface expression and increased fusion activity, clearly shown in the quantitative fusion assay (Fig. 3B). These results suggest mechanistic differences in fusion promotion between the Hendra virus and Nipah virus F proteins and F proteins from other paramyxoviruses.

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REFERENCES


