

# Role of Cellular Glycosaminoglycans and Charged Regions of Viral G Protein in Human Metapneumovirus Infection<sup>∇</sup>

Sutthiwan Thammawat, Tania A. Sadlon, Peter G. Hallsworth, and David L. Gordon\*

Department of Microbiology and Infectious Diseases, Flinders University and Flinders Medical Centre, Bedford Park, Adelaide, Australia

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**Human metapneumovirus (hMPV) is an important cause of lower respiratory tract disease, particularly in infants and young children. hMPV has two major glycoproteins, G and F, which are responsible for virus attachment and membrane fusion, respectively. We investigated the role of cellular glycosaminoglycans (GAGs) and G protein in hMPV infection. The pretreatment of hMPV with soluble heparin markedly inhibited the infection of HEP-2 cells. Recombinant G protein, comprising the extracellular domain of G, bound to heparin-agarose columns and also to HEP-2 cells. hMPV infection and G protein binding to HEP-2 cells was inhibited by other soluble GAGs, including chondroitin sulfates, by the enzymatic removal of cell surface GAGs with GAG lyases or by the pretreatment of cells with basic fibroblast growth factor. The role of cellular GAGs was confirmed by the binding of G protein to wild-type CHO cells but not to GAG-deficient CHO-pgsA745 cells. An analysis of the G protein sequence revealed two adjacent clusters of positively charged amino acids (<sup>149</sup>EKKKTRA<sup>155</sup> and <sup>159</sup>QRRGKGKE<sup>166</sup>). Truncated G fragments were expressed, and only the fragment containing these putative heparin binding domains retained heparin binding. The alanine mutagenesis of charged residues in either of these regions resulted in the loss of binding to heparin and to HEP-2 cells, suggesting that both sites are likely to be required for hMPV attachment. These results, taken together with the inhibition of hMPV infection by soluble G protein, indicate an important role for G protein and cellular GAGs in hMPV infection.**

Human metapneumovirus (hMPV) is a recently recognized paramyxovirus and is a major cause of respiratory infection, particularly in infants and young children (34). Respiratory syncytial virus (RSV), another paramyxovirus, is the most closely related human virus to hMPV. Although belonging to a different genus, hMPV shares many epidemiological and clinical features with RSV (18). Similarly to all *Paramyxoviridae*, hMPV is an enveloped, single-stranded, negative-sense RNA virus containing three envelope glycoproteins, the fusion (F), attachment (G), and small hydrophobic (SH) proteins (33, 34). The F and G proteins are potential targets for neutralizing and protective immune responses, with F protein being one of the most conserved proteins. In contrast, an analysis of G protein from a number of isolates reveals a high level of genetic diversity, with two major groups, designated A and B, and minor subgroups 1 and 2 (2, 4, 26). hMPV G protein is a type II membrane protein comprised of extracellular, transmembrane, and intracellular domains (2, 33). It is heavily glycosylated with O- and N-linked sugars (2, 4, 25). G protein is thought to play an important role in viral attachment, and recombinant virus lacking G protein has reduced replicative ability *in vivo* (3, 5).

Glycosaminoglycans (GAGs) are linear, unbranched polymers of repeating disaccharide units composed of glucuronic acid or its epimer, iduronic acid, linked to an amino sugar, glucosamine or galactosamine. They are found on the surface of most mammalian cell types, in tissue, and in the extracellular matrix. Several viruses, including RSV, have been shown to

utilize GAGs, particularly heparan sulfate (HS), for cellular attachment and entry (1, 10, 17, 37). Previous studies indicated that RSV infection is inhibited in the presence of soluble GAGs such as heparin and by the enzymatic removal of GAGs from the cell surface (20). The RSV G attachment protein binds specifically to heparin, and a linear heparin binding domain has been identified (9, 10). hMPV infection also has been shown to be inhibited by heparin (35), suggesting that the cellular attachment of hMPV may be similarly mediated by a G protein-GAG interaction. In this study, in an experimental approach analogous to that used for RSV, we have determined the role of GAGs in hMPV infection. Furthermore, using recombinant G proteins and mutants, we have characterized the functional domains in the hMPV G protein involved in this interaction.

