Human Metapneumovirus (HMPV) Binding and Infection Are Mediated by Interactions between the HMPV Fusion Protein and Heparan Sulfate

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Human metapneumovirus (HMPV) is a major worldwide respiratory pathogen that causes acute upper and lower respiratory tract disease. The mechanism by which this virus recognizes and gains access to its target cell is still largely unknown. In this study, we addressed the initial steps in virus binding and infection and found that the first binding partner for HMPV is heparan sulfate (HS). While wild-type CHO-K1 cells are permissive to HMPV infection, mutant cell lines lacking the ability to synthesize glycosaminoglycans (GAGs), specifically, heparan sulfate proteoglycans (HSPGs), were resistant to binding and infection by HMPV. The permissiveness to HMPV infection was also abolished when CHO-K1 cells were treated with heparinases. Importantly, using recombinant HMPV lacking both the G and small hydrophobic (SH) proteins, we report that this first virus-cell binding interaction is driven primarily by the fusion protein (HMPV F) and that this interaction is needed to establish a productive infection. Finally, HMPV binding to cells did not require β 1 integrin expression, and RGD-mediated interactions were not essential in promoting HMPV F-mediated cell-to-cell membrane fusion. Cells lacking β 1 integrin, however, were less permissive to HMPV infection, indicating that while β 1 integrins play an important role in promoting HMPV infection, the interaction between integrins and HMPV occurs after the initial binding of HMPV F to heparan sulfate proteoglycans.

Human metapneumovirus (HMPV) is a major worldwide respiratory pathogen first isolated in 2001 from children with respiratory syncytial virus (RSV)-like infection symptoms (67). Several studies have since confirmed the importance of HMPV, generally placing it as the second or third most common cause of severe acute upper and lower respiratory tract disease in children. Though children and infants, the elderly, people with underlying cardiopulmonary conditions, and immunocompromised individuals are more susceptible to severe disease from this virus, HMPV affects people in all age groups (reviewed in reference 45). Seroprevalence studies have shown that most individuals have been exposed to this virus by the age of 5 years, though reinfections with this virus are frequent (67). HMPV infection results in a range of disease severities from mild cold-like symptoms to bronchiolitis, pneumonia, and febrile seizures and can potentially lead to death (28, 45).

Most paramyxoviruses express two major surface glycoproteins: an attachment protein and a fusion (F) protein. Some paramyxoviruses, including HMPV, express an additional putative membrane-spanning protein: the small hydrophobic (SH) protein (33). For a paramyxovirus to infect a cell, the virus must attach to a cellular receptor, usually through the attachment protein, and then fuse the viral and cellular membranes, a process driven by the F protein (33). Paramyxovirus F proteins are synthesized as a precursor (F_0) form which is then proteolytically cleaved to the fusogenically active F_1 - F_2 form (33). For HMPV, this cleavage is accomplished by an exogenous protease (53, 54). This proteolytic cleavage primes the F protein for triggering, which, for some clades of HMPV, is driven by low pH (27, 53). There is no evidence that the SH protein plays a role in viral entry. In fact, HMPV SH protein is dispensable for virus growth *in vitro* and *in vivo* (4).

The paramyxovirus attachment protein is a type II integral

membrane protein called either HN, H, or G (33). Paramyxoviruses with a G protein do not bind to sialic acid but instead bind to cellular factors such as ephrin B2 for the henipaviruses (7, 41). Members of the *Pneumovirinae* subfamily express a functionally different G protein which has been shown to interact with cell surface proteoglycans in the case of RSV and HMPV (31, 65). Although it has been shown that most paramyxoviruses require the attachment protein for binding and infection, a role for HMPV G protein in receptor binding has not been confirmed.

Interestingly, while the attachment protein is essential for virus attachment and subsequent membrane fusion in the *Paramyxovirinae* subfamily, studies have shown that some members of the *Pneumovirinae* subfamily can be infectious in the absence of the attachment protein. RSV lacking G (Δ G) can be propagated *in vitro* but could not replicate efficiently *in vivo* (21, 63), and bovine respiratory syncytial virus (BRSV) lacking G can still infect its host (51). Similarly, a recombinant avian metapneumovirus (AMPV), the closest relative of HMPV, lacking the SH and G proteins (Δ SH/ Δ G) was able to grow, albeit slower than wild-type AMPV, in cell culture (40). Studies from our laboratory and others indicate that the G protein of HMPV is also dispensable for attachment and fusion, as cell-cell fusion can be accomplished in the absence of G and recombinant HMPV particles lacking G are in-

Received 1 November 2011 Accepted 23 December 2011

Published ahead of print 11 January 2012

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doi:10.1128/JVI.06706-11

fectious *in vitro* (53). Furthermore, a mutant virus devoid of the G protein can efficiently infect African green monkeys (5), suggesting that the F protein of HMPV is capable of performing both the attachment and fusion steps *in vivo*. Indeed, the HMPV F protein has been shown to be an important marker of human infection (47).

Most cells express a large number of different surface carbohydrates that mediate multiple functions such as adhesion, growth, and signaling (49). The glycan synthesis of glycoproteins is based on two main processes: N-glycosylation, which involves an addition of N-acetylglucosamine (GlcNAc) to the nitrogen atom of an asparagine residue, or O-glycosylation, which is the addition of N-acetylgalactosamine (GalNAc) or xylose to the oxygen atom of a serine or a threonine residue. GalNAc addition leads to the synthesis of mucins, whereas xylose addition leads to the synthesis of glycosaminoglycans (GAGs). GAGs can be classified into six different groups on the basis of their composition: chondroitin sulfate, heparan sulfate (HS), dermatan sulfate, keratan sulfate, hyaluran, and heparin (3, 48, 60).

HS is the most often implicated GAG in virus-cell interactions. HS has been described to be a receptor for multiple herpesviruses and is also necessary during the first step of adhesion of other viruses such as HIV and influenza virus (reviewed in reference 56). HS plays an important role in the initial steps of entry of RSV, the human pathogen most closely related to HMPV (8, 24, 25, 31). This interaction, however, appears to be largely mediated by RSV G, and viruses lacking G show a significant decrease in binding to Chinese hamster ovary (CHO-K1) cells (63). A recent study showed that the HMPV G protein also has the ability to bind GAGs and suggested that this interaction might be a significant factor for virus-cell interactions (65).

Integrins are extracellular matrix binding proteins expressed in nearly all cell types and play multiple roles, including the regulation of cell adhesion, tissue growth, and migration (reviewed in reference 2). These heterodimers, composed of at least 18 different α and 10 different β subunits, bind to a myriad of proteins, including HS proteoglycans (HSPGs), and many proteins that express the amino acid sequence Arg-Gly-Asp (RGD) (50). Due to their broad distribution on the surfaces of many cell types, several integrins have been implicated in viral entry. Integrins are part of the receptor complex of some picornaviruses, hantaviruses, and papillomaviruses and are also involved in adeno-associated virus, Ebola virus, and reovirus entry, though not as cellular receptors (16, 38, 52, 59). Furthermore, it has recently been reported that the $\alpha\beta 1$ integrin plays a major role in promoting HMPV infection (15).

