Receptor Binding, Fusion Inhibition, and Induction of Cross-Reactive Neutralizing Antibodies by a Soluble G Glycoprotein of Hendra Virus

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Hendra virus (HeV) and Nipah virus (NiV) are closely related emerging viruses comprising the Henipavirus genus of the Paramyxovirinae, which are distinguished by their ability to cause fatal disease in both animal and human hosts. These viruses infect cells by a pH-independent membrane fusion event mediated by their attachment (G) and fusion (F) glycoproteins. Previously, we reported on HeV- and NiV-mediated fusion activities and detailed their host-cell tropism characteristics. These studies also suggested that a common cell surface receptor, which could be destroyed by protease, was utilized by both viruses. To further characterize the G glycoprotein and its unknown receptor, soluble forms of HeV G (sG) were constructed by replacing its cytoplasmic tail and transmembrane domains with an immunoglobulin κ leader sequence coupled to either an S-peptide tag (sG<sub>κ</sub><sub>Light</sub>) or myc-epitope tag (sG<sub>myc</sub>-tag) to facilitate purification and detection. Expression of sG was verified in cell lysates and culture supernatants by specific affinity precipitation. Analysis of sG by size exclusion chromatography and sucrose gradient centrifugation demonstrated tetrameric, dimeric, and monomeric species, with the majority of the sG released as a disulfide-linked dimer. Immunofluorescence staining revealed that sG specifically bound to HeV and NiV infection-permissive cells but not to a nonpermissive HeLa cell line clone, suggesting that it binds to virus receptor on host cells. Preincubation of host cells with sG resulted in dose-dependent inhibition of both HeV and NiV cell fusion as well as infection by live virus. Taken together, these data indicate that sG retains important native structural features, and we further demonstrate that administration of sG to rabbits can elicit a potent cross-reactive neutralizing antibody response against infectious HeV and NiV. This HeV sG glycoprotein will be exceedingly useful for structural studies, receptor identification strategies, and vaccine development goals for these important emerging viral agents.

Two newly recognized and closely related paramyxoviruses have been identified from cases of severe respiratory and encephalitic diseases in animals and humans; these viruses are known now as Hendra virus (HeV) and Nipah virus (NiV) (reviewed in reference 15). HeV appeared in eastern Australia in 1994 and was transmitted to humans by close contact with infected horses; NiV emerged in 1998. The HeV outbreaks in Bangladesh have been confirmed, totaling some 53 human cases of infection, and HeV reappeared in northern Australia in late 2004, with two cases of fatal infection in horses and one nonfatal human case (2). Several significant observations in the most recent NiV outbreaks have been made, including a higher incidence of acute respiratory distress syndrome, possibly a higher incidence of person-to-person transmission, significantly higher case fatality rates (60 to 75%), and no direct link to infected livestock or domestic animals (1, 3, 9, 17). The development of therapeutic or intervention strategies to deal with these emerging viral agents is now of importance.

Paramyxoviruses are negative-sense RNA-containing enveloped viruses and contain two major membrane-anchored envelope glycoproteins that are required for infection of a receptive host cell. All members contain an F glycoprotein, which mediates pH-independent membrane fusion between the virus and its host cell, while the second attachment glycoprotein can be either a hemagglutinin-neuraminidase protein (HN), a hemagglutinin protein (H), or a G protein, depending on the particular virus (reviewed in reference 24). The F proteins are type I membrane glycoproteins existing as trimeric oligomers with considerable hydrophobicity, while the attachment glycoproteins are oligomeric type II membrane glycoproteins, and evidence has shown that both dimeric and/or tetrameric (a dimer of dimers) configurations exist from studies on the HN glycoprotein of Newcastle disease virus and simian virus 5...
supplemented with 10% cosmic calf serum (HyClone, Logan, UT) and 2 mM L-glutamine (DMEM-10). PCI 13 cells were maintained in DMEM-10 supplemented with 10% cosmic calf serum (HyClone). All cell cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Haemagglutination (HA) and neuraminidase (NA) activity were determined by incubating infected cells at 31°C overnight (4). Cell fusion reactions were conducted with the various cell mixtures in 96-well plates at 37°C. Typically, the ratio of infected cells to uninfected cells was 1:1 (2 × 10⁵ total volume of virus stock used) for infection of all cell lines. For quantiﬁcation of both hemagglutinin and neuraminidase activities in greater than 25 different cell lines examined to date we devised a quantitative assay for studying the structural and functional characteristics of HeV and NiV glycoprotein-mediated membrane fusion (reviewed in references 5 and 16) and also possess proportional cell fusion activities for each receptor-positive cell line tested (6, 7). Several cell lines non-permissive for fusion, some derived from the same animal species, have been identiﬁed, and protease treatment of permissivemembrane fusion and also possess proportional cell fusion activities for each receptor-positive cell line tested (6, 7). To further characterize the viruses’ G glycoprotein and its unknown host cell receptor, we have developed epitope-tagged versions of soluble HeV G (sHeV G) with the transmembrane-cytoplasmic tail deleted was constructed by PCR ampliﬁcation of HeV G using primers sHGS, 5'-GTCGACACCATGCACGTGGTAC-3' and sHGAS, 5'-GGTTAAGCTTCGACATTACACGCTCTGAAATCTTGACATGGCGAGATC-3'. All PCR products were performed using Accupol DNA polymerase (PGS Scientiﬁcs Corp., Gaithersburg, MD). These primers generated a PCR product for the sHeV G open reading frame framed by Sall sites. PCR products were gel puriﬁed (QIAGEN, Valencia, CA) and subcloned into the TOPO vector (Invitrogen Corp., Carlsbad, CA). The TOPO sHeV G construct was then sub-cloned into pSeTag2B (Invitrogen Corp.). The pSeTag2-HeV G construct was then modiﬁed by inserting either the S-peptide tag or a myc-epitope tag using repetitive overlapping oligonucleotides as follows. Overlapping oligonucleotides were synthesized that encoded the sequence for the S-peptide along with KpnI and EcoRI restriction sites: SPEPS, 5'-CAAGGAGACGCCTGCCTGCTAAGTGCAAGCGCCACATGATGCCTCT-3'; SPEPS, 5'-ATTAGAAGCTCTAGGTTGCTTACATGGCGAGGCCTCCTTGGTAC-3'. The annealed oligonucleotides were cloned into KpnI-EcoRI-digested pSeTag2B-HeV G to generate sGtag or sGmyc-tag. The HeV sGtag and sGmyc-tag constructs were then subcloned into the vaccinia vector pMC02 (10) to generate recombinant vaccinia viruses vKB6 (sGtag) and vKB15 (sGmyc-tag).