## MATERIALS AND METHODS

**Cells and viruses.** The human epithelial tumor cell line HEP-2 and rhesus monkey kidney cells (LLC-MK2) were grown in medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). Wild-type Chinese hamster ovary cells (CHO) and GAG-deficient CHO-pgsA745 cells (8) were grown in Hams F12 medium (Invitrogen) supplemented with 10% FBS. hMPV genotype B2 was a clinical isolate obtained from a child with lower respiratory tract infection. Stocks of hMPV were prepared by the inoculation of LLC-MK2 cells with hMPV and incubation for 14 to 21 days at 37°C in 5% CO<sub>2</sub>. hMPV stocks were stored at -70°C until use. The hMPV infectivity titer was determined using an immunofluorescence assay. Briefly, cells were incubated with dilutions of the virus and counted after being stained with a monoclonal antibody (MAb) to hMPV matrix protein (Chemicon, Temecula, CA) and fluorescein isothiocyanate-labeled secondary antibody. The virus titer was calculated assuming each fluorescent focus represented 1 infectious unit of virus and is reported as fluorescent focus-forming units (FFU) per milliliter.

**Chemicals and enzymes.** The following chemicals were purchased from Sigma (St. Louis, MO): dextran from *Leuconostoc mesenteroides* (average molecular mass, ~10 kDa); dextran sulfate; chondroitin sulfates A, B, and C (CS-A, CS-B, and CS-C, respectively); heparin (sodium salt IA); HS (porcine intestinal mu-

\* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, Flinders Medical Centre, Bedford Park, SA 5042, Australia. Phone: 61 8 82045293. Fax: 61 8 82044773. E-mail: D.Gordon@flinders.edu.au.

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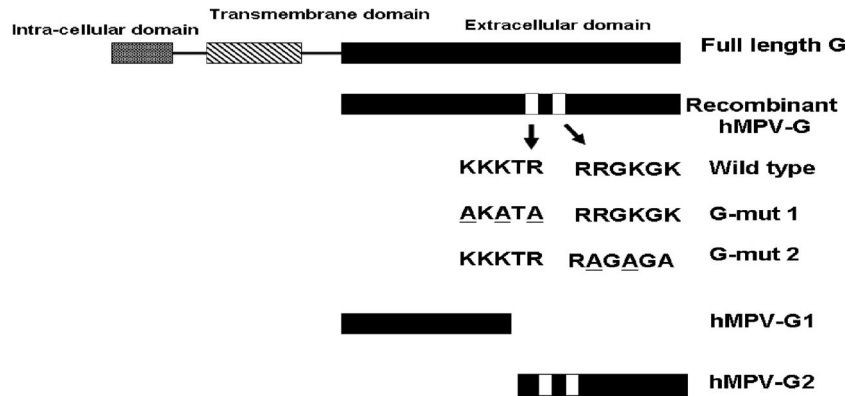


FIG. 1. Schematic diagram of recombinant G protein constructs. The putative heparin binding regions are indicated by the white bars. G-mut1 contained alanine substitutions of K151A, K153A, and R155A, and G-mut2 included alanine substitutions of R161A, K163A, and K165A. The lysine and arginine residues mutated to alanine are underlined. The truncated G constructs hMPV-G1 and hMPV-G2 used in this study also are shown.

cosa); de-*N*-sulfated heparin sodium salt; *N*-acetyl-de-*O*-sulfated heparin sodium salt; recombinant human basic fibroblast growth factor (bFGF); heparinase I; heparitinase; and chondroitinase ABC.

**hMPV infectivity ELISA.** A cell-based enzyme-linked immunosorbent assay (ELISA) was used to quantitate hMPV infection. HEp-2 cell monolayers in 96-well plates (Linbro; ICN Biomedicals, Aurora, OH) were inoculated with hMPV at a multiplicity of infection of 1 FFU per cell and incubated for 2 h at 37°C, 5% CO<sub>2</sub>. Control wells were mock inoculated with no virus. Cells were washed with medium 199 to remove unbound virus. Medium 199 containing 1 µg/ml trypsin then was added, and cells were cultured for 48 h. Medium was removed, and cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. Cells were washed twice with PBS and permeabilized with 0.02% Triton X-100-PBS for 30 min at 4°C, followed by two washes with PBS. Nonspecific sites were blocked with 5% skim milk-PBS for 1 h. The wells then were incubated with hMPV matrix protein MAB diluted 1:320 (vol/vol) in 0.5% Tween 20-PBS, followed by 1:10,000 (vol/vol) horseradