Detection of Receptor-Induced Glycoprotein Conformational Changes on Enveloped Virions by Using Confocal Micro-Raman Spectroscopy

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Conformational changes in the glycoproteins of enveloped viruses are critical for membrane fusion, which enables viral entry into cells and the pathognomonic cell-cell fusion (syncytia) associated with some viral infections. However, technological capabilities for identifying viral glycoproteins and their conformational changes on actual enveloped virus surfaces are generally scarce, challenging, and time-consuming. Our model, Nipah virus (NiV), is a syncytium-forming biosafety level 4 pathogen with a high mortality rate (40 to 75%) in humans. Once the NiV attachment glycoprotein (G) binds the cell receptor ephrinB2 or -B3, G triggers conformational changes in the fusion glycoprotein (F) that result in membrane fusion and viral entry. We demonstrate that confocal micro-Raman spectroscopy can, within minutes, simultaneously identify specific G and F glycoprotein signals and receptor-induced conformational changes in NiV-F on NiV virus-like particles (VLPs). First, we identified reproducible G- and F-specific Raman spectral features on NiV VLPs containing M (assembly matrix protein), G, and/or F or on NiV/vesicular stomatitis virus (VSV) pseudotyped virions via second-derivative transformations and principal component analysis (PCA). Statistical analyses validated our PCA models. Dynamic temperature-induced conformational changes in F and G or receptor-induced target membrane-dependent conformational changes in F were monitored in NiV pseudovirions in situ in real time by confocal micro-Raman spectroscopy. Advantageously, Raman spectroscopy can identify specific protein signals in relatively impure samples. Thus, this proof-of-principle technological development has implications for the rapid identification and biostability characterization of viruses in medical, veterinary, and food samples and for the analysis of virion glycoprotein conformational changes in situ during viral entry.

Conformational changes in the glycoproteins of enveloped viruses are essential for membrane fusion, a critical step during both viral entry and the pathognomonic cell-cell fusion (syncytia) associated with many viral infections, such as those of the paramyxoviruses. However, technologies that detect glycoprotein conformational changes on actual enveloped virions are difficult and time-consuming for those viruses for which such detection is possible (1–6). Additionally, there is a great need for rapid identification and characterization of virions in medical, veterinary, or food samples. Having a method with these capabilities would advance the fields of virus diagnosis and analysis.

Our model, Nipah virus (NiV), is an enveloped virus in the important Paramyxoviridae family, which comprises human and veterinary enveloped viruses such as measles virus, mumps virus, Newcastle disease virus, respiratory syncytial virus, canine distemper virus, the metapneumoviruses, the human parainfluenza viruses, Hendra virus (HeV), and NiV (6–8). NiV is an emerging zoonotic virus in the Henipavirus genus that causes severe illness in humans, characterized by encephalitis and respiratory disease associated with syncytium formation (7, 9). Although NiV causes 40 to 75% mortality in humans, there is no approved treatment; thus, NiV is classified as a biosafety level 4 agent and a priority pathogen in the NIH/NIAID agenda. Additionally, because paramyxoviruses are relatively stable in aerosols and NiV is capable of animal-to-animal, animal-to-human, and human-to-human transmission, NiV is considered a potential agro- and/or bio-terrorism agent (7).

Conformational changes of the viral glycoproteins are required for viral entry and cell-cell fusion. However, it is difficult to obtain X-ray crystal structural information from intact full-length glycoproteins because their hydrophobic transmembrane regions are embedded in a lipid membrane. Therefore, structural studies have been skewed toward ectodomain glycoprotein forms because it is relatively more straightforward to obtain structural information for them. Viral glycoprotein conformational changes have typically been observed either by analysis of viral glycoprotein soluble ectodomain forms (e.g., see references 10–12) or by analysis of full-length wild-type glycoproteins expressed on cell surfaces (e.g., see reference 13). Although soluble ectodomains normally bind their respective cell receptors, it is often not possible to assess how accurately their structures and structural changes compare with those of their membrane-bound full-length wild-type counterparts. Moreover, the tendency of soluble glycoprotein ectodomains to adopt postfusion conformational changes in many cases limits our ability to detect and characterize essential glycoprotein receptor-induced conformational changes (12, 14). Analysis of receptor-induced conformational changes of full-length wild-type glycoproteins embedded in cellular or viral membranes is preferred, and even for such analyses, there may be differences in the roles of the receptor-induced conformational changes in cell-cell versus...
virus-cell membrane fusion. Therefore, detection of conformational changes on the surface of actual viral particles is highly desirable but currently not common due to technical limitations (1–5) and to our knowledge has not been accomplished for the paramyxoviruses.

Furthermore, current available techniques for identification of viruses include transmission electron microscopy (TEM) (15), PCR (16), cell culture (17), and enzyme-linked immunosorbent assays (ELISAs) (18). However, these techniques require complicated and/or time-consuming methods for sample preparation and relatively large numbers of virions for identification. Raman spectroscopy is widely used for protein characterization, characterizing analytes by compiling vibrational properties of a wide range of functional groups together and providing information about chemical constituents of biological samples (19). Raman spectroscopy has also been employed to investigate nonviral protein structural variations (20). Recently, surface-enhanced Raman scattering (SERS) spectroscopy and tip-enhanced Raman scattering (TERS) spectroscopy have been employed to identify and discriminate different types of viruses, such as Poxviridae virions (21), measles viruses (22), rotavirus (23), respiratory syncytial viruses (24, 25), tobacco mosaic virus (26), and avipoxvirus (27). Moreover, for nonenveloped viruses, Raman spectroscopy has made identification of single viral particles possible (26). However, to our knowledge, the identification of the exact viral protein to which each Raman spectral signal corresponds has not been reported for viruses that contain one or multiple surface glycoproteins, such as the paramyxoviruses.

In this study, we employed confocal micro-Raman spectroscopy to identify and characterize the individual glycoproteins on the membranes of either NiV virus-like particles (VLPs) or NiV/vesicular stomatitis virus (VSV) pseudotyped virions. For NiV, F and G are the envelope glycoproteins essential for membrane fusion during both viral entry into host cells and pathognomonic cell-cell fusion (syncytium formation) following an infection (28–31). The G glycoprotein recognizes and binds to ephrinB2 and ephrinB3 receptors on host cell membranes (32–35). We previously reported the kinetics for the conformational cascade in NiV-F that starts with cell receptor binding to NiV-G, which triggers F from a prefusion form to a prehairpin intermediate and subsequently to a six-helix bundle conformation that mediates membrane fusion (13, 36).

In the current study, we employed Raman spectroscopy, principal component (PC) analysis (PCA), and chemometric methods to successfully discriminate glycoproteins (i.e., F and G) on the membrane of NiV VLPs or NiV/VSV pseudotyped virions (19, 37). In addition, dynamic receptor- or temperature-induced conformational changes of these glycoproteins on NiV pseudovirions were monitored in situ by using a Raman spectroscopy-based two-dimensional (2D) correlation synchronous plotting method. Our ability to produce NiV VLPs and pseudovirions that contained either none, one, or both glycoproteins allowed us to, as a proof-of-principle, both identify individual F and G spike “fingerprints” on these virus particles and demonstrate that Raman spectroscopy can be used to study receptor- and/or temperature-induced glycoprotein conformational changes on these viral particles. Our results promise to expedite the important studies of glycoprotein conformational changes that occur during membrane fusion and viral entry on actual viral particles for the paramyxoviruses and for other enveloped virus families.

**Materials and Methods**

**Expression plasmids.** PCDNA3.1 plasmids expressing the NiV-M (38), NiV-F, and NiV-G (39) codon-optimized genes were previously constructed and reported to express relatively high levels of their respective proteins (29, 34, 38, 39). The green fluorescent protein (GFP) expression plasmid was also previously reported (13, 36).

**Cell culture.** Chinese hamster ovary (CHO) cells (CHOPgaA745) and African green monkey kidney epithelial cells (Vero) were cultivated in minimal essential medium alpha with 10% fetal bovine serum (FBS). 293T cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS. PK13-B2 cells were selected for stable expression of ephrinB2 using G418, and a syncytium assay was used to select a PK13-B2 subclone that expressed high levels of ephrinB2.

**Virus preparation and purification.** NiV VLPs were produced in different cell lines, CHO, 293T, or Vero cells, as previously described (38). Briefly, cells were transfected with equal amounts of codon-optimized NiV-M, -F, and -G expressed in PCDNA3.1 expression plasmids, and viruses were purified from the viral supernatants, as described below. NiV/VSV pseudotyped virions were produced as we previously reported (28, 29, 34, 35). Briefly, 293T cells were transfected with equal amounts of F and G expression plasmids and 24 h later were infected with VSV virions that contained the Renilla luciferase gene in place of VSV-G (VSV-rLuc). Virus supernatants were collected at 24 h posttransfection or postinfection for the NiV VLPs or NiV/VSV pseudotyped virions, respectively, and viral particles were purified by ultracentrifugation through a sucrose cushion of 20% in NTE buffer (150 mM NaCl, 40 mM Tris-HCl [pH 7.5], and 1 mM EDTA [pH 8.0]) and subsequently resuspended in NTE buffer.

**Electron microscopy and Western blot analysis.** The incorporation of M, F, and G into the virus-like particles was confirmed by electron microscopy (uranyl acetate and phosphotungstic acid staining, as described previously [38]) and by Western blot analysis using specific anti-AU1 and anti-hemagglutinin (HA) tag antibodies at 1:2,000 and 1:4,000 dilutions, respectively, as previously described (28, 29).

**Virus sample preparation for spectral collection.** The purified viral samples were transferred from an ice bath directly onto a glass microarray slide coated with a thin film of gold (Thermo Scientific Inc., Waltham, MA). This gold-coated microarray slide has low fluorescence, providing a high signal-to-noise ratio, and is highly compatible with green laser (532.5 nm) biophotonics applications. The viral samples on the gold-coated microarray slide were immediately put onto the confocal microscope stage for Raman spectral collection.

**Raman instrumentation.** Confocal micro-Raman spectroscopic analysis was performed by using a WITec alpha300 Raman microscope (WITec, Ulm, Germany) equipped with a UHTS-300 spectrometer. The spectrometer has an entrance slit of 50 μm and a confocal length of 300 μm and is equipped with a 600-line/mm grating with a 532.5-nm laser power of 12-mW incident light on the viral samples. The Raman-scattered light was detected by using a 1,600- by 200-pixel charge-coupled-device (CCD) array detector. The size of each pixel was 16 μm by 16 μm. A Nikon 100× objective focused the laser light onto the viral samples. An integration time of 15 s (3-s integration time with 5 signal averages) was used for virus spectral collection. The z displacement was controlled by a piezotransducer on the objective. WITec Control v1.5 software (WITec, Ulm, Germany) was employed over a simultaneous wave number shift range of 3,700 to 200 cm⁻¹ in an extended mode.