In this study, we investigated the role of GAGs and the integrin $\alpha\beta 1$ in HMPV infection using wild-type HMPV, a mutant virus lacking the G protein, and a mutant virus devoid of G and SH (5). We show that the $\alpha\beta 1$ integrin is not essential for HMPV F-mediated fusion or for the initial binding step. Rather, our data indicate that integrins promote infection at a step downstream of the initial binding. Additionally, we demonstrate that the G and SH proteins do not play a major role in viral attachment, making F the main viral determinant for HMPV binding. Finally, we show that HMPV F interacts specifically with heparan sulfate and that HS is an indispensable component of the HMPV binding receptor complex.

MATERIALS AND METHODS

Cell lines. Vero cells, BSR cells (provided by Karl-Klaus Conzelmann, Max Pettenkofer Institut) (9), and GD25 and $\beta 1$ GD25 cells (provided by Deane Mosher, University of Wisconsin, Madison, WI) (68) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S). CHO-K1, pgsA745 (20), and Lec1 (58) cells, which were obtained from ATCC, IdIDLec1 cells (19, 57), which were derived from IdID cells (30) (provided by Craig Vander Kooi, University of Kentucky), and pgsD677 cells (35) (provided by Jeff Esko, University of California, San Diego, CA) were grown in HyClone Ham's F-12, Kaighn's modification medium (Thermo Scientific, Waltham, MA) supplemented with 10% FBS and 1% P/S. BSR cells were selected for T7 polymerase-expressing cells every third passage with 0.5 mg/ml G-418 sulfate (Gibco Invitrogen, Carlsbad, CA). The medium of $\beta 1$ GD25 cells was supplemented with 10 μ g/ml puromycin (Gibco Invitrogen, Carlsbad, CA) every third passage to select for the $\beta 1$ -expressing cells.

Plasmids, mutagenesis, and antibodies. The HMPV F gene within the pGEM-3Zf(+) vector was constructed with permission from Guy Boivin (Centre Hospitalier Universitaire de Québec, Québec City, Québec, Canada). The HMPV F D331A (F RGA) protein mutant was created using QuikChange site-directed mutagenesis (Stratagene). The HMPV F wild-type (WT) and D331A genes were released from pGEM-3Zf(+), ligated into the pCAGGS mammalian expression vector, and sequenced in their entirety. Antipeptide antibodies against HMPV F (Genemed Synthesis, San Francisco, CA) were generated using amino acids 524 to 538 of HMPV F. 9EG7 anti-mouse $\beta 1$ integrin was purchased from BD-Pharmingen (San Diego, CA). MAB2021Z anti- αv integrin was purchased from Millipore (Billerica, MA). NBP1-21631 anti-HMPV N monoclonal antibody was purchased from Novus Biologicals (Littleton, CO). Goat anti-mouse DyLight649 secondary antibody was purchased from KPL Inc. (Gaithersburg, MD).

Viruses. Recombinant, green fluorescent protein (GFP)-expressing HMPV (rgHMPV) strain CAN97-83 (genotype group A2) and the mutant viruses HMPV Δ G and HMPV Δ G/ Δ SH with a codon-stabilized SH gene (4) were kindly provided by Peter L. Collins and Ursula J. Buchholz (NIAID, Bethesda, MD). Recombinant WT, Δ G, and Δ G/ Δ SH HMPVs were propagated in Vero cells (starting multiplicity of infection [MOI], 0.01 to 0.03) and incubated at 32°C with Opti-MEM, 200 mM L-glutamine, and 0.3 μ g/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin, replenished every day. On the seventh day, cells and medium were collected and frozen at -80°C . The medium was thawed at 37°C the next day and subjected to centrifugation at 2,500 $\times$ g for 20 min at 4°C on a Sorval RT7 tabletop centrifuge. The supernatant was then subjected to centrifugation on a 20% sucrose cushion for 2 h and 15 min at 27,000 $\times$ g and 4°C using a SW28 swinging-bucket rotor on a Beckman Optima L90-K ultracentrifuge. Following centrifugation, the supernatant was removed and the pellet was resuspended in 300 μ l of either 10% sucrose or Opti-MEM and left at 4°C overnight. Aliquots of the samples were made the next morning and stored at -80°C . Recombinant GFP-expressing parainfluenza virus 5 (PIV5) was kindly provided by Robert Lamb (Howard Hughes Medical Institute, Northwestern University) and Jessica Robach (Northwestern University). GFP was inserted between the P/V and M genes with a duplicate of the EIS (end/intergenic/start) sequence that precedes M. PIV5 was propagated in MDBK cells as described previously (46) and stored in 1 $\times$ SPG (218 mM sucrose, 0.0049 M L-glutamic acid, 0.0038 M KH_2PO_4 , 0.0072 M K_2HPO_4). Aliquots were frozen in a dry ice-methanol mix and thawed twice prior to storage at -80°C .

Virus titer calculation. Viral titers were calculated by creating serial dilutions of the viral samples on a 96-well plate and counting the number of GFP-expressing cells the following day. For non-GFP-expressing viruses (recombinant HMPV [rHMPV]), a control rgHMPV was used as a reference. Five hundred thousand Vero cells plated the day before were infected with the nonfluorescent virus and control rgHMPV by serial

dilution. Infected cells were suspended the following day, fixed in 4% formaldehyde, permeabilized in 0.02% Triton X-100 at 4°C, and labeled with 70 μ l of anti-HMPV N antibody for 90 min, after resuspending the cells in 70 μ l FBS. Samples were analyzed by fluorescence-activated cell sorting (FACS) following incubation with a DyLight649 reagent-conjugated goat anti-mouse secondary antibody. The ratio of rgHMPV to rgHMPV at the same dilution was used to calculate the titer for the non-GFP-expressing HMPV.

Expression, metabolic labeling, and biotinylation of surface proteins. Cells in 60-millimeter dishes were transfected with 1.10 μ g pCAGGS-HMPV F wild type or mutants or empty pCAGGS as a control using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's protocol. At 18 to 24 h posttransfection, cells were starved in methionine- and cysteine-deficient DMEM for 1 h and then metabolically labeled with Tran³⁵S label (100 μ Ci/ml; Perkin Elmer) with 0.3 μ g/ml TPCK-trypsin for 4 h. Following radiolabeling, cells were washed three times with ice-cold phosphate-buffered saline (PBS; pH 8) and incubated with 1 mg/ml EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL) diluted in PBS (pH 8) for 30 min with rocking at 4°C and then for 20 min at room temperature. Cells were again washed three times with pH 8 PBS and then lysed in 1 ml radioimmunoprecipitation assay buffer containing 100 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% deoxycholic acid, protease inhibitors (1 Kallikrein inhibitory unit of aprotinin [Calbiochem, San Diego, CA], 1 mM phenylmethylsulfonyl fluoride [Sigma, St. Louis, MO]), and 25 mM iodoacetamide (Sigma). The lysates were subjected to centrifugation at $136,500 \times g$ for 10 min at 4°C, and supernatants were collected. Antipeptide sera and protein A-conjugated Sepharose beads (Amersham, Piscataway, NJ) were used to immunoprecipitate the F proteins as previously described (46). Immunoprecipitated protein was released from the beads by boiling using a total of 100 μ l of 10% SDS (40 μ l for first boil and 60 μ l for second boil). Fifteen percent of total protein collected was removed for total expression analysis, and the remaining 85% was diluted in 500 μ l biotinylation dilution buffer (20 mM Tris-HCl [pH 8], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% bovine serum albumin) and incubated with immobilized streptavidin (Pierce) for 1 h at 4°C. Samples were washed, resolved by SDS-15% polyacrylamide gel electrophoresis (PAGE), and visualized using a Typhoon imaging system. The intensities of the bands were obtained using ImageQuant software, background corrected, and normalized to expression of HMPV F WT.

Syncytium assay in transfected cells. Subconfluent monolayers of Vero cells plated in 6-well plates were transiently transfected with a total of 0.5 μ g of DNA consisting of pCAGGS-HMPV F WT or F-protein mutant or the empty pCAGGS vector (control) using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions. The next morning, confluent cell monolayers were washed twice with PBS and incubated at 37°C in 1 ml Opti-MEM with 0.3 μ g/ml TPCK-trypsin for 1.5 h. Cells were then rinsed once with PBS (pH 7.3) before adding 1 ml of PBS of the indicated pH buffered with 10 mM HEPES and 10 mM morpholineethanesulfonic acid (MES). Cells were incubated for 4 min at 37°C under the indicated pH conditions. Medium (1 ml Opti-MEM with 0.3 μ g/ml TPCK-trypsin) was replaced, and cells were incubated at 32°C until the next pH pulse. The pH pulse was repeated a total of four times (2 to 3 h apart) throughout the day, and cells were incubated overnight at 32°C in order to allow final cellular rearrangements to take place. Digital photographs of syncytia were taken the next morning with a Spot Insight Firewire digital camera mounted on a Carl-Zeiss Axiovert 100 inverted microscope using a $\times 10$ objective (Thornwood, NY).

Reporter gene fusion assay. Vero cells in 60-millimeter dishes were transfected with 0.55 μ g pCAGGS-HMPV F wild-type or mutant F protein and 0.55 μ g T7 plasmid containing luciferase cDNA under the control of the T7 promoter (Promega, Madison, WI) using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions. The following day, Vero cells in one 60-millimeter dish were lifted from the plate surface with 1 ml of trypsin, resuspended in 2 ml of DMEM

plus 10% FBS and 1% P/S, and subjected to centrifugation at $400 \times g$ and 4°C in a Sorval RT7 tabletop centrifuge for 5 min. Pelleted cells were resuspended in 2 ml of DMEM plus 10% FBS and 1% P/S and overlaid onto two 35-millimeter dishes of confluent BSR cells, which constitutively express the T7 polymerase (1 ml of Vero cells per 35-millimeter dish). Cells were incubated at 32°C for 1.5 h to allow Vero cells to attach. Afterwards, cells were rinsed once with PBS before adding 1 ml of PBS of the indicated pH (one 35-millimeter dish per pH) buffered with 10 mM HEPES and 10 mM MES and incubated for 4 min at 37°C. Buffered PBS was replaced by 1 ml of Opti-MEM with 0.3 μ g/ml TPCK-trypsin after the pH pulse, and cells were incubated at 37°C for 1 h. Treatment with low pH was repeated one more time, and cells were incubated afterwards in 2 ml DMEM plus 10% FBS and 1% P/S at 37°C for 4 h to allow luciferase production. Finally, cell lysates were analyzed for luciferase activity using a luciferase assay system (Promega) according to the manufacturer's protocol. Light emission was measured using an Lmax luminometer (Molecular Devices, Sunnyvale, CA) for 5 s with a 1.6-s delay between each measurement.

Flow cytometry analysis of rgHMPV infection. One hundred thousand cells plated in a 24-well plate the day before (except for β 1GD25, for which 50,000 cells were plated instead, to account for the difference in growth rate) were infected with different MOIs of rgHMPV diluted to a final volume of 200 μ l/well. The cells were exposed to the virus for a total of 4 h at 37°C. Following an overnight incubation, cells were resuspended, fixed in 1% formaldehyde diluted in PBS with 50 mM EDTA, and analyzed with a BD FACSCalibur flow cytometer, for which the GFP expression and intensity of at least 10,000 cells were determined. Data analysis was performed using FCS Express (version 3) software, and data presented in graphs represent the percentage of GFP-expressing cells in the total population or as a percentage of the control.

Enzyme-linked immunosorbent assay (ELISA) for HMPV binding. Various amounts of HMPV were added to a confluent monolayer of cells (see figures for specific cell types) plated in a 96-well plate at 4°C and spinoculated at $1,000 \times g$ and 4°C in a Beckman Coulter (Brea, CA) Allegra X-15R tabletop centrifuge for 1 h to allow virus binding. Immediately after binding, cells were thoroughly washed with ice-cold PBS, fixed with 4% paraformaldehyde, and permeabilized with 1% Triton X-100 at 4°C. Following fixation and permeabilization, cells were blocked in 1% normal goat serum for 1 h and blotted with 50 μ l of mouse anti-HMPV N monoclonal antibody (Novus Biologicals) diluted 1:1,500 for 1 h. Cells were thoroughly washed with PBS and then blotted with 50 μ l of horseradish peroxidase (HRP)-conjugated goat antimouse monoclonal antibody diluted 1:500 for 1 h. Cells were thoroughly washed with PBS and assayed using a tetramethylbenzidine 2-component microwell peroxidase substrate kit (KPL Inc., Gaithersburg, MD) according to the manufacturer's protocol. Absorbance of each well was read at 460 nm using a μ Quant microplate reader (Bio-Tek Instruments Inc., Winooski, VT), and values were normalized to the number of cells in a well which was obtained using a Beckman Coulter Z1 Coulter particle counter.

Western blot binding assay. A constant number of cells (40×10^3 for β 1GD25 and 60×10^3 for the other cell types, to account for differences in growth rate) were plated in a 24-well plate and exposed to rgHMPV, HMPV Δ G, or HMPV Δ G/ Δ SH at an MOI of 10 or 100 for 1.5 h at 4°C to prevent internalization. Cells were lysed with 0.5% NP-40 and centrifuged at $136,500 \times g$, and a volume corresponding to 6×10^4 cells (determined by counting a duplicate well of each cell type at the time of lysis) was loaded onto a 10% SDS-polyacrylamide gel. The gel was analyzed using a rabbit anti-HMPV F antiserum, followed by an IRDye680-conjugated goat antirabbit antibody, and visualized using an Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE). Signal intensities were quantified using Odyssey application software, corrected for background, and normalized to WT binding to Vero cells.

Protease treatment. Cells plated the day before (2.4×10^5 cells for trypsin treatment and 6×10^5 for proteinase K treatment) were resuspended with PBS with 50 mM EDTA (pH 8) and treated with different

concentrations of trypsin for 10 min or 1.25% trypsin combined with 90 μ l of FBS as a control (0% trypsin). Following trypsin neutralization by addition of 90 μ l of FBS, the cells were transferred into a 15-ml conical tube, washed with 13 ml PBS, and exposed to virus (rgPIV5 at an MOI of 20 and rgHMPV at an MOI of 5) for 1 h at 4°C. Cells were then washed with 14 ml PBS and plated in 35-mm dishes. The cells were detached the following day, fixed in 1% formaldehyde, and analyzed by flow cytometry. For proteinase K treatment, cells were detached from the plate either with 2.5 mM EDTA-PBS (pH 8) or with 300 μ l of 300 μ g/ml proteinase K in Tris-buffered saline with 3 mM CaI₂. For control cells (0 μ g/ml proteinase K), 1 ml of PBS–2.5 mM EDTA–1% phenylmethylsulfonyl fluoride (PMSF) and 300 μ l of 300 μ g/ml proteinase K were added simultaneously. Proteinase K was neutralized by the addition of 1% PMSF before being exposed to virus (MOIs, 10, 50, and 50 for HMPV, PIV5, and vesicular stomatitis virus G* Δ G, respectively). Cells were incubated with the virus for 45 min at 4°C before being plated in a 12-well plate overnight. The cells were detached, fixed in 1% formaldehyde, and analyzed by flow cytometry the following day.

Heparinase treatment. Cells plated the day before were treated with 2 mIU/ml of heparinase 1 or heparinase 3, or both (Sigma), or 20 mIU/ml chondroitinase ABC as a control for 1 h at 37°C, followed by the standard protocol for binding or infection assays (described above).

Homology modeling. A model of the prefusion conformation of the HMPV F protein from the molecular coordinates (Molecular Modeling Database accession number 37132) determined from the crystal structure of the prefusion form of PIV5 F (70) was generated using DeepView Swiss-PdbViewer (version 4.0.1) software (www.expasy.org/spdbv/). The HMPV F sequence was loaded and fitted to model on each monomer of PIV5 F. The modeled HMPV F monomers were merged, and the structure was energy minimized with 100 steps of steepest descent. For the postfusion HMPV F-protein model, the F₁ and F₂ sequences of HMPV F were loaded to model the F₁ and F₂ domains of each NDV F monomer (Molecular Modeling Database accession number 82359) (61), fitted onto the structure, and then merged.

RESULTS

The RGD motif in HMPV F is not required for cell-to-cell membrane fusion. Integrin α v β 1 has been shown to play a role in promoting HMPV infection, potentially by interacting with the RGD motif of HMPV F (15). To determine whether RGD-dependent binding of HMPV F to α v β 1 integrin is required for fusion activity of HMPV F, we mutated the aspartic acid residue in the RGD motif of HMPV F to alanine (F RGA, D331A) using site-directed mutagenesis. Since the RGD-integrin interaction is dependent on the presence of all three residues (50), an RGA mutation should abolish interactions between HMPV F and α v β 1 integrin through this motif. To verify that this mutation did not affect protein folding and expression, the cell surface expression and proteolytic processing of WT F and mutant HMPV F RGA were determined. Vero cells were transiently transfected with the pCAGGS expression vector (42) encoding the genes for the HMPV WT F or HMPV F RGA mutant. After metabolic labeling in medium with TPCK-treated trypsin to proteolytically cleave the F protein (53), the surface proteins were labeled covalently with biotin. The F proteins were immunoprecipitated with an F-specific antiserum following cell lysis, the surface population was separated with streptavidin beads, and samples were analyzed by SDS-PAGE. Both HMPV F WT and the HMPV F RGA mutant were expressed at the cell surface (Fig. 1A and B), and the addition of trypsin to HMPV F RGA produced the lower-molecular-mass F₁ form. Higher-molecular-mass forms consistent with oligomeric HMPV F were also observed for both the WT and RGA mutant. Although the total and surface expression levels of the

inactive F₀ precursor form of HMPV F RGA mutant were slightly greater than those for the WT, the fusogenically active, cleaved F₁ form was expressed at WT levels. The proper surface expression of both the F₀ and F₁ forms of HMPV F RGA indicates that this mutant adopts an intracellular transport-competent conformation and the cleavage site remains accessible to trypsin, suggesting that the HMPV F RGA mutant adopts a similar conformation as HMPV F WT.

To assess the fusogenic activity of HMPV F RGA, syncytium assays were performed (53). Briefly, Vero cells expressing HMPV F WT or HMPV F RGA were subjected to four 4-min pH 5 pulses (pH 7 as a control) to trigger the trypsin-activated F protein; pictures of syncytium formation were then taken the next day. We have previously shown that expression of HMPV F alone is sufficient for promoting syncytium formation (53). Both HMPV F WT and the HMPV F RGA mutant were triggered by pH 5 treatment, leading to the formation of similar numbers of syncytia, which were of similar size (Fig. 1C). Neither HMPV F WT nor the HMPV F RGA mutant was fusogenically active at neutral pH. These results confirm that the RGA mutation does not affect the pH dependency of the fusion protein and that expression of the HMPV F RGA mutant is still sufficient to induce membrane fusion in the absence of HMPV G (53).

The fusogenic activity of the RGA mutant was quantified using a firefly luciferase reporter gene assay. Vero cells expressing a luciferase plasmid under the control of the T7 promoter and either the HMPV F WT or HMPV F RGA mutant were overlaid onto BSR cells, which constitutively express the T7 polymerase (9). Luciferase activity of the cell lysates was assayed following two 4-min pH pulses (53). Luciferase expression directly correlates to fusion activity, as production of luciferase occurs only after Vero cells carrying a luciferase plasmid and expressing a fusogenically active F protein fuse with BSR cells. The HMPV F RGA mutant efficiently promoted membrane fusion at levels approximately 80% of those of the wild type at pH 5 (Fig. 1D). As in our syncytium assay, neither the HMPV F WT nor the HMPV F RGA mutant promoted fusion at neutral pH, and the HMPV G protein was not required for promotion of cell-to-cell membrane fusion. These results indicate that the RGD motif in HMPV F is not essential for the promotion of cell-to-cell membrane fusion.

β 1 integrin is required for efficient infection by HMPV but not for the initial binding step. To determine whether interaction of HMPV F with α v β 1 integrin is necessary for initial virus-cell binding, we directly analyzed virus binding to cells lacking β 1 integrin. GD25 cells are mouse embryonic fibroblasts deficient in β 1 integrin expression due to the introduction of a null mutation within the β 1 integrin gene. Expression of β 1 integrin was rescued by stable transfection of a wild-type β 1 integrin gene (β 1GD25 cells) (68). The expression levels of α v and β 1 integrins for GD25 and β 1GD25 were confirmed using fluorescence-activated cell sorting (Fig. 2A). GD25 and β 1GD25 cells were spinoculated with different MOIs (between 0.5 and 50) of HMPV at 4°C to prevent internalization. After thorough washes, cell-bound viruses were labeled with an anti-HMPV N monoclonal antibody, followed by an HRP-conjugated secondary antibody. Binding efficiency of HMPV to GD25 cells and β 1GD25 cells did not vary significantly, indicating that the expression of β 1 integrin does not play a critical role in the initial virus-cell binding (Fig. 2B). HMPV bound to GD25 and β 1GD25 cells at similar levels compared to CHO-K1

FIG 1 HMPV F RGA mutant promotes cell-to-cell membrane fusion at levels similar to those for HMPV F WT. (A) Representative SDS-polyacrylamide gel of lysates from metabolically labeled and biotinylated Vero cells transfected with F WT versus F RGA. The total amount of F was immunoprecipitated with an F-specific antiserum, and the surface population was separated using streptavidin beads. (B) Quantification of SDS-PAGE bands ($n = 3$) corresponding to total and surface expression of HMPV F WT and RGA mutant. Data represented as percentage of surface or total WT F₀ or F₁ expression. (C) Representative pictures ($n = 4$) of syncytium assays in Vero cells transfected with F WT or the HMPV F RGA mutant are shown. (D) Luciferase reporter gene assay ($n = 3$) used to compare fusion activity between HMPV F WT and HMPV F RGA mutant. MCS, multiple-cloning site, empty vector pCAGGS control. Error bars, mean $\pm$ standard error of the mean.

cells, which express $\beta 1$ integrin but nondetectable levels of αv integrin.

The results from the first binding assay were verified with a different binding assay using Western blots (Fig. 2C and D). In addition to WT HMPV, the binding efficiency of recombinant mutant viruses lacking the putative attachment protein HMPV G (ΔG) or both HMPV G and the small hydrophobic protein HMPV SH ($\Delta G/\Delta SH$) (5) was also assessed to determine which viral surface glycoprotein is involved in the initial binding step. Gene deletions for each virus were verified by reverse transcription-PCR, and the quantity of HMPV F incorporated into the mutant viruses compared to that incorporated into WT virus was similar (Fig. 2E). As previously reported (6), infectivity of these mutant viruses was similar to that of WT HMPV (data not shown). HMPV WT, ΔG , and $\Delta G/\Delta SH$ viral particles were then added to cells at an MOI of 7 and incubated at 4°C for binding. After thorough washes, the cells were lysed and a volume corresponding to 6×10^4 cells for each cell line was loaded onto an SDS-polyacrylamide gel and analyzed by Western blotting, and the band intensities were quantified using the Odyssey application software.

While WT HMPV binding to Vero cells was 5 times higher than binding to $\beta 1$ GD25 and GD25 cells, the amount of HMPV bound to GD25 did not significantly differ from the quantity of virus bound to $\beta 1$ GD25 (Fig. 2D, black bars). The absence of G alone or both G and SH did not significantly affect the binding

efficiency of HMPV (Fig. 2D, gray and white bars, respectively; $P > 0.05$), providing direct evidence that the initial binding step for HMPV to the cell is predominantly mediated by the fusion protein. Similar results were obtained when cells were treated with an MOI of 100 (data not shown). These observations were further supported by a binding assay using biotinylated HMPV and quantified with streptavidin-fluorescein isothiocyanate (FITC) by flow cytometry (data not shown). These results strongly indicate that the initial binding step for HMPV does not require $\beta 1$ integrin.

As $\beta 1$ integrin had been reported to play a role in HMPV infection but did not significantly alter HMPV binding or HMPV F-promoted membrane fusion, we assessed whether $\beta 1$ integrin expression promotes infection by HMPV. Recombinant HMPV strain CAN97-83 expressing the green fluorescent protein (rgHMPV) was used to infect the various cell lines at different MOIs. Both $\beta 1$ GD25 and GD25 cells were permissive to HMPV infection, though a much higher MOI was required to achieve significant infection levels in cells lacking $\beta 1$ integrin (Fig. 3). Approximately 70% of Vero and $\beta 1$ GD25 cells were infected at an MOI of 10, whereas only approximately 20% of GD25 cells were infected at the same MOI. Increasing the MOI to 50 resulted in an additional 10% increase in the number of GD25 cells infected by rgHMPV. Taken together, our results confirm that $\beta 1$ integrin expression is indeed important for HMPV infection, though the initial binding step does not require expression of $\beta 1$ integrin.

FIG 2 Initial HMPV binding is independent of $\beta 1$ integrin expression. (A) Expression levels of αv and $\beta 1$ integrins in Vero, GD25, $\beta 1$ GD25, and CHO-K1 cells assessed by flow cytometry. Control (gray), secondary antibody only. (B) Different MOIs (0.5, 1, 2, 5, 10, 25, and 50) of HMPV were bound to GD25 (closed circles), $\beta 1$ GD25 (closed squares), and CHO-K1 (open circles) cells. HMPV binding was assessed by HRP activity following immunolabeling of the HMPV N protein. Data were normalized to those for virus bound to Vero cells at an MOI of 10. Results shown are averages of duplicate wells for 3 independent experiments. (C) Representative Western blots used for quantification in panel D. WT rgHMPV (lanes 2, 6, and 10), ΔG mutant HMPV (lanes 3, 7, and 11), and $\Delta G/\Delta SH$ mutant HMPV (lanes 4, 8, and 12) at an MOI of 7 were bound to Vero (lanes 1 to 4), GD25 (lanes 5 to 8), and $\beta 1$ GD25 (lanes 9 to 12) cells. Lysates of virus bound to cells were collected, and a volume corresponding to an equal number of cells was analyzed with an anti-HMPV F antibody. The control cell lane without virus is shown in lanes 1, 5, and 9. (D) The signal intensity of specific bands for HMPV F protein on the Western blot was quantified for WT (black bars), ΔG (grey bars), and $\Delta G/\Delta SH$ (white bars) HMPV bound to the different cell lines and normalized to WT Vero cell binding efficiency ($n = 6$). Error bars, mean $\pm$ standard error of the mean; brackets, $P > 0.05$, analysis of variance. (E) An equal number of WT, ΔG , and $\Delta G/\Delta SH$ viral particles concentrated by a sucrose cushion were lysed and blotted for HMPV F using anti-HMPV F antisera. Quantification of the signal of the specific bands on the Western blot is shown ($n = 10$). Data were normalized to those for control WT GFP-expressing HMPV. Error bars, mean $\pm$ standard error of the mean.

FIG 3 HMPV infection is enhanced by the expression of $\beta 1$ integrin. Vero, GD25, $\beta 1$ GD25, and CHO-K1 cells were infected with different amounts of WT rgHMPV. GFP expression of at least 10,000 cells was analyzed by FACS the following day. Results shown are averages for 3 independent experiments done in duplicate. No statistical significance between CHO-K1 and Vero cell infectivity was seen. Error bars, mean $\pm$ standard error of the mean.

Productive HMPV infection is dependent on a proteinaceous receptor. Our data clearly show that HMPV binding is primarily driven by HMPV F and does not require $\beta 1$ integrin, but the nature of the cellular receptor is still unclear. To test whether a proteinaceous receptor is needed for HMPV infection, Vero and CHO-K1 cells pretreated with trypsin (Fig. 4A and C) or proteinase K (Fig. 4B and D) were infected with control parainfluenza virus 5 (Fig. 4A and B) or rgHMPV (Fig. 4C and D). Cellular binding of PIV5 occurs through the interactions of the PIV5 attachment protein HN with sialic acid moieties at the cell surface. Consistent with this, pretreatment of cells with trypsin or proteinase K did not significantly affect PIV5 infection (Fig. 4A and B). However, rgHMPV infectivity was decreased by 50% or more after trypsin treatment. Proteinase K treatment resulted in a more drastic reduction of infection (Fig. 4D), likely due to the more efficient digestion of proteins by proteinase K than trypsin. Cell surface integrins have been reported to be insensitive to protease treatment (26). Therefore, to verify that $\alpha v\beta 1$ integrin was not the protease-sensitive component involved in HMPV infection, the amounts of αv and $\beta 1$ integrin on the cell surface were measured after trypsin or proteinase K treatment. As expected, protease treatments did not remove the antibody epitopes from the cell surface (Fig. 4E and F). Together these results indicate that efficient HMPV infection requires the proper expression of a trypsin- and proteinase K-sensitive proteinaceous receptor.

Heparan sulfate is important for HMPV F-dependent virus-cell binding. Glycoconjugates, including GAGs, are involved in many virus-cell interactions (56), and a previous report showed that the HMPV G protein interacts with GAGs (65). Since our results indicated that HMPV F is the major viral binding protein, we directly analyzed the role of GAGs in virus binding and infection to the mutant CHO cell lines pgsA745, Lec1, and ldlDLec1 (Table 1). pgsA745 cells carry a mutation in the xylosyltransferase gene abolishing expression of any GAG. Lec1 cells carry a mutation in GlcNAc transferase 1, resulting in the inability of these cells to express complex N-linked glycans, whereas the ldlDLec1 cells carry an additional mutation (4-epimerase) preventing them from expressing complex N-linked glycans, mucins, and GAGs in the absence of galactose and GalNAc (30). However, because

ldlDLec1 cells were cultured with serum, these cells produce heparan sulfate as their only GAG (Table 1) (11, 19).