Recombinant vaccinia viruses. For expression of recombinant sGtag or sGmyc glycoprotein, the following recombinant vaccinia viruses were generated and employed: vKB6:V and NiV and F glycoproteins, the following recombinant vaccinia viruses were generated and employed: vKB 7 (NIV F), vKB 8 (NIV G), vKB 1 (HeV F), and vKB 2 (HeV G) (6, 7). Bacteriophage T7 RNA polymerase was produced by infection with vTF7-3 (19). The Escherichia coli lacZ gene linked to the T7 promoter was introduced into cells by infection with vCE21R-LacZ (18).

Construction and analysis of soluble HeV G glycoprotein. HeLa cells were infected with vKB16 (multiplicity of infection [MOI] of 5) for 2 h. After infection the virus was removed and serum-free OptiMEM medium (Invitrogen Corp.) was added. After 36 h, the supernatants were removed and clariﬁed by centrifugation. An S-protein column was poured with 15 ml of S-protein agarose (Novagen, Inc., San Diego, CA) in an XK26 column (Amersham Pharmacia Biotech, Piscataway, NJ), and the column was washed with 10 bed volumes of phosphate-buffered saline (PBS). The supernatant from vKB16-infected cells was passed over S-protein agarose, and the column was washed with 10 bed volumes of PBS. The sGtag was eluted with 1 bed volume of 0.2 M citrate, pH 2, into 20 ml of 1 M Tris, pH 8. The eluate was then concentrated using 30-kDa Centricon centrifugal ﬁlter units (Millipore, Billerica, MA). Protein concentrations were determined by sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE), Coomassie brilliant blue R-250 staining, and densitometry analysis with NIH Image 1.62 software by comparison to a standard amount of puriﬁed sGtag previously analyzed by quantitative amino acid analysis.

Molecular weight determinations. A Superdex 200 gel ﬁltration column 10/300 (Amersham Pharmacia Biotech, Piscataway, NJ) was purchased. Void volume (Vo) was determined using blue dextran (1 mg/ml) in degassed PBS, pH 7.4 (Quality Biologicals, Inc., Gaithersburg, MD). A panel of high-molecular-weight proteins (Amersham Pharmacia Biotech) was used to calibrate the column. Each protein marker, and their elution volumes (Ve) were determined. Kav values for fractions containing sGtag were calculated, and the molecular weights were determined. Fractions containing sGtag as determined by UV absorption was scanned by SDS-PAGE and Coomassie G-250 staining.

Cell fusio assays. Fusion between envelope glycoprotein-expressing and target cells was measured by a reporter gene assay in which the cytoplasm of one cell population contained vaccinia virus-encoded T7 RNA polymerase and the cytoplasm of the other contained the E. coli lacZ gene linked to the T7 promoter. β-galactosidase (β-Gal) is synthesized only in fused cells (8, 29). Vaccinia virus-encoded proteins were produced by infecting cells at an MOI of 10 and incubating infected cells at 31°C overnight (4). Cell fusion reactions were conducted with the various cell mixtures in 96-well plates at 37°C. Typically, the ratio of envelope glycoprotein-expressing cells to target cells was 1:1 (2 × 10⁵ total cells per 0.2 ml total volume of virus stock used) for infection of all cell lines. The fusion reaction mixture to reduce nonspeciﬁc β-Gal production (4). Quantitative analyses, Nonidet P-40 was added (0.5%) ﬁnal) at 2.5 h, and aliquots of the lysates were assayed for β-Gal at ambient temperature with the substrate chlorophenol red-t-o-galactopyranoside (Roche Diagnostics Corp.). For inhibition
by sG preparations, serial dilutions of sG were performed and added to target cell populations 30 min prior to addition of effector cell populations. All assays were performed in duplicate, and fusion results were calculated and expressed as the ratio of β-Gal activity (change in optical density at 570 nm per minute × 1,000) (29).

**Metabolic labeling and immunoprecipitation.** For [35S]methionine labeling of HeV glycoproteins expressed by recombinant vaccinia viruses, HeLa cells were infected at an MOI of 10. At 6 h postinfection, monolayers were washed, overlaid with methionine- and cysteine-free minimal essential medium (Invitrogen, Corp.) containing 2.5% dialyzed fetal calf serum (Invitrogen, Corp.) and 100 μCi of [35S]methionine-cysteine (ProMix)/ml (Amersham Pharmacia Biotech, Pisca-taway, NJ), and incubated overnight. Supernatants were removed and clarified by centrifugation. Lysis of cells was performed in 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Triton X-100, and once with DOC buffer (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS). Proteins were separated by SDS-PAGE (10%) and visualized by autoradiography.

**Sucrose gradient analysis.** The oligomeric forms of soluble and full-length HeV G glycoproteins were analyzed by sucrose gradient centrifugation. Nonmetabolically labeled material was used for the analysis of HeV sGtag. The serum-free culture medium (12 ml of OptiMEM) containing sGtag, produced from 1 × 106 3BS-infected HeLa cell culture flasks (40 h at 37°C), was collected, clarified by centrifugation, buffer-exchanged into PBS, and concentrated 40-fold using 30-KDA Centricron centrifugal filter units (Millipore, Bil-lerica, MA). The final volume of 1 ml, containing approximately 100 μg, was divided into two equal portions of 0.5 ml each. One portion of the sGtag was cross-linked with the reductive reagent 3,3′-dithiobis-(sulfosuccinimidylpropi- onate) (DTSSP; Pierce Biotechnology, Rockford, IL) at a final concentration of 4 mM for 30 min at room temperature. The reaction was then quenched with 100 μl PBS. One half (0.2 ml) of the cell suspension was cross-linked with 1 μl of DTSSP and incubated for 30 min at room temperature. The reaction was then quenched with 100 μM Tris, pH 7.5, for 15 min at room temperature. The cell surface cross-linked and un-cross-linked HeV G-expressing cells were lysed in Triton-X-containing buffer (100 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.25% 3BS-100) and metabolically labeled with [35S]methionine (Amersham Biosciences) for 18 h at 37°C. The culture was recovered by using cell dissociation buffer (Invitrogen Corp.), and resuspended in 0.4 ml PBS. One half (0.2 ml) of the cell suspension was cross-linked with 1 μl of DTSSP and incubated for 30 min at room temperature. The reaction was then quenched with 100 mM Tris, pH 7.5, for 15 min at room temperature. The cell surface cross-linked and un-cross-linked HeV G-expressing cells were lysed in Triton-X-containing buffer (100 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.25% 3BS-100) and metabolically labeled with [35S]methionine (Amersham Biosciences) for 18 h at 37°C. The culture was recovered by using cell dissociation buffer (Invitrogen Corp.), and resuspended in 0.4 ml PBS. One half (0.2 ml) of the cell suspension was cross-linked with 1 μl of DTSSP and incubated for 30 min at room temperature. The reaction was then quenched with 100 mM Tris, pH 7.5, for 15 min at room temperature. The cell surface cross-linked and un-cross-linked HeV G-expressing cells were lysed in Triton-X-containing buffer (100 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.25% 3BS-100) and metabolically labeled with [35S]methionine (Amersham Biosciences) for 18 h at 37°C. The culture was recovered by using cell dissociation buffer (Invitrogen Corp.), and resuspended in 0.4 ml PBS. One half (0.2 ml) of the cell suspension was cross-linked with 1 μl of DTSSP and incubated for 30 min at room temperature. The reaction was then quenched with 100 mM Tris, pH 7.5, for 15 min at room temperature. The cells were washed three times with PBS-Tween. Ten micrograms of sGtag, in 200 μl of IFA buffer was added to each well and incubated at 37°C for 1 h. The cells were washed three times with PBS-Tween. Ten micrograms of sGtag, in 200 μl of IFA buffer was added to each well and incubated at 37°C for 1 h. The cells were washed three times with PBS-Tween and allowed to air dry in the dark. The chambers were removed from the slides, and 90% glycerol–10% PBS-Tween was used as a mounting medium for the slide coverslips. Samples were examined with an Olympus BX50 system microscope with a BX-FLA reflected light fluores-cence attachment and an Olympus U-M4010 filter. All images were obtained at an original magnification of ×40 with a SPOT RT charge-coupled device digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and images were imported into Adobe Photoshop 7.0. Image size was adjusted individually, but brightness and contrast were only adjusted after all images had been merged.

**HeV and NiV infection and IFA.** All live virus experiments were conducted under strict biosafety procedures in a BSL-4 laboratory. S67-Infected Vero cell monolayers were seeded onto 6-well plates at 6 × 105 cells in 300 μl and grown to 90% confluence in EMEM-10 at 37°C under a humidified 5% CO2 atmosphere. For inhibition experiments, the sGtag was diluted twofold in EMEM-10 and monolayers were preincubated with various sGtag concentrations for 30 min prior to virus inoculation. The medium was discarded, and 100 μl of diluted virus combined with the same amount of sGtag used in the preincubation was added per well and cultured at 37°C for 1 h. After the wash, plates were washed twice with PBS–0.05% Tween 20 and the cells were infected with 100 μl of NiV for 1 h, incubated at 37°C for 1 h. The cells were washed three times with PBS-Tween. Twenty microliters of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit anti-serum (ICN Pharmaceuticals, Costa Mesa, CA) diluted 1:200 in PBS-BSA, containing 1% BSA for 5 min. After the wash, a secondary antibody, a goat anti-rabbit antibody (1:500 in PBS-BSA), was added for 30 min. After washing, an Alexa Fluor 488-conjugated donkey anti-rabbit immunoglobulin G (Fab)-specific antibodies (1:200; Molecular Probes, Eugene, OR) in 200 μl IFA buffer were added to each well and incubated at 37°C for 1 h. The cells were washed three times with PBS-Tween and allowed to air dry in the dark. The chambers were removed from the slides, and 90% glycerol–10% PBS-Tween was used as a mounting medium for the slide coverslips. Samples were examined with an Olympus BX50 system microscope with a BX-FLA reflected light fluores-ence attachment and an Olympus U-M4010 filter. All images were obtained at an original magnification of ×40 with a SPOT RT charge-coupled device digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and images were imported into Adobe Photoshop 7.0. Image size was adjusted individually, but brightness and contrast were only adjusted after all images had been merged.