**Spectral preprocessing.** Raw micro-Raman spectra contain spectral interferences, including fluorescence background of the biological samples, CCD background noise, Gaussian noise, and cosmic noise (40). The background correction was first performed by using a polynomial background fit, as described previously by Lieber and Mahadevan-Jansen (41). This procedure can minimize the effect of different background profiles caused by fluorescence of the biological samples on gold-coated microarray slides and the thermal fluctuations on the CCD detector. Spectral binning (2 cm⁻¹) was performed, followed by smoothing using a Savitzky-Golay algorithm (9 point).
**Spectral reproducibility.** Spectral reproducibility was determined by calculating the differentiation index ($D_{1,10}$) value, as described in our previous studies (19, 37). Lower $D_{1,10}$ values indicate greater reproducibility of the Raman spectra for biological samples (19). Generally, $D_{1,10}$ values of $<1.000$ correspond to adequate reproducibility. The spectral reproducibility of each group (MFG, MF, MG, MPCDNA3, and PCDNA [M is matrix protein, F is fusion protein, G is attachment protein, and PCDNA is PCDNA 3.1+; DNA vector]) was calculated by using $D_{1,10}$ values. The MFG, MF, MG, and MPCDNA3 $D_{1,10}$ values were all $<1.000$, indicating significant reproducibility of the Raman spectral features (19). Furthermore, spectra of both fresh NiV samples and frozen and then thawed NiV samples were collected (data not shown), and spectral reproducibility was suitable ($D_{1,10}$ value of $<300$). These data indicated that the Raman spectral features were not affected by a single freeze-thaw cycle.

**Spectral selectivity.** Selectivity was calculated as previously described (19). Selectivity values greater than 1 were obtained and thus were considered to be significant for segregating biological samples based upon differences in their spectral features (19).

**Second-derivative transformations of Raman spectra.** The variations between the spectral features of closely related biological samples are small, and second-derivative transformations visually separate overlapping bands, eliminate baseline offsets, increase the apparent spectral resolution, and magnify minor spectral variations, making interpretation of spectral results easier (42).

**Classification models.** Principal component analysis (PCA) was employed to establish a two-dimensional cluster model for segregation of viral glycoproteins on the basis of the identified major principal components responsible for spectral variation, with the first principal component (PC1) contributing more than the second principal component (PC2), etc., to the observed differences between spectra (43). This classification procedure maximizes the variance between groups and minimizes the variance within a group. The variance can be quantified as a Mahalanobis distance, which measures intergroup variation and is defined as $M_{1,2} = (x_1 - x_2)S^{-1}(x_1 - x_2)^T$, where $S$ is the pooled estimate of the within-group covariance matrix and $x_1$ and $x_2$ are mean vectors for the two groups. Thus, $M_{1,2}$ is the distance between groups in units of within-group standard deviations (44). The relative locations of the signals on the plot are a result of the relative concentrations of the sources of each signal (e.g., F, G, and M, in each total sample) and do not correlate with the identity of the signal. Therefore, the locations in the PCA plot will vary for the specific protein signals among viral preparations (for example, from distinct cell types). The parameter that measures the distinctiveness between signals is the Mahalanobis distance between given signal groups, as explained above.

Following this analysis, a Bayesian probability approach was employed to validate the selection of principal components using PCA for a multivariate chemometric cluster model to discriminate glycoproteins on the NiV virions. The formula of Bayes’ rule for conditional probabilities can be expressed as

$$P(B_i | A) = \frac{P(B_i)P(A | B_i)}{\sum_{k=1}^{n} P(B_k)P(A | B_k)} (i = 1, 2, \ldots, n)$$

where $A$ and $B$ are two events (i.e., $A$ is the hypothesis and $B$ is data), and the basic principle is that a factor with a large variance has a higher probability for construction of a chemometric model than a factor with a small variance (45). The application of the Bayesian probability approach to construction of chemometric cluster-based models for infrared and Raman spectroscopy of bacteria has been extensively demonstrated by our group and others (37, 46). Additionally, Monte Carlo estimation was performed to determine the stability of the cluster models created by the Bayesian probability approach. In this study, random models were constructed, and the variability of the intracluster distances was calculated. The inverse of the average variance was expressed as stability (47).

**Two-dimensional correlation Raman spectroscopy.** Two-dimensional (2D) correlation Raman spectroscopy, a cross-correlation method, was employed to monitor dynamic behavior of glycoproteins on NiV virions, indicating the conformational changes of these glycoproteins (i.e., G and F glycoproteins) under specific perturbation and observed changes in Raman spectral features. This correlation method can amplify minor spectral variations by dispersing the spectral data in a second dimension (42). Two types of 2D correlation Raman spectroscopy are common, synchronous and asynchronous. For synchronous spectra, the autocorrelation bands are derived from self-correlated Raman signals and are on-diagonal ($v_1 = v_2$), indicating the degree of sensitivity of Raman spectra under a specific external perturbation; the cross-peaks are off-diagonal ($v_1 \neq v_2$) and indicate the intramolecular or intermolecular interactions among functional groups. Autocorrelation bands are always positive, while cross-peaks may be positive or negative (48). If the two overlapped Raman scattered bands in one-dimensional spectra are derived from different functional groups, the variations can be shown as two clearly independent bands in the two-dimensional spectra.

The 2D correlation method described previously by Noda (48) was employed in the current study, and calculations were programmed and performed with Matlab, using the following algorithm:

$$\Phi(v_1, v_2) = \frac{1}{n-1} y^T(v_1) \cdot y(v_2)$$

where $\Phi$ is the 2D synchronous spectrum, $v_1$ and $v_2$ are two spectral channels, $y$ is the vector, and $n$ is the number of signals in the original data set. The one-dimensional spectra used for the 2D correlation analysis are the averages of three replicates, with spectral collection at different locations within a biological sample.

**Statistical analysis of replicate samples.** The experiments were performed for at least three independent replicate trials. Significant difference ($P < 0.05$) was determined by one-way analysis of variance (ANOVA) following the t test in Matlab.

**Validating the feasibility of the Raman spectral subtraction method.** The wave number region of 1,800 cm$^{-1}$ to 650 cm$^{-1}$ (i.e., fingerprint region) (Fig. 1C) was selected for further study because this region had the best signal reproducibility, as evidenced by the lowest $D_{1,10}$ values ($<300$). For example, an addition of the aliphatic band at 2,850 cm$^{-1}$ or the division of this fingerprint region into subregions resulted in a significant ($P < 0.05$) increase of $D_{1,10}$ values ($>1,000$), reducing spectral reproducibility. To compensate for potential variations in the total amount of organic matter in the various viral samples, normalization was performed based upon the most intensive band (in the fingerprint region) at 1,460 cm$^{-1}$.

**Bayesian probability analysis.** We used Bayesian probability analysis to compare the top 10 significant features of M, F, and G expressed as principal components (PCs) determined by using a PCA model. PCs are a construct that compares the importance of certain variations in spectral features to the difference between two biochemical substances, in this case glycoproteins on the surface of the viral membrane. We found substantial agreement between the results of the two methods. The stability of the PCA model was determined by using three different Monte Carlo estimations, one using the 10 most significant features, a second with the 10 least significant features, and one in which all features were selected and compared. The highest stability of the PCA model was derived from the use of the 10 most significant features (0.58 ± 0.08). A model with a slightly lower stability (0.51 ± 0.1) was achieved when all features were selected, indicating the contribution of nonsignificant features and possibly interference and noise. The lowest-stability model was obtained when the 10 least significant features (stability, 0.05 ± 0.02) were used.

**F-triggering assay.** We previously reported an F-triggering assay and performed it here basically as previously described (13, 36). Briefly, CHO cells cotransfected with NiV-F, NiV-G, and GFP expression plasmids were mixed with PK13 cells stably expressing ephrinB2 (PK13B2 cells) to allow NiV-G/ephrinB2 receptor binding and to synchronize the membrane fusion cascade at 4°C for 90 min. These cell mixtures were then incubated at 37°C, 25°C, or 4°C for 60 min in the presence or absence of a biotinylated
HR2 NiV-F-mimicking peptide. HR2 peptide binding was detected by using the secondary fluorescent reagent neutravidin Dylight 649 (Thermo-Fisher) by flow cytometry. Live cells highly expressing GFP (well transfected) were gated to enrich the strengths of the Dylight 649 fluorescent signals. The amounts of biotinylated HR2 peptide bound to NiV-F were calculated for each temperature as the difference between the Dylight 649 fluorescence signals in the presence and absence of the biotinylated HR2 peptide. These differences were then normalized to the result obtained at 37°C, set at 100%.

**RESULTS**

Raman spectral features of Nipah virus glycoproteins F and G on virus-like particles are reproducible.

We and others previously reported that NiV VLPs can be purified from supernatants of mammalian cells transiently expressing NiV-M, -F, and -G (38, 49–51). Thus, we produced and purified NiV VLPs by transfecting 293T cells with equal amounts of codon-optimized NiV-M, -F, and -G expression plasmids, just as previously reported (38). Conventional negative-staining transmission electron microscopy of purified NiV VLPs revealed their glycoprotein spikes (Fig. 1A). Importantly, we previously showed the functionality of these NiV VLPs in virus binding and virus-cell entry assays (38). The incorporation of the M, F, and G proteins into the purified NiV VLPs was confirmed by Western blotting (Fig. 1B).

The Raman spectral features of three distinct NiV MFG VLP sample preparations and a negative-control sample (MPCDNA3), obtained from an identical sucrose purification of supernatants from 293T cells transfected with only the M and mock PCDNA3.1 expression plasmids, are shown in Fig. 1C. Visually, the reproducibility of the Raman signals appeared adequate. To statistically assess the reproducibility of the Raman spectral features of VLPs containing MFG, MF, MG, or the negative-control MPCDNA3 VLPs, the Raman signals were subjected to principal component analyses (PCAs) (Fig. 1D). In these analyses, the higher the reproducibility, the tighter the clusters of data obtained from replicate samples. PCAs revealed tight clusters for the different data groups (MFG, MF, MG, and MPCDNA3), showing that the reproducibility of the Raman signals for the various samples tested was statistically significant. For example, Fig. 1D reveals that the intragroup spectral variation of the MPCDNA3 VLP samples (comparison of multiple MPCDNA3 samples) was significantly ($P < 0.01$) smaller than the intergroup spectral variation (comparison of MPCDNA3 samples to MFG, MF, or MG samples). The Mahalanobis distances computed between groups ranged from 23.07 to 44.36. Clusters with Mahalanobis interclass distance values higher than 3 are considered to be significantly different from each other (44). Therefore, the PCA model validated the feasibility of

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**FIG 1** Raman spectroscopy signals of NiV VLPs. (A) Electron micrograph of NiV VLPs obtained from 293T cells expressing NiV-M, -F, and -G glycoproteins. The NiV-F and -G spikes are marked by black arrows. (B) Western blot analysis of total proteins in NiV VLPs. Briefly, VLPs generated by 293T cells were denatured and detected with a rabbit anti-AU1 monoclonal antibody against AU1-tagged NiV-F (top), a mouse anti-flag monoclonal antibody against flag-tagged NiV-M (middle), and a mouse anti-HA monoclonal antibody against HA-tagged NiV-G (bottom). One of three representative experiments is shown. (C) Raman spectral features of NiV VLPs. The spectrum in red is PCDNA3 (negative control), and spectra in black, green, and blue are three independently produced NiV VLP samples generated in 293T cells. (D to F) Principal component analysis (PCA) validates the significant variations of Raman spectra of different types of NiV VLPs (i.e., MFG, MF, MG, and M) produced in various cell lines: 293T cells (D), Vero cells (E), and CHO cells (F). The relative locations of the signals on the plot are a result of the relative concentrations of the sources of each signal (e.g., F, G, and M, in each total sample) and do not correlate with the identity of the signal. Therefore, the locations in the PCA plot will vary for the specific protein signals among viral preparations (for example, from distinct cell types). The parameter that measures the distinctiveness between signals is the Mahalanobis distance between given signal groups (explained in Materials and Methods). Data points are circled to simplify their identification. Red, MPCDNA3; blue, MF; black, MG; green, MFG.
Raman spectroscopy to obtain reproducible viral sample spectral features (Fig. 1D).

In addition, to assess whether the Raman spectral features of NiV VLPs were cell dependent, NiV virions were prepared and purified from two additional cell lines (Chinese hamster ovary [CHO] cells and African green monkey kidney epithelial [Vero] cells), and their corresponding Raman spectra were collected and analyzed. We observed similar overall NiV VLP Raman spectral features regardless of the cell line used to produce the VLPs, and PCA models could discriminate among different VLP samples (MFG, MF, MG, and MPCDNA3), regardless of their different cell line sources (293T cells [Fig. 1D], Vero cells [Fig. 1E], and CHO cells [Fig. 1F]). The intergroup spectral variations among MFG, MF, MG, and MPCDNA3 (computed between the centroids of groups) were significantly (P < 0.05) larger than intragroup spectral variations based on the calculated Mahalanobis distance (17.52 to 52.28). These statistical analyses showed the feasibility of obtaining reproducible viral glycoprotein Raman spectra regardless of the cell line utilized to produce the NiV VLPs.

Validating the feasibility of the Raman spectral subtraction method. The wave number region of 1,800 cm\(^{-1}\) to 650 cm\(^{-1}\) (i.e., fingerprint region) (Fig. 1C) was selected for further study because this region had the best signal reproducibility (see Materials and Methods). Spectral subtraction was performed to obtain specific NiV protein signals (i.e., M = MPCDNA3 – PCDNA3, F = MF – MPCDNA3, and G = MG – MPCDNA3). The Raman spectral features of the M, F, and G proteins on NiV VLPs produced in distinct cell lines (i.e., 293T, Vero, and CHO cells) are shown in Fig. 2. Additional subtraction formulas, such as F = MFG – MG or G = MFG – MF, confirmed the data obtained with the original subtraction formulas (data not shown). The spectral reproducibility of the Raman signals of the M, F, and G proteins on NiV VLPs were calculated as described in Materials and Methods. Di values of <1,000 correspond to adequate reproducibility. The reproducibility of the specific protein signals from NiV VLPs produced in different cell cultures was significant based upon the Di values obtained (<270) (Fig. 2A to C). These values indicate the feasibility of relying upon the reproducibility of Raman spectra to obtain specific signals for specific NiV VLP proteins even when these were obtained from VLP samples produced in distinct cell types. To further illustrate this conclusion, Fig. 2D shows the average of the Raman spectral features obtained for M, F, and G from the three different cell lines, revealing an adequate spectral reproducibility (Di value of <650).

In addition, to discern whether the presence of core viral proteins or RNA would have an effect on the specific NiV-F and -G viral glycoprotein signals, we produced NiV/VSV pseudotyped virions from 293T cells, just as previously reported (28, 29, 34, 35). We then subjected the NiV/VSV pseudovirions to Raman spectroscopic analysis and obtained the Raman spectral features of NiV-F and -G from those pseudovirions (Fig. 2E). The reproducibility of
the NiV-F and -G spectral features between the NiV VLPs and the NiV/VSV pseudotyped virions produced in 293T cells was pronounced, with a $D_{MAX}$ value of 400. These data indicate that the Raman spectral features of the NiV-F and -G glycoproteins are reproducible independently of the type of viral particles that contain them and independently of the presence of other viral proteins or nucleic acid in those particles.

Importantly, wavelength number differences were observed among the Raman spectral features of different viral proteins (i.e., M, F, and G) (Fig. 2). Standard band assignments (wavelength numbers) of Raman spectra for NiV structural features are listed in Table 1. These band assignments corresponded to both protein and glycan features, in agreement with those originating from the NiV M protein and the F and G glycoproteins. The differences in the M, F, and G Raman features were further analyzed to (i) confirm whether these features could be used to reproducibly distinguish among M, F, and G signals and (ii) study whether these signals could be used to study conformational changes in the F and G glycoproteins induced by temperature or by receptor binding to the VLPs or pseudovirions.

### Differentiation of Raman spectral features for specific NiV glycoproteins.

Second-derivative-transformed Raman spectral features of different NiV glycoproteins are shown in Fig. 3. Spectral selectivity was calculated before performing second-derivative transformations (see Materials and Methods), and the spectral selectivity values obtained indicated the feasibility of using second-derivative transformations for differentiating distinct proteins on NiV VLPs (37). Significant ($P < 0.01$) band variations between the M, F, and G viral proteins are summarized in Table 1. These bands have the potential of serving as indicators for real-time monitoring of conformational changes of glycoproteins on NiV membranes. In general, the wave number region from 1,500 to 1,200 cm$^{-1}$ contained spectral features corresponding mainly to secondary structural features of proteins (Fig. 3A and Table 1), while wave numbers of 1,200 to 900 cm$^{-1}$ contained spectral features corresponding mainly to polysaccharides (Fig. 3B and Table 1). Comparison of Fig. 3B and A shows that, in general, secondary

### TABLE 1 Assignment of Raman bands of NiV glycoproteins

<table>
<thead>
<tr>
<th>Spectrum and wave no. (cm$^{-1}$)</th>
<th>Assignment</th>
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<tr>
<td>Raw</td>
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<tr>
<td>840</td>
<td>Glucose-saccharide band</td>
</tr>
<tr>
<td>1,071</td>
<td>Glucose-saccharide band</td>
</tr>
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<td>C-C skeletal stretch transformation</td>
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<td>Amide III bands in protein</td>
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<tr>
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<td>2nd derivative</td>
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<tr>
<td>990</td>
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<td>1,460</td>
<td>CH$_2$/CH$_3$ deformation of lipids and proteins</td>
</tr>
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FIG 3 Second-derivative transformation analyses of Raman spectral features reveal clear specific M, F, and G spectral peaks. (A) Second-derivative transformation of Raman spectral features (1,500 to 1,200 cm$^{-1}$) of M, F, and G proteins on NiV VLPs (from the Vero cell line). (B) Second-derivative transformation of Raman spectral features (1,200 to 900 cm$^{-1}$) of M, F, and G proteins on NiV VLPs (from the Vero cell line). (C) A representative PCA classification model was established and cross-validated to differentiate NiV proteins ($n = 4$). Data points are circled to simplify their identification. Blue, F protein; black, G protein; red, M protein.
protein structural variations appeared to be more important for differentiating NIV glycoproteins than polysaccharide variations. The clear separation ($P < 0.05$) between the M, F, and G glycoprotein Raman signals by PCA (Fig. 3C) indicates that the spectral features of these glycoproteins are unique and have discernible Raman spectra. The Mahalanobis interclass distances ranged from 13.26 to 29.71, computed between the centroids of groups for each glycoprotein. In addition, we employed Bayesian probability analysis to compare the top 10 significant features of M, F, and G (principal components [PCs]) determined by using a PCA model (see Materials and Methods). Taken together, these mathematical analyses validated the choice of the selected M, F, and G spectral features for PCA model establishment and for the following studies.

**Monitoring temperature-induced conformational changes of G and F glycoproteins on NIV VLPs and NIV/VSV pseudovirions.** Confocal micro-Raman spectroscopy was employed to monitor the conformational changes of NiV-G and -F glycoproteins on NIV/VSV pseudovirions. We looked for potential temperature- and/or receptor-induced conformational changes in the glycoproteins on the NIV/VSV pseudovirions, and we found both. Temperature treatment alone has been reported to be able to induce conformational changes in viral glycoproteins, as shown, for example, in a previous study for a soluble paramyxovirus F glycoprotein (12). By technical necessity, in this study, we had to subject our pseudovirions to room temperature while looking for receptor-induced conformational changes in their glycoproteins. Therefore, we compared the Raman signal changes of the NiV/ VSV pseudovirion glycoproteins induced by temperature alone (as an important control) to the receptor-induced Raman signal changes. If significant differences were observed between the two treatments, by definition those differences would be due to receptor-induced conformational changes in the glycoproteins. In addition, viral glycoprotein conformational changes usually occur rather quickly; for example, we previously reported that at 37°C, receptor-induced conformational changes in full-length NiV-F (prehairpin intermediate formation) on cell surfaces occurred within a few minutes (36).

Thus, we first monitored the temperature-induced conformational changes of G and F glycoproteins on NiV/VSV pseudovirions preincubated at 4°C over a 20-min period at 25°C as changes in the second-derivative transformations of the G- or F-specific Raman spectral features. We focused our analysis on bands at 1,409 cm$^{-1}$ ($\nu_2$ COO$^-$ chemical group from proteins) and 1,302 cm$^{-1}$ [$\delta$(CH$_2$) for protein twisting or wagging] for the F glycoprotein and on bands at 1,321 cm$^{-1}$ (α-helix of amide III) and 1,337 cm$^{-1}$ (amide III and CH$_2$ wagging vibrations from the glycine backbone) for the G glycoprotein, shown in Fig. 3A to be prominent spectral features for differentiation of F and G on pseudovirus surfaces. For F, the band intensity at 1,409 cm$^{-1}$ increased (upward movement) while the band intensity at 1,302 cm$^{-1}$ decreased (downward movement) with increasing temperature treatment times (Fig. 4A). For G, the band intensity at 1,321 cm$^{-1}$ decreased (upward movement) while the band intensity at 1,337 cm$^{-1}$ increased (downward movement) with increasing treatment times (Fig. 4B). Taken together, these data indicate that confocal micro-Raman spectroscopy can monitor temperature-induced conformational changes in both G and F glycoproteins on NiV/VSV pseudovirions in situ. Thus, this method provides a significant advantage over other methods, by monitoring conformational changes of intact viral glycoproteins on actual enveloped virions in their native state and in real time. These results may have implications for the future monitoring of the biostability of viruses in medical, veterinary, and/or food samples, even at room temperatures.

To further explore the potential of employing confocal micro-Raman spectroscopy to monitor dynamic conformational changes of F and G glycoproteins on NIV particle surfaces, 2D correlation synchronous spectra were investigated (Fig. 4C and D; also see Materials and Methods). Previous works by Ashton et al. (42) and Brewster et al. (43) validated the feasibility of performing 2D correlation Raman spectroscopy to monitor the dynamic conformational changes of glycosylation and phosphorylation in proteins. 2D correlation Raman spectroscopy data indicate the relative levels of change of a given spectral feature (or chemical group) in time and space. For the F glycoprotein, two autopeaks were observed at ∼1,409 and 1,302 cm$^{-1}$ (Fig. 4C), indicating that the intensities of these bands changed with an increase in treatment time (0 to 20 min) at 25°C. For the G glycoprotein, two adjacent autopeaks were observed at ∼1,321 and 1,337 cm$^{-1}$ (Fig. 4D), indicating a similar conclusion as that drawn for F. These results correlated well with the band variations observed using second-derivative transformations (Fig. 4A and B). In addition, another cross-peak was observed at ∼1,210 cm$^{-1}$ for the F glycoprotein (Fig. 4C), corresponding to a C–C–H stretching mode in tyrosine and phenylalanine (20). This spectral variation was not apparent in the second-derivative transformations of one-dimensional confocal micro-Raman spectra, showing the increased selectivity of 2D correlation spectroscopy to monitor dynamic conformational changes of glycoproteins.

**Monitoring receptor-induced conformational changes on NIV glycoproteins.** We previously reported that ephrinB2-containing VSV pseudovirions (VSV-B2 or reversed pseudotyped virions) were capable of inducing membrane fusion and viral entry on cells expressing NiV-F and -G (28). Thus, we mixed NiV/VSV pseudovirions with VSV-B2 pseudovirions for 1 h at 4°C to allow G/B2 receptor binding, as we previously reported that incubation at 4°C allows receptor binding but not membrane fusion. Therefore, an incubation of F/G-containing membranes with receptor-containing membranes at 4°C can be used to synchronize the membrane fusion and viral entry events (13, 36). To distinguish with certainty Raman spectral feature changes induced by temperature from receptor-induced conformational changes relevant to membrane fusion, we additionally replaced wild-type NiV/VSV pseudovirions with NiV/VSV-G mutant pseudovirions. The mutant pseudovirions contained a G mutant glycoprotein (28, 29) that we previously characterized as capable of being incorporated into NiV/VSV pseudovirions and capable of binding the ephrinB2 receptor similarly to wild-type NiV-G but incapable of triggering F to induce membrane fusion or viral entry (13).

Raman spectral features were collected for wild-type NiV/VSV pseudovirions and NiV-G mutant NiV/VSV pseudovirions, and the spectral subtraction described above was performed, yielding spectral features of NiV-F and -G glycoproteins. Figure 5 shows the second-derivative-transformed Raman spectral features of F and G glycoproteins (for either the wild-type or G 4-5 fusion mutant NiV/VSV pseudovirions) with or without incubation with ephrinB2 receptor at 4°C and then with incubation at 25°C. For the wild-type NiV/VSV pseudovirions, spectral variations at 1,321 cm$^{-1}$ and 1,337 cm$^{-1}$ for the G glycoprotein (Fig. 5A) were ob-
served to be similar to those induced by temperature treatment in Fig. 4B. However, for NiV-F, the conformational change induced by receptor binding at 1,409 cm$^{-1}$ (receptor plus temperature condition) occurred in the opposite direction (downward) (Fig. 5B) from that observed with temperature treatment alone (upward) (Fig. 4A, left). These data suggest that receptor binding induced a specific and different F glycoprotein Raman signature compared to the conformational change induced by temperature alone. Importantly, this experiment suggests that confocal micro-Raman spectroscopy can detect a receptor-triggered conformational change in a full-length fusion glycoprotein embedded in a pseudovirus particle.

To confirm that this change in the 1,409-cm$^{-1}$ F glycoprotein Raman signal direction was induced by receptor binding to the G glycoprotein (subsequently triggering the F glycoprotein), we subjected the G 4-5 mutant glycoprotein pseudovirions to an identical Raman spectroscopy analysis as that for the wild-type NiV/VSV pseudovirions. We observed that for the G 4-5 mutant pseudovirions, the band variations at 1,321 cm$^{-1}$ and 1,337 cm$^{-1}$ for the G glycoprotein were similar to those of the wild-type pseudovirions although to a somewhat lesser extent (Fig. 5C). However, there was no change observed for the F glycoprotein Raman band at 1,409 cm$^{-1}$ (Fig. 5D). This result supports the notion that although the G glycoprotein mutant was capable of binding to ephrinB2 (13), it did not induce a conformational change in the F glycoprotein that resulted in membrane fusion. Therefore, a receptor-induced conformational change in F was detectable by Raman spectroscopy in wild-type NiV pseudovirions but not in NiV-G fusion mutant pseudovirions, suggesting that confocal micro-Raman spectroscopy can monitor the receptor-induced con-

![FIG 4](https://example.com/figure4.png)
formational changes of glycoproteins on viral particles in situ and in real time.

A receptor-induced conformational change in NiV-F can occur at 25°C, and it depends on the presence of a target membrane. The receptor-induced conformational changes that we observed by Raman spectroscopy for NiV-F on NiV/VSV pseudovirions were detected at 25°C. Conformational changes of soluble fusion proteins were previously detected at temperatures higher than 37°C (e.g., see reference 12). However, it is well known that class I fusion proteins (such as HIV-1 gp41) can undergo a conformational change from the prefusion to the prehairpin intermediate at 23°C to 27°C (52, 53). Thus, to confirm the biological relevance of the Raman spectroscopic conformational change that we observed for NiV-F, we performed a NiV-F-triggering assay to confirm that a receptor-induced conformational change in NiV-F can occur at 25°C. We previously reported a NiV-F-triggering assay capable of trapping and quantifying the receptor-induced formation of the prehairpin intermediate of NiV-F at 37°C but not at 4°C. This flow cytometry cell-based assay quantifies the binding of a biotinylated peptide that mimics the HR2 region of NiV-F to NiV-F under triggering conditions. Here, we utilized this assay to learn whether the NiV-F glycoprotein can be triggered to undergo a conformational change from a prefusion to a prehairpin intermediate at 25°C. As observed for other class I fusion proteins, NiV-F underwent conformational changes from a prefusion to a prehairpin intermediate at 25°C, at levels of approximately 50% of the levels of NiV-F triggering at 37°C (Fig. 6A). These data further suggest that the conformational change detected for NiV-F in pseudovirions at 25°C by Raman spectroscopy is likely prehairpin intermediate formation.

To further investigate whether prehairpin intermediate formation for NiV-F can be induced by soluble ephrinB2 unanchored to a target membrane, we performed our F-triggering assay using a soluble ephrinB2-Fc molecule that we previously reported to be capable of binding NiV-G and inducing a conformational change in NiV-G (13, 34). In our previous report, soluble ephrinB2 binding to NiV-G induced a conformational change detected by 3 independent assays: enhanced binding of monoclonal antibody
Mab45 to NiV-G, circular dichroism, and detection of enhanced F/G dissociation (13). Interestingly, we observed that soluble ephrinB2 was incapable of inducing prehairpin intermediate formation in NiV-F (Fig. 6A). These data suggest that in the absence of a target membrane, although conformational changes in NiV-G can occur (13), the prehairpin intermediate of NiV-F either does not occur or is not stable. The latter may be likely, because the prehairpin intermediate may need to insert its hydrophobic fusion peptide into the target membrane to be stable.

To corroborate the need for a target membrane for the NiV-F conformational changes in virions, we subjected our NiV/VSV pseudovirions to our Raman spectroscopy analysis using soluble ephrinB2. As with the F-triggering cell-based assays, soluble ephrinB2 induced conformational changes in G (Fig. 6B) but was unable to induce a conformational change in NiV-F (Fig. 6C). Our results suggest that for both cell-based and virus-based assays, induction of the NiV-F prehairpin intermediate requires the presence of a target membrane.