WT HMPV was unable to bind to pgsA745 cells as efficiently as it bound to CHO-K1 cells (Fig. 5A). Surprisingly, binding to ldlDLec1 and CHO-K1 cells was similar (Fig. 5A). The binding profile of HMPV was similar for the HMPV ΔG (Fig. 5B) and the HMPV $\Delta G/\Delta SH$ mutants (Fig. 5C), confirming that the primary viral binding factor is HMPV F. The profile of HMPV binding to the different cell lines was confirmed by the Western blots. While binding of WT and mutant HMPV to pgsA745 decreased by 90% compared to the binding to Vero cells, binding to Lec1 and ldlDLec1 cells was not significantly decreased (Fig. 5D and E). The differences in binding efficiencies between Lec1 and ldlDLec1 cells in the two binding assays could be due at least in part to the spin-oculation step performed only in the ELISA-based assay, as ldlDLec1 cells have a higher tendency to aggregate, resulting in artificially higher normalized values in an ELISA-based assay, where the initial number of cells employed is already small. Nonetheless, our data confirm that HMPV F is the predominant viral protein involved in the initial binding step for HMPV and that cellular binding of HMPV depends on the proper synthesis of GAGs.

The need for proper GAG synthesis for HMPV binding and infection was confirmed by the inability of wild-type rgHMPV to infect pgsA745 even at high MOIs, despite a high degree of infectivity of CHO-K1, Lec1, and ldlDLec1 cells (Fig. 6). At an MOI of 10, for instance, nearly 100% of CHO-K1, Lec1, and ldlDLec1 cells were infected, whereas only 3% of pgsA745 cells were infected. The infectivity of pgsA745 did not significantly increase even at an MOI of 50. Importantly, the absence of GAGs did not affect PIV5 infection, as this virus was able to infect pgsA745 and CHO-K1 cells with similar efficiency (Fig. 7C), indicating that not all paramyxoviruses require proper synthesis of GAGs for binding and infection. These data demonstrate that HMPV F-GAG interaction is specific for HMPV and that this interaction is essential for infection.

The fact that ldlDLec1 cells were permissive for binding and infection at levels equivalent to those for CHO-K1 cells suggested that the primary GAG involved in these processes is heparan sulfate. To confirm that heparan sulfate is indeed the specific GAG required for HMPV binding and infection, pgsD677 cells were exposed to HMPV at different MOIs and assessed for binding and infectivity. pgsD677 cells are unable to synthesize heparan sulfate, but chondroitin sulfate synthesis is enhanced 3- to 4-fold due to a deficiency in *N*-acetylglucosaminyltransferase and glucuronyltransferase (35). Wild-type HMPV efficiency of binding to pgsD677 cells, using both our ELISA and Western blot analyses, was comparable to that to pgsA745 cells (data not shown). Importantly, HMPV ΔG (data not shown) and HMPV $\Delta G/\Delta SH$ (Fig. 7A and B) also showed a drastic decrease in efficiency of binding to these cells, providing additional evidence that F is the primary binding factor for HMPV. As expected, HMPV was unable to infect pgsD677 cells since the virus cannot bind to these cells (Fig. 7C). Importantly, no significant differences in the levels of control PIV5 infectivity were seen in pgsA745 and pgsD677 cells (Fig. 7C). Since PIV5 is known to bind to sialic acid, our results suggest that binding to HS is an event specific for HMPV.

To further confirm that heparan sulfate is indeed the primary binding partner for HMPV F, CHO-K1, ldlDLec1, pgsD677, and pgsA745 cells were treated either with a mixture of 2 mIU/ml of

FIG 4 Effective HMPV infection requires the expression of a trypsin- and proteinase K-sensitive cellular surface protein. Vero or CHO-K1 cells were treated with 0%, 0.0625%, or 0.125% trypsin (A, C, and E) or 0 μ g/ml or 300 μ g/ml of proteinase K (B, D, and F) prior to either infection by control rgPIV5 (A and B) or rgHMPV (C and D) or analysis of α v and β 1 integrin expression (E and F; $n = 3$). GFP expression or FITC fluorescence intensity of at least 10,000 cells was analyzed by FACS the following day ($n = 5$ for trypsin and $n = 6$ for proteinase K treatment). Data in panels A and B are expressed as percentage of total infected cells, whereas data in C and D are expressed as percentage of cells infected normalized to the results for Vero cells not treated with proteinase K. Error bars, mean $\pm$ standard error of the mean.

heparinases 1 and 3 or with 20 mIU/ml of chondroitinase ABC for 2 h prior to analysis of binding for both wild-type HMPV and HMPV Δ G/ Δ SH. Binding efficiency for wild-type HMPV was greatly reduced after pretreatment with heparinases 1 and 3 but not after pretreatment with chondroitinase ABC (data not shown). This drastic reduction in binding efficiency after treatment with heparinases 1 and 3 was reproduced using HMPV Δ G/ Δ SH (Fig. 7A and B) and was also seen after heparinase treatment of Vero cells (data not shown), whereas pretreatment with chondroitinase ABC did not have an effect on binding. As seen in Fig. 7B, treatment with a combination of heparinases 1 and 3 decreased HMPV binding to both CHO-K1 and ldlDLec1 cells to levels equivalent to those to pgsD677 and pgsA745 cells. Similarly, HMPV infection was drastically reduced below 5% for CHO-K1

cells after pretreatment with either 2 mIU/ml heparinase 1 or heparinase 3 for 1 h but not after treatment with 20 mIU/ml chondroitinase ABC (Fig. 7D). Importantly, PIV5 infection was unaffected by heparinase 1, heparinase 3, or chondroitinase ABC treatment, confirming that the interaction between HS and HMPV F is specific to HMPV. Additionally, the lack of HMPV binding and infection of heparinase-treated β 1 integrin-expressing CHO-K1 cells further confirms that the initial binding step of productive infection is dependent on heparan sulfate and that the F-heparan sulfate interaction cannot be replaced with integrins.

DISCUSSION

The results presented in this study indicate that the initial interaction between HMPV and its target cell occurs through heparan

TABLE 1 Cell lines used in this study

Name	Mutation	Phenotype under culture conditions		
		Complex N-linked glycans	O-linked glycans	
			Mucins	GAGs
Vero	NA ^a	Yes	Yes	Yes
CHO-K1	NA	Yes	Yes	Yes
pgsA745	Xylosyltransferase	Yes	Yes	No
pgsD677	N-Acetylglucosaminyltransferase- and glucuronyltransferase-deficient CHO mutant	Yes	Yes	No HS
Lec1	GlcNAc transferase 1-deficient CHO mutant	No	Yes	Yes
ldlDLec1	GlcNAc transferase 1- and 4-epimerase-deficient CHO mutant	No	No	Only HS
GD25	β 1 integrin null mouse embryonic fibroblast			
β 1GD25	GD25 cells stably transfected with human β 1 integrin			

^a NA, not applicable.

sulfate moieties expressed at the cell surface. Importantly, we provide direct evidence that the critical virus-cell attachment step is predominantly mediated by the fusion protein of a paramyxovirus. Furthermore, our results support the report from Cseke et al. (15) that α v β 1 integrin is important for HMPV infection, though our studies suggest that the role of this integrin is not as the initial binding receptor. Rather, our results indicate that β 1 integrin promotes efficient HMPV infection after the virus binds to cell surface heparan sulfate.

In this study, we confirmed that the fusion protein of HMPV is the primary entry factor of the virus, promoting both virus-cell binding and membrane fusion. No differences in binding or infectivity were observed between the WT virus and the mutant viruses lacking HMPV G or both HMPV G and SH (Fig. 2 and 5). A recent report showed that cell surface nucleolin is a functional receptor for RSV F (62). This interaction, however, is likely preceded by interactions between GAGs and RSV G, as G expression is still required for efficient growth *in vivo* (29, 31, 62, 63). Both

FIG 5 Initial HMPV binding is dependent on GAG expression and is mediated by the F protein. (A to C) CHO-K1, pgsA745, Lec1, and ldlDLec1 cells were exposed to different MOIs (0, 1, 10, and 50) of HMPV WT (A), Δ G (B), or Δ G/ Δ SH (C) at 4°C. Results shown are averages of 6 independent experiments done in duplicate. (D) Representative Western blot shown for quantification in panel E. WT rgHMPV (lanes 2, 6, 10, and 14), HMPV Δ G (lanes 3, 7, 11, and 15), and HMPV Δ G/ Δ SH (lanes 4, 8, 12, and 16) at an MOI of 7 were bound to Lec1 (lanes 1 to 4), ldlDLec1 (lanes 5 to 8), pgsA745 (lanes 9 to 12), and control CHO-K1 (lanes 13 to 16) cells. Lysates of virus bound to cells were collected, and a volume corresponding to an equal number of cells was analyzed with an anti-HMPV antibody. The control cell line without virus is shown in lanes 1, 5, 9, and 13. (E) Quantification of Western blot signals showing binding activity of the WT, Δ G mutant, and Δ G/ Δ SH mutant HMPV. Data were normalized to those for WT Vero binding efficiency ($n = 10$). Error bars, mean $\pm$ standard error of the mean; asterisks, $P < 0.001$.

FIG 6 HMPV infection is greatly reduced in the absence of GAGs. CHO-K1, pgsA745, Lec1, and IdlDLec1 cells were infected with different MOIs (0, 1, 10, and 50) of rgHMPV, and GFP expression of at least 10,000 cells was analyzed by FACS the following day. Results shown are averages of 3 independent experiments done in duplicate. Error bars, mean $\pm$ standard error of the mean.

our previous observation that HMPV F-mediated cell-to-cell fusion occurs in the absence of HMPV G (53) (Fig. 1) and our current study indicating that HMPV binding is very different from binding of other paramyxoviruses support the idea that the

HMPV F protein is the major factor driving both viral attachment and entry. We cannot, however, rule out the possibility that the HMPV G could confer a selective advantage for viral attachment in some tissues, as it has been demonstrated previously that the HMPV Δ G mutant is less efficient at infecting the lower respiratory tract (6).

It has been reported that all strains of HMPV isolated clinically have a conserved Arg-Gly-Asp (RGD) sequence in their F protein, raising the possibility of an interaction between F and integrins (15). Indeed, α v β 1 integrin has been shown to be a major factor promoting HMPV infection (15). A direct interaction between α v β 1 integrin and the prefusion conformation of HMPV F, however, has not been confirmed. In this study, we investigated the role of β 1 integrin and the RGD sequence in HMPV F by performing functional studies with an HMPV F RGA mutant and assessing the binding and infectivity of recombinant HMPV in cells lacking β 1 integrin. Our data show that the RGD motif of HMPV F is not necessary for cell-to-cell membrane fusion (Fig. 1), suggesting that this motif is not needed for F-mediated membrane fusion. Additionally, three independent approaches to assess virus binding clearly indicate that expression of β 1 integrin or lack thereof does not significantly affect binding (Fig. 2). Cells lacking β 1 integrin, however, are significantly more resistant to HMPV infection. Interestingly, infectivity of CHO-K1 cells is equivalent to that

FIG 7 HMPV binding and infection are decreased in the absence of heparan sulfate. (A and B) HMPV Δ G/ Δ SH binding to CHO-K1 (lanes 1 to 4), IdlDLec1 (lanes 5 to 8), pgsD677 (lanes 9 to 12), and pgsA745 (lanes 13 to 16) cells was assessed after a 2-h treatment with heparinases 1 and 3 (lanes 4, 8, 12, and 16) or chondroitinase ABC (lanes 3, 7, 11, and 15) or mock treatment (lanes 2, 6, 10, and 14). The control cell line without virus is shown in lanes 1, 5, 9, and 13. The signal for the specific bands on the Western blots represented in panel A ($n = 3$) were quantified and shown in panel B. (C) pgsD677 cells were exposed to HMPV or PIV5 at an MOI of 10. Data shown are normalized to the infectivity of CHO-K1 cells and represent the averages of 3 independent experiments done in duplicate ($n = 3$). (D) CHO-K1 cells treated for 1 h at 37°C with 2 mIU/ml of heparinase 1 or heparinase 3 or 20 mIU/ml of chondroitinase ABC (mock treatment as a control) were exposed to 1 MOI of either HMPV or PIV5 at 4°C, followed by normal incubation to allow infection to occur. pgsA745 cell infection is shown as a control. Data shown are normalized to the infectivity of CHO-K1 cells in the absence of any treatments and represent the averages of 3 independent experiments done in duplicate ($n = 3$). Error bars, mean $\pm$ standard error of the mean.

of Vero cells (Fig. 3) and LLC-MK2 cells (data not shown), even though there were no detectable levels of α v integrin in CHO-K1 cells. This suggests that the β 1 integrin subunit plays a more important role in HMPV infection than the α v subunit. Thus, our data show that β 1 integrin expression dramatically enhances the infectivity of HMPV but is not required for the initial binding of the virus.

It is not clear what role β 1 integrin plays in HMPV entry, but our data suggest that β 1 integrin is involved after initial binding and not as a direct initial cellular receptor for the virus. Analysis of a homology model of HMPV F in its prefusion and postfusion conformations, based on the crystal structure of the prefusion form of PIV5 F (70) and postfusion form of Newcastle disease virus F, respectively (61), suggests that the RGD motif in HMPV F is covered by other stretches of the protein in its prefusion form (Fig. 8A; RGD shown in red). Although partially buried, the RGD motif appears to be more accessible for protein-protein interaction in its postfusion conformation (Fig. 8B), suggesting that an RGD-dependent integrin interaction is more likely to occur in an intermediate step of the viral entry process, after a change in conformation from the prefusion form of F that exposes the RGD motif (2, 59). Integrins are well-known for their signaling capabilities and their ability to orchestrate a myriad of cellular processes. Indeed, the involvement of integrins in these processes has been shown to influence viral infectivity. The regulation of endosomal cathepsins by the α 5 β 1 integrin, for instance, mediates Ebola virus entry (52), and the presence of a cellular receptor is presumably dependent on membrane trafficking of proteins mediating cell adhesion (17, 18). Since HMPV infectivity is decreased without changes in cell binding when β 1 integrin expression is altered, it is possible that, after a conformational change that results from the initial binding step, HMPV F interacts with α v β 1 integrin, triggering a signal that increases cell permissiveness. Alternatively, α v β 1 integrin could direct HMPV to a more efficient route of entry without necessarily interacting with the virus, as it has been hypothesized for HSV (23). Future studies with HMPV recombinants containing the F-RGA mutation should provide important insights into the role of F-integrin interactions in HMPV infection.