**HeV and NiV neutralization assay.** All live virus experiments were conducted under strict biosafety procedures in a BSL-4 laboratory. S67-Infected Vero cell monolayers were seeded onto 6-well plates at 6 × 105 cells in 300 μl and grown to 90% confluence in EMEM-10 at 37°C under a humidified 5% CO2 atmosphere. For inhibition experiments, the sGtag was diluted twofold in EMEM-10 and monolayers were preincubated with various sGtag concentrations for 30 min prior to virus inoculation. The medium was discarded, and 100 μl of diluted virus combined with the same amount of sGtag used in the preincubation was added per well and cultured at 37°C for 1 h. After the wash, plates were washed twice with PBS–0.05% Tween 20 and the cells were infected with 100 μl of NiV for 1 h, incubated at 37°C for 1 h. The cells were washed three times with PBS-Tween. Twenty microliters of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit anti-serum (ICN Pharmaceuticals, Costa Mesa, CA) diluted 1:200 in PBS-BSA, containing 1% BSA for 5 min. After the wash, a secondary antibody, a goat anti-rabbit antibody (1:500 in PBS-BSA), was added for 30 min. After washing, an Alexa Fluor 488-conjugated donkey anti-rabbit immunoglobulin G (Fab)-specific antibodies (1:200; Molecular Probes, Eugene, OR) in 200 μl IFA buffer were added to each well and incubated at 37°C for 1 h. The cells were washed three times with PBS-Tween and allowed to air dry in the dark. The chambers were removed from the slides, and 90% glycerol–10% PBS-Tween was used as a mounting medium for the slide coverslips. Samples were examined with an Olympus BX50 system microscope with a BX-FLA reflected light fluores-ence attachment and an Olympus U-M4010 filter. All images were obtained at an original magnification of ×40 with a SPOT RT charge-coupled device digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and images were imported into Adobe Photoshop 7.0. Image size was adjusted individually, but brightness and contrast were only adjusted after all images had been merged.

**FTTC immunofluorescence was visualized using an Olympus IX71 inverted microscope (Olympus Australia, Mt. Waverley, Australia) coupled to an Olym-pus DP70 high-resolution color camera, and all images were obtained at an original magnification of ×85. Image analysis was then performed using Analy-sis image analysis software (Soft Imaging System GmbH, Munster, Germany). Briefly, individual virus syncytia were detected by threshold analysis followed by “hole filling” and subsequently measured to determine the area of each syncy-ium. To ensure repeatability between images, all procedures were performed as a macro function with fixed parameters. Nine images were analyzed for each sGtag concentration, resulting in the collation of syncytial area data for between 9 and 79 foci per sGtag concentration (average, ~15). Measurements were collated, and nonlinear regression analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA) to determine the 50% inhibitory concentration (IC50).
first inoculation contained 50 μg of sGS-tag in CSIRO triple adjuvant (60% [vol/vol] Montanide, 40% [vol/vol] S0S-tag combined with Quil A, 3 mg/ml, and DEAE-dextran, 30 mg/ml) in water), in a total volume of 200 μl injected in two intramuscular sites. The second and third inoculations contained 25 μg of sGS-tag in CSIRO triple adjuvant in a total volume of 200 μl injected in two intramuscular sites. All injections were done 3 weeks apart. Sera were collected 2 weeks after the third inoculation. For both rabbits, prebleeds were also collected prior to inoculation. Sera were diluted in EMEM-10 by doubling dilution starting at 1:10. Fifty microliters of sera was added to wells in duplicate in a 96-well plate to inoculation. Sera were diluted in EMEM-10 by doubling dilution starting at 1:10, Fifty microliters of sera was added to wells in duplicate in a 96-well plate followed by 50 μl virus containing 200 TCID50 of either HeV or NiV and incubated at 37°C for 30 min. A total of 2 × 10^6 Vero cells were infected with WR (a control vaccinia virus), sGS-tag, sGmyc-tag, or full-length HeV G-encoding vaccinia viruses. The various preparations were affinity precipitated with either virus-specific antiserum, anti-myc MAb, or S protein agarose as indicated in the figure. The anti-HeV antiserum and either the anti-myc MAb or the S-protein agarose were able to precipitate sGmyc-tag and sGS-tag, respectively, from both the lysates and the supernatants from the appropriate expressing cells. The cell lysate precipitates of sGS-tag and sGmyc-tag had apparent molecular masses of ~80 kDa, similar to that seen with full-length G (7). The sGS-tag and sGmyc-tag differ from full-length HeV G by 5 and 10 amino acid residues, respectively; thus, there was not a significant shift in the electrophoretic mobility of these sG glycoproteins. The sGS-tag and sGmyc-tag glycoproteins precipitated from the cell culture supernatants had apparent molecular weights slightly larger than those of the sG glycoproteins recovered in the cell lysates or full-length HeV G. We speculate that this could be due to a putative N-linked glycosylation site located at the beginning of the protein’s predicted ectodomain that may not be available for posttranslational modification in full-length membrane-anchored G because of its proximity to the TM domain. Previous studies have demonstrated that removal of this potential N-linked glycosylation site by mutagenesis had no effect on expression or function of full-length HeV G or on its apparent molecular weight (K. N. Bossart and C. C. Broder, unpublished observations). These observations suggest that this site is not normally modified, but in the context of the soluble form of the protein with TM deleted, it may be accessible, and such alteration is apparent when comparing sG that has been released from cells to the G glycoprotein still present within the cell. However, since both epitope-tagged constructs were efficiently secreted and

![Diagram](https://via.placeholder.com/150)

**FIG. 1.** HeV sG constructs. The HeV coding sequence with the transmembrane-cytoplasmic tail deleted was generated by PCR and cloned in frame into the pSecTag2B vector, which contained the Ig k leader sequence. The modified epitope-tagged pSecTag2B vectors were subsequently generated using overlapping oligonucleotides and cloned in frame into pSecTag2B-HeV sG. The figure shows the S-peptide-tagged and c-myc-tagged versions of HeV sG. The linker amino acids were derived from vector sequences.
precipitated with whole virus-specific antisera, we presumed that the addition of the Ig κ leader and the epitope tag did not appear to significantly alter the native structure of G. Moreover, the S-protein agarose affinity precipitations demonstrated high specificity with little nonspecific binding of proteins detected in the control WR or full-length HeV G samples. There was also more sGmyc-tag recovered in comparison to sGmyc-tag-encoding viruses, or a recombinant vaccinia virus encoding HeV G and incubated 16 h at 37°C. Beginning at 6 h postinfection, the cells were labeled overnight with [35S]methionine-cysteine. Supernatants were removed and clarified by centrifugation. Lysates were precipitated for both the unreduced and reduced SDS-PAGE analysis, whereas for fraction 39, 0.5 ml was precipitated using centrifugal filter units. Approximately 100 µg was divided in two equal portions of 0.5 ml each. One portion was cross-linked with the reducible reagent DTSSP at a final concentration of 4 mM, and each of the sG preparations was overlaid and boiled in SDS sample buffer, one set with and one set without β-mercaptoethanol, and all four sets of fractions were then analyzed by 4-to-12% gradient SDS-PAGE. Figure

Characterization of soluble HeV G. We next sought to determine if the secreted sG was oligomeric in nature. The apparent molecular weight of purified sG material was first examined using size exclusion chromatography with a calibrated Superdex 200 analytical grade column 10/300. A 500-µg aliquot of affinity-purified sG was passed over the Superdex 200, and fractions were collected using the same methods employed for the molecular weight standards. The locations of the protein standards and the elution profile of purified sG are shown in Fig. 3A. Figure 3B shows the analysis of the sG from several fractions across the elution profile separated by 3-to-8% gradient SDS-PAGE under both reducing and nonreducing conditions. These results demonstrate tetrameric, dimeric, and monomeric species of sG. In addition, we note that the majority of sG comprising the bulk of both the first and second peak zones appears as an SDS-stable dimer, which can be seen in fractions 42, 45, and 48. Further, in order to visualize each of the sG species with comparative intensities by staining (Fig. 4), the dimer elutes with an estimated molecular mass of ~542 ± 20 kDa. A similar elution profile is observed with lectin affinity-purified sG when separated on the Superdex 200 column (not shown). However, from prior experience in the preparation and analysis of engineered soluble virus-derived membrane glycoproteins, such as gp120 from human immunodeficiency virus type 1, the molecular mass calculations derived from size exclusion chromatography analysis are likely overestimated. Specifically, the human immunodeficiency virus type 1 gp120 glycoproteins (~120 kDa in SDS-PAGE) from multiple isolates have apparent molecular masses of >210 kDa when separated on Superdex 200 and are generally in agreement with data from other groups (11, 41). Not surprisingly, the apparent molecular masses of the sG species derived by size exclusion chromatography are about twice those observed by SDS-PAGE. Nevertheless, these results clearly demonstrate that the majority of the soluble and secreted G glycoprotein is oligomeric and appears to be a dimer.

To further characterize the oligomeric species of sG, we separated purified sG using sucrose gradient centrifugation. Figure 4 depicts the oligomeric profiles of sG on 5-to-20% continuous sucrose gradients. Nonmetabolically labeled sG was harvested from cultures of HeLa cells expressing the protein under serum-free conditions as detailed in Materials and Methods. The supernatant was clarified by centrifugation, buffer exchanged into PBS, and concentrated 40-fold using centrifugal filter units. Approximately 100 µg was divided in two equal portions of 0.5 ml each. One portion was cross-linked with the reducible reagent DTSSP at a final concentration of 4 mM, and each of the sG preparations was overlaid and separated on 5-to-20% continuous sucrose gradients. After fractionation of each gradient, the fractions were split into two tubes, precipitated with S-protein–agarose, washed, resuspended, and boiled in SDS sample buffer, one set with and one set without β-mercaptoethanol, and all four sets of fractions were then analyzed by 4-to-12% gradient SDS-PAGE.
4A and B are un-cross-linked sG S-tag separated on the sucrose gradient, fractionated, and affinity precipitated. In Fig. 4A the fractions were prepared in the absence of β-mercaptoethanol, whereas in Fig. 4B the fractions were prepared under reducing conditions with β-mercaptoethanol. Figure 4C and D show cross-linked sG S-tag separated in a parallel sucrose gradient, fractionated, and affinity precipitated. In Fig. 4C the cross-linked sG S-tag fractions were prepared in the absence of β-mercaptoethanol, whereas in Fig. 4D each fraction was prepared with β-mercaptoethanol and resolved on the gradient gels. From the data shown in Fig. 4A and C we can determine that for both the un-cross-linked and cross-linked sG S-tag there are
three distinct species of sG present. Based on the apparent molecular weights of sG in each of the fractions across each of the gradients, these three species represent monomer, dimer, and most likely tetramer. In addition, the immediate cross-linking of the sG with DTSSP prior to sucrose gradient centrifugation did not significantly alter its oligomeric profile and revealed that only a small portion of tetrameric sG is an SDS-sensitive dimer of dimers. The comparative analysis of the non-cross-linked, unreduced, and reduced sG indicates that the sG dimer is disulfide linked, which we anticipated based on data derived from several other paramyxovirus attachment glycoproteins (24, 27). The dimers of other paramyxovirus attachment glycoproteins have also been shown to exist as tetramers and dimers on the surface of infected cells, and it is generally believed that the native oligomeric structure of the paramyxovirus attachment glycoprotein is a dimer of dimers. In addition, the tetrameric species sG we observed also appears to be disulfide linked and is stable when boiled in SDS sample buffer. To contrast these observations on sG to the native full-length G glycoprotein, we expressed and metabolically labeled full-length HeV G in HeLa cells (a receptor-negative cell line) and performed a similar cross-linking experiment and sucrose gradient analysis. Following the cross-linking procedure, or no treatment, of surface-expressed HeV G on intact cells, the cells were lysed with nonionic detergent, lysates were clarified by centrifugation, and each preparation was analyzed by sucrose gradient centrifugation. Fractions were immunoprecipitated with a polyclonal anti-HeV G mouse serum followed by protein G-Sepharose and resolved on 3-to-8% gradient SDS-PAGE under reducing and nonreducing conditions. Shown in Fig. 5 are the results of sucrose gradient analysis of surface-expressed un-cross-linked full-length metabolically labeled HeV G. Here we observed that in the non-reduced fractions, ~70% of the full-length HeV G exists as the apparent tetrameric species (Fig. 5A, lanes 2 to 5), and this oligomeric species is also clearly dependent on disulfide bonds as illustrated by the corresponding reduced fractions which are monomeric (Fig. 5B, lanes 2 to 5). Parallel sucrose gradient analysis of DTSSP (1 mM) cross-linked G resulted in the majority of the dimer migrating at ~160 kDa shifting to the tetramer position in the gradient gel (Fig. 5C). The calculation of the ratios of tetramer to dimer in the un-reduced samples in these first 4 lanes of the gradient gel analysis of the cross-linked G shows a range of 2.2 to 2.8 (Fig. 5C), whereas those ratios in the un-cross-linked G in those lanes (Fig. 5A) range from 1.3 to 1.5. These results strongly suggest that the natural oligomeric form of G in the membrane is a tetramer or dimer of dimers and because of the stability of some of the non-cross-li...
linked tetramer complexes when boiled and analyzed by SDS-PAGE under nonreducing conditions, suggests that there may be disulfide bonds linking them together. Repeated experiments using higher amounts of DTSSP (3 and 4 mM) resulted in higher molecular weight aggregates which could not be satisfactorily resolved in the gel unless treated with reducing reagent. In contrast, depending on the particular preparation, the majority (80 to 90%) of the engineered sGS-tag glycoprotein released from expressing cells is oligomeric, of which ~95% is dimer and ~5% tetramer, and suggests that it may retain important and useful native structural features.