**DISCUSSION**

It is well established that the glycoproteins of enveloped viruses, regardless of the family to which they belong, are responsible for cell-cell fusion and viral entry. Membrane fusion is therefore essential in the life cycle of enveloped viruses (reviewed in references 9 and 6). However, many of the steps in the cascade of conformational changes that glycoproteins undergo during viral entry and the syncytium formation that follows an infection remain unclear (6, 8, 9). The receptor-induced conformational changes that enveloped glycoproteins undergo during membrane fusion have remained of great interest in the last couple of decades. An understanding of such glycoprotein conformational changes can lead, and is already leading, to the development of antiviral drugs that block viral entry. For example, enfuvirtide is an FDA-approved drug currently used against HIV-1 to block a conformational change of the fusion protein gp41, required for membrane fusion (54, 55). Therefore, an understanding of the receptor-induced conformational changes that enveloped virus glycoproteins undergo during membrane fusion will likely expand the repertoire of potential antiviral therapeutic targets and agents. Identifying such conformational changes in a rapid fashion offers many potential applications in the field of virology. To our knowledge, this is the first study to employ confocal micro-Raman spectroscopy to detect distinct glycoproteins and simultaneously analyze conformational changes of these glycoproteins while they are embedded on enveloped VLPs or pseudovirions. This new technology is particularly suitable for studies of viral glycoprotein conformational changes, as instrument responses are rapid.

Methods that can monitor the processes that glycoproteins on actual virions undergo after cell receptor binding are generally lacking. These processes are critical to our understanding of the pathology of enveloped viruses (6). For example, for the paramyxoviruses, what we currently know about receptor-in-
duced conformational changes in their enveloped glycoproteins has been studied primarily by using soluble glycoprotein forms (6, 12) or glycoproteins expressed on cell surfaces (6, 9, 13, 56). Although those studies are quite valuable, they do not necessarily replicate what happens in a virion. In contrast, the glycoproteins in the virion envelope have a truly native conformation: full length, embedded in the membrane, and in the proper context with other envelope proteins. Although there are viruses for which assays have been developed to study conformational changes in virions (e.g., see references 1–5), by far, these methods tend to be complex and time-consuming. In this study, we developed a method for studying viral glycoprotein conformational changes in virions in situ that is selective, accurate, and rapid enough to be appropriate for dynamic conformational studies conducted in real time.

Raman spectroscopy has been widely employed to investigate protein dynamics and conformational changes but rarely in studies of viruses. For example, Raman spectroscopic methods have been used to investigate protein structure (57); alpha-helix hydration in polypeptides, proteins, and viruses (58–62); polypeptide and carbohydrate structures of glycoproteins (63); carbohydrate characterization (64); and the molecular structures of proteins (62, 63, 65–67). Raman spectroscopy has been validated as a highly accurate and reliable method to monitor and determine protein secondary structural variations in situ, such as folding and orientation (68, 69), glycosylation status (43), phosphorylation status (42, 70), and posttranslational modifications (71). The overall secondary structures of the glycosylated and deglycosylated forms of soluble glycoprotein C of herpes simplex virus were differentiated by using conventional Raman spectroscopy (72). In recent studies, surface-enhanced Raman scattering (SERS) was able to detect binding of viral nucleoproteins to anti-influenza virus aptamers (73, 74).

However, to our knowledge, this is the first study to employ confocal micro-Raman spectroscopy to simultaneously detect distinct glycoproteins and analyze specific viral glycoprotein conformational changes on enveloped VLPs, pseudovirions, or any type of viral particles in situ. The instrument response is rapid. For example, structural changes of proteins occurring within a picosecond-to-millisecond range have been successfully monitored by using UV Raman spectroscopy (75). Other important protein conformational changes, such as alpha-helix peptide folding and unfolding, can be monitored by using UV resonance Raman spectroscopy on a nanosecond time scale (68). Although the present study involved measurements at less frequent intervals, measurements on the order of milliseconds are clearly possible and may be appropriate in studies where detailed kinetics of glycoprotein binding and/or conformational changes is important.

Additionally, Raman spectroscopy is amenable to applications such as cell receptor binding to viral particles, where the ability to obtain dynamic measurements is important. Green laser (510- to 570-nm) light, as employed in this study (532 nm), has been shown to be well suited to studies measuring protein conformational changes, including flow-induced conformational changes to proteins (76), as well as investigations of conformational changes to proteins on the sarcoplasmic reticulum membrane (77). These studies provided evidence that Raman spectroscopy using this frequency range would be appropriate for studies of conformational changes in proteins occurring on the surface of virus particles and that dynamic and time-dependent measure-
mational changes in NIV-F required the presence of a target membrane; thus, we speculate that the prehairpin intermediate requires a target membrane to insert into and stabilize. Importantly, similar results were shown by both cell-based (F-triggering) and virus-based (Raman) assays, underlining the biological significance of a target membrane to stabilize the prehairpin intermediate of the membrane fusion cascade.

In summary, in the present study, we show the feasibility of performing confocal micro-Raman spectroscopy to detect and discriminate glycoproteins on NIV particles with multiple glycoproteins in situ. In addition, we observed that dynamic conformational variations of glycoproteins on NIV pseudovirions could be monitored in real time by using Raman spectroscopic techniques. The technologies described here provide fast and sensitive methods to study both temperature- and receptor-induced conformational changes. These technologies open new avenues for examination of viral glycoproteins during syncytium formation and/or viral entry. Our studies have implications for the examination of inactivation of virions upon temperature or chemical treatment during vaccine development and can measure the biostability of virions in medical, veterinary, or food samples. Therefore, our discoveries have the potential of expediting advances in the fields of virus identification, virus decontamination, viral vaccine development, and virus entry.

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