We report that the primary cellular binding partner for HMPV is proteinaceous in nature, as infection is decreased by protease treatment (Fig. 4). Importantly, integrins, including β 1, are known to be trypsin resistant (26), and we confirmed that neither α v nor β 1 integrin is sensitive to cleavage by trypsin or proteinase K since the antibody epitope was not affected by the proteases (Fig. 4). Since pretreatment of Vero or CHO cells at trypsin concentrations higher than 0.0625% does not further reduce HMPV infectivity (Fig. 4 and data not shown), HMPV entry appears to involve more than one component, of which at least one is trypsin sensitive and at least another is trypsin resistant. While the antibody epitopes were unaffected by protease treatment, there remains a possibility that the interaction sites with HMPV F were destroyed.

Our analysis of HMPV binding and infectivity of mutant cell lines derived from CHO-K1 cells indicates that binding and infectivity are highly dependent on the presence of GAGs, especially heparan sulfate. The drastic reduction of binding and infectivity of pgsA745 and pgsD677 cells compared to WT suggests that HMPV specifically interacts with HS (Fig. 5 to 7). Importantly, this robust reduction in binding and infection was reproduced by treating the parental, β 1 integrin-expressing CHO-K1 cells with heparinases

FIG 8 Homology model of the prefusion and postfusion form of HMPV F. (A) The amino acid sequence of HMPV F was threaded onto the crystal structure of the prefusion form of PIV5 F (70) using DeepView Swiss-PdbViewer (version 4.0.1) software. The RGD sequence of HMPV (red) is covered by two stretches of amino acid (yellow and green in inset). (B) Individual sequences of the F₁ and F₂ subunits of HMPV F were threaded onto the crystal structure of the postfusion form of Newcastle disease virus F (61).

(Fig. 2A and 7). Since β 1 integrin is not an HSPG, it should not be affected by heparinase treatment. The dramatic loss in both binding and infection of CHO cells after treatment with heparinase confirms that the heparan sulfate-F interactions are needed for productive infection and cannot be replaced by the presence of integrins. Altogether our data strongly suggest that the inhibition of HMPV infection with heparin treatment reported by Wyde et al. (69) was due to a direct and specific interaction of the virus with

FIG 9 Potential mechanisms for the initial steps of HMPV infection via interactions with HS. (A) HMPV could bind to any heparan sulfate proteoglycan or any specific modification in the HS moiety through HMPV F, concentrating the virus and allowing HMPV F to interact with a more specific viral receptor or coreceptor, which could also be an HSPG. This interaction, in turn, could allow the virus to interact with a potential fusion receptor that will activate HMPV F. (B) HMPV could bind to a specific HSPG or any specific modification in the HS moiety through HMPV F, and that interaction could be sufficient for HMPV F triggering and viral entry. (C) HMPV could bind to any HSPG or any specific modification in the HS moiety through HMPV F, and that interaction could be sufficient for HMPV F triggering and viral entry. Integrin $\alpha v\beta 1$ expression renders the cell more permissive to HMPV infection.

HS. Importantly, our data show that the dependence on HS for infection is driven by HS interactions with the F protein rather than the G protein, as previously hypothesized (69). The use of the F protein as the primary viral protein for cellular attachment highlights the uniqueness of HMPV among the paramyxoviruses, as other human pathogens in this family use the attachment protein for this step.

Carbohydrates play an important role in entry for many viruses, including RSV, the human pneumovirus most closely related to HMPV (25, 31, 36). Indeed, earlier studies have suggested that both RSV F and, primarily, RSV G are capable of binding GAGs (21, 31, 32). Here we report that, unlike RSV, the fusion protein of HMPV is the main GAG binding protein. It has been reported that a recombinant HMPV G protein has the ability to bind GAGs (65). In light of our findings, this interaction is not required for the initial viral attachment step. Nonetheless, since HMPV G is present in all clinical isolates sequenced so far (5, 6) and studies by Biacchesi et al. demonstrated that HMPV lacking the G protein is significantly attenuated for infection in the upper respiratory tract of hamsters (6), this protein could confer an advantage for the virus to grow *in vivo* either by facilitating infection of certain cells with a specific GAG (or a specific protein) recognized by G at a higher affinity or by providing a protective mechanism against the host immune defenses (5, 6, 64).

Since the sequence of the F protein from the virus used in this study was verified to be identical to the original strain CAN97-83 F-protein sequence directly isolated from the patient, the interaction between HS and the HMPV F protein is likely relevant *in vivo*. Some studies have shown that cell surface HS is primarily located on the basolateral side of differentiated ciliated airway epithelial

cells (10, 39, 71). Thus, binding to HSPG may be used to infect nonciliated cells in the respiratory tract or to promote cell-to-cell spreading from the basolateral side. Moreover, the small amount of HSPG present on the apical side (10, 13) could be enough for HMPV to establish an initial infection in the upper respiratory tract. Since apical levels of HSPG are upregulated upon injury and basolateral HSPGs can be exposed when the monolayer is disrupted (including during tissue regeneration) (10), this initial infection would in turn increase the accessibility of HSPGs to the virus.

By examination of viruses using HS as their first attachment factor (44, 72), it is possible to distinguish three potential mechanisms leading to infection (Fig. 9). Like most viruses that bind HS, HMPV could attach to this specific GAG to concentrate at the cell surface and facilitate a subsequent interaction with a more specific secondary receptor, which could be another HSPG (Fig. 9A) (1, 22, 34, 37). Alternatively, HMPV could interact with a specific chemical modification of HS which then directly triggers fusion, as it has been reported to be one of the entry pathways for herpes simplex virus type 1 (HSV-1) (Fig. 9C) (43, 55, 66). While not yet reported, the high-affinity attachment and promotion of fusion could also be carried out by one specific HSPG (Fig. 9B). Like HSV (12), HMPV could also be interacting with different cellular proteins and using different entry pathways depending on the cell type. While the data reported in this study indicate that HMPV binds to HS, whether HS alone is sufficient to trigger fusion remains unknown.

In this study, we demonstrated that the F protein is the major protein driving attachment of HMPV. We also showed that $\alpha v\beta 1$ integrin is not responsible for viral attachment but instead that the

cell-virus interaction is highly dependent on the presence of HS. Several HSPGs with a wide range of function, including basement membrane barrier organization, cell signaling, and cellular cross talk, have been identified to date (14). Interestingly, some HSPGs have the ability to interact with integrins. Since interactions with integrins enhance HMPV infection and HMPV binds to HS, a specific HSPG could mediate the interaction between the virus and integrins, allowing the virus to efficiently infect its target cell.

ACKNOWLEDGMENTS

This work was supported by NIH grant R01AI051517 and NIH grant 2P20 RR020171 from the National Center for Research Resources to R.E.D., by AHA Great Rivers Affiliate predoctoral fellowship 10PRE4230022 to A.C., and with funds from the NIAID Division of Intramural Research to U.J.B.

We thank Deane Mosher (University of Wisconsin, Madison, WI) for providing us with the GD25 and β 1GD25 cells and Craig Vander Kooi (University of Kentucky, Lexington, KY) for providing us with the ldlDlec1 cells. We also thank Jeffrey Esko (UCSD, San Diego, CA) for providing us with the pgsD677 cells and for helpful discussion. In addition, we thank members of the Dutch lab, Peter Collins (NIH, Bethesda, MD), Skip Waechter, and Katherine O'Connor for their helpful suggestions and for critical review of the manuscript.

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