**Immunofluorescence staining of receptor-positive cells by soluble HeV G.** Previously, we identified putative receptor-positive and receptor-negative cell types based on their ability to support HeV- and NiV-mediated fusion (6, 7). HeLa cells were the first putative receptor-negative cell line identified, and our data were further confirmed by the resistance to infection by HeV and NiV (unpublished data). Of all the cell lines examined to date, human U373 and PCI 13 have exhibited the highest levels of both HeV- and NiV-mediated fusion and may have a corresponding high level of surface-expressed viral receptor. Vero cells used to grow and maintain HeV and NiV stocks are also fusion permissive. Since G mediates binding to the host cell, we used our sGS-tag construct in an adapted indirect IFA to show specific binding to receptor-positive cells, the results of which are shown in Fig. 6. Cells were cultivated in Lab-Tek II chamber slides for 3 days and then briefly fixed with acetone. The cells were stained as described in Materials and Methods. The anti-HeV G peptide-specific rabbit serum was used because it does not block HeV-mediated fusion (data not shown) and, therefore, should not interfere with the interaction of sGS-tag and its putative receptor. In Fig. 6A, the anti-HeV G peptide-specific rabbit antiserum was omitted from each of the cell types stained to show any nonspecific
staining of the cells by sGS-tag or the fluorescent conjugate. In Fig. 6B each of the cell types was stained with sGS-tag the anti-HeV G peptide-specific rabbit antiserum and the anti-rabbit conjugate. Clearly, sGS-tag bound to the receptor-positive cells, U373, PCI 13 and Vero, whereas the HeLa cells did not show any positive staining. These data strongly suggest that the sGS-tag can specifically bind to its host cell receptor and that the HeLa cell line we routinely employ in our studies does not express a functional receptor.

Soluble HeV G blocks virus-mediated cell fusion. HeV- and NiV-mediated fusion have identical cell tropism characteristics, suggesting that these viruses use the same host cell receptor (7). Since sGS-tag could bind the viral receptor, we hypothesized that sG should therefore be capable of blocking HeV and NiV cell fusion. Using our HeV and NiV cell fusion reporter gene assay, we assessed the inhibitory activity of sG. Figure 7 shows the quantitation of cell fusion reactions mediated by HeV and NiV glycoproteins in the presence of sGS-tag.

All supernatants or purified sG were added to target cells for 30 min at 37°C prior to the addition of effector cells. The data shown in Fig. 7A and B were generated using a sGS-tag supernatant and a WR-infected culture supernatant (control), which were collected and processed identically. The culture supernatants were diluted and added to either U373 (Fig. 7A) or PCI 13 (Fig. 7B) target cells. sGS-tag was capable of inhibiting HeV-mediated fusion in a dose-dependent manner, whereas the WR supernatant had no effect. In Fig. 7C and D, purified sGS-tag was tested for its ability to inhibit HeV- and NiV-mediated fusion in both U373 (Fig. 7C) and PCI 13 (Fig. 7D) cells, and it was capable of potently inhibiting HeV-mediated fusion in both cell lines. As expected, NiV-mediated fusion was also inhibited, providing further evidence of a shared cellular receptor. The IC_{50} of sG in these experiments ranged from 0.2 to 0.4 µg/ml with U373 cells and 0.8 to 3.0 µg/ml with PCI 13 cells for NiV and HeV infection, respectively. Like sGS-tag, lectin-purified sGS-myc-tag was also capable of inhibiting both HeV- and NiV-mediated fusion in a dose-dependent manner (data not shown).

Inhibition of HeV and NiV infection by soluble HeV G. We next evaluated the sGS-tag effects on live virus infection of Vero cells in culture. Here, following preincubation of Vero cells with various concentrations of sGS-tag, the cells were infected with 1.5 × 10^7 TCID_{50}/ml and 7.5 × 10^2 TCID_{50}/ml of live HeV or NiV, respectively, in the presence of sGS-tag for 30 min, followed by removal of the virus inoculum and incubation with sGS-tag. After 24 h in culture, the number of HeV and NiV infection foci was quantified by specific immunostaining of cell monolayers with a cross-reactive anti-phosphoprotein (P) antisem as detailed in Materials and Methods. Representative examples of infected Vero cells in the presence or absence of sGS-tag are shown in Fig. 8. Typically, infection of Vero cells with live HeV or NiV produces characteristic syncytium morphologies for each virus. Immunofluorescence for HeV P protein in HeV syncytia demonstrated that HeV reproducibly infects and incorporates surrounding cells into each syncytium, with cell nuclei and viral protein equally detectable throughout the majority of infected cells (Fig. 8A). NiV-infected cells initially showed a similar appearance to HeV syncytia, but ultimately incorporated nuclei within each giant cell were sequestered together towards the periphery while the remaining cellular debris was also arranged around the outside, leaving the central region largely empty. Thus, immunofluorescence for NiV P protein in NiV syncytia often appears as hollow spheres coated in viral antigen (Fig. 8B) and, by comparison, the untreated control HeV infections produce smaller syncytia relative to the untreated NiV control (Fig. 8A and B). Figure 8C and D are representative examples of HeV- and NiV-infected Vero cells in the presence of 100 µg/ml sGS-tag. Although there were still some infected cells present as detected by immunofluorescence, syncytium formation was completely blocked in both HeV- and NiV-infected cells (Fig. 8C and D, respectively). Furthermore, quantitative analysis of the inhibition of HeV and NiV infection by purified sGS-tag glycoprotein revealed a dose-dependent response, further demonstrating its specificity, as shown in Fig. 9. Together these data provide strong evidence that HeV and NiV utilize a common receptor on the surface of the host cell. Additionally, the specific inhibition of both viruses by sGS-tag further demonstrated that the
sGS-tag construct maintains important native structural elements. Interestingly, HeV infection was inhibited significantly better than NiV, such that the IC50 determined for sGS-tag was fourfold greater for NiV (13.20 μg/ml) than for HeV (3.3 μg/ml) (Fig. 9). Given the current evidence suggesting both viruses utilize a common receptor, the reasons for the differences observed in sGS-tag inhibition of virus infection versus cell fusion remain unknown. We did not observe a similar difference in the ability of sGS-tag to inhibit HeV- and NiV-mediated fusion, as demonstrated in Fig. 6. Although HeV-mediated fusion was more potent than NiV-mediated fusion, illustrated by the higher levels of substrate turnover, the sGS-tag IC50 in both cell fusion assays remained constant. In previous reports we have demonstrated through heterotypic function that the difference in cell fusion rates between HeV and NiV was dependent on the fusion protein. In this study, we demonstrated that natural NiV infection appears to be more vigorous than HeV infection. We can only speculate that other viral proteins present during infection are perhaps influencing the kinetics of infection, thus altering the inhibition susceptibility, or there may be differences in the affinities of HeV sG versus NiV G to the cell surface-expressed receptor.

Soluble HeV G elicits a potent virus-neutralizing polyclonal antibody response. With few exceptions, it is the envelope glycoproteins of viruses to which virtually all neutralizing antibodies are directed and all successful human viral vaccines induce neutralizing antibodies that can cross-react with immunologically relevant strains of a virus (34). More specifically, virus-neutralizing antibodies are the key vaccine-induced protective mechanism in the case of the paramyxoviruses mumps and measles (reviewed in references 20 and 30), and it has been shown that vaccinia virus-expressed full-length envelope glycoproteins from NiV can elicit virus-neutralizing antibodies (38). Our data indicate that the sGS-tag glycoprotein retains important structural features based on its abilities to specifically bind receptor-positive cells and block both HeV- and NiV-mediated fusion and infection. Thus, the immunization of animals with sG should potentially generate potent virus-neutralizing antibodies. To test this possibility, purified sGS-tag was used to immunize rabbits, and the resulting anti-G antiserum.
the presence of 100 infections; (B) untreated NiV control infections; (C) HeV infections in
rescence of anti-P-labeled HeV and NiV. (A) Untreated HeV control
tion of
color camera, and all images were obtained at an original magnifica-
inverted microscope coupled to an Olympus DP70 high-resolution
digital microscopy. Images were obtained using an Olympus IX71
fixed in methanol, and immunofluorescently labeled for P protein prior
to digital microscopy. Images were obtained using an Olympus IX71
and NiV-mediated fusion, suggesting that these closely related
viruses utilize the same host cell receptor for infection (6, 7). The
receptor for HeV and NiV is likely a specific cell surface-
expressed protein, and there are several lines of evidence to
support this notion. First, HeV does not contain any hemag-
glutinating activity (28) or neuraminidase activity (42). In ad-
dition, neuraminidase treatment of Vero cells (the cell line
used to propagate HeV and NiV stocks) does not inhibit HeV or
NiV infection or cell fusion, but it can abrogate their sus-
ceptibility to Newcastle disease virus and influenza virus A, two

was evaluated in virus neutralization assays with both HeV and
NiV. Table 1 summarizes the neutralization of HeV and NiV
infection by the polyclonal rabbit anti-G sera. The sera from
both rabbits were capable of complete neutralization of HeV
at a dilution of 1:1,280. NiV was also neutralized by the sG S-tag
antisera, with complete neutralization at a dilution of 1:640.
A twofold difference in titer is consistent with partial antibody
cross-reactivity of the HeV and NiV G glycoproteins. Pre-
bleeds from both rabbits were also tested for their abilities to
neutralize HeV and NiV. Although there was slight neutral-
ization at the highest concentration, this activity was com-
pletely abrogated upon dilution of the sera (data not shown).
Previous studies have demonstrated that HeV and NiV anti-
sera do cross-neutralize, with each serum being slightly less
effective against the heterotypic virus (13, 38). Moreover, in
previous work we demonstrated a similar trend in cross-neu-
tralization in a cell fusion assay for HeV and NiV (6, 7).
Because sG S-tag is able to elicit such a potent immune response
with high levels of neutralizing antibodies, it may provide an
avenue for vaccine development strategies.

**DISCUSSION**

Previously, we examined the functional activities of the fu-
sion and attachment envelope glycoproteins of HeV and NiV
by using a vaccinia virus-based expression system and a quan-
titative assay for measuring membrane fusion. These studies
revealed the same pattern of host-cell tropism for both HeV-

![FIG. 8. Immunofluorescence-based syncytium assay of HeV and NiV. Vero cells were plated into 96-well plates and grown to 90% confluen
ce. Cells were pretreated with sG S-tag for 30 min at 37°C prior to
fection with 1.5 × 10^3 TCID_{50}/ml and 7.5 × 10^2 TCID_{50}/ml of live
HeV or NiV (combined with sG S-tag). Cells were incubated for 24 h,
in fixed methanol, and immunofluorescently labeled for P protein prior
to digital microscopy. Images were obtained using an Olympus IX71
inverted microscope coupled to an Olympus DP70 high-resolution
color camera, and all images were obtained at an original magnification
of 85. Shown are representative images of FITC immunofluores-
ence of anti-P-labeled HeV and NiV. (A) Untreated HeV control
fections; (B) untreated NiV control infections; (C) HeV infections in
the presence of 100 μg/ml sG S-tag, (D) NiV infections in the presence
of 100 μg/ml sG S-tag.](image)

![FIG. 9. Inhibition of HeV and NiV infection by sG. Vero cells were
plated into 96-well plates and grown to 90% confluence. Cells were
pretreated with sG S-tag for 30 min at 37°C prior to infection with 1.5 ×
10^3 TCID_{50}/ml and 7.5 × 10^2 TCID_{50}/ml of live HeV or NiV (com-
combined with sG S-tag). Cells were incubated for 24 h, fixed in methanol,
and immunofluorescently labeled for P protein prior to digital micros-
copy and image analysis to determine the relative area of each synec-
tium (see Materials and Methods). The figure shows the relative syn-
cytial area (pixels squared) versus sG S-tag concentration for HeV
(circles) and NiV (triangles).](image)

**TABLE 1. Neutralization of HeV and NiV infection**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>HeV</th>
<th>NiV</th>
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*Anti-HeV G antisera were generated in rabbits by three inoculations with
purified sG S-tag. Sera collected 2 weeks after the third injection were analyzed in
a virus neutralization assay against HeV and NiV. Serum neutralization titers (in
duplicate) were determined by the presence of CPE (indicated by +) and re-
corded as the serum dilution where at least one of the duplicate wells showed
CPE. –, no CPE.
viruses which employ sialic acid as receptors (16). Also, certain cell lines from the same species, most notably human cell lines, can be clearly positive or negative for fusion, and protease treatment prevents fusion of an otherwise permissive target cell (6, 7, 16). To further characterize the HeV attachment G glycoprotein and its unknown host cell receptor, we have constructed two soluble forms of the protein (sG).

The first description of an engineered soluble and secreted type II membrane glycoprotein from a virus was derived by a novel strategy whereby the signal sequence and membrane anchor domain elements of the influenza virus neuraminidase glycoprotein were replaced with the hydrophobic amino-terminal domain of the F1 subunit of the SV5 F glycoprotein, which could serve as a signal peptide (32). Using a similar strategy, the HN glycoprotein of SV5, another type II membrane glycoprotein, was successfully engineered into a soluble and secreted product (31). Aside from HeV and NiV, the only other paramyxovirus possessing a G envelope glycoprotein as its attachment glycoprotein is respiratory syncytial virus (RSV) (reviewed in reference 21). However, the HeV and NiV G glycoproteins are quite distinct from RSV G, lacking homology and, at 604 and 602 amino acids, respectively, double the length of RSV G (reviewed in references 22 and 40). It has been previously observed that RSV can produce both a membrane-associated and a secreted truncated form of its attachment G glycoprotein, where alternative methionine start sites are utilized (25, 35). Our initial attempts to design a soluble HeV G construct implemented either a truncation strategy of the molecule’s N terminus or a sequential deletion strategy through the TM based on the observations of soluble RSV G, but these attempts proved unsuccessful. Here we devised a mutagenesis strategy whereby the protein’s predicted signal sequence, cytoplasmic tail, and transmembrane domains were replaced with an Ig κ leader signal sequence coupled to either an S-peptide tag (sGS-tag) or the myc epitope tag (sGmyc) to facilitate purification and detection.

These constructs were efficiently expressed, and the sG glycoprotein products could be readily purified from the culture supernatants of cells infected with an sG-encoding vaccinia virus. Although both tagged versions appeared to be expressed equally well, were oligomeric, were recognized by polyclonal anti-HeV antiserum, and could block HeV and NiV cell fusion, we found that the sGS-tag version was more readily expressed and purified to higher levels in a cost-effective manner using S-protein affinity matrices than the myc-tagged version of sG. Analysis of purified sG by fractionation using sucrose gradient centrifugation and analysis by gradient SDS-PAGE under both reducing and nonreducing conditions revealed a monomeric species of ~380 kDa. Based on the stability of both the full-length and soluble HeV G dimer and tetrameric species in boiled SDS sample buffer that is observed using SDS-PAGE analysis under nonreducing conditions and in the absence of cross-linker, we conclude that the dimer and some tetramer species are disulfide linked.

The retention of the protein’s oligomeric characteristics suggested that the purified sG glycoprotein also retained important biological characteristics as well. Indeed, the sG glycoprotein could specifically bind to the surfaces of cell fusion-permissive cell types, whereas there was little evidence of binding to fusion-nonpermissive cell types, most notably the HeLa cell line clone used here. Presumably, these binding data are a direct reflection of surface-expressed HeV receptor. These observations were further supported by the ability of the sG glycoprotein preparations to potently block both HeV- and NiV-mediated cell fusion. The mechanism of this inhibition is likely due to occupancy of virus receptor sites on the target cells and further supports the likelihood that both HeV and NiV utilize the same receptor. We confirmed these observations through an examination of the glycoprotein’s ability to block infectious virus entry, and we found that it was capable of blocking both HeV and NiV infection of Vero cells. Whether there could be differences in the ability of a dimeric sG versus its monomeric form in the ability to presumably bind cell-surface receptor and block virus-mediated fusion is presently unknown, but it could be that a dimeric form would have greater potency though avidity.

Finally, the sG glycoprotein could serve as a potential subunit vaccine, and we also demonstrated here that administration of HeV sG to rabbits is capable of eliciting a potent and cross-reactive virus-neutralizing antibody response against both HeV and NiV. Taken together, the sG glycoprotein derived from HeV appears to retain several important functional and biochemical characteristics and will facilitate structural studies on this important Henipavirus membrane glycoprotein. The optimization of such a soluble glycoprotein strategy to henipavirus vaccine development could prove highly successful as one means of combating these emerging viral threats.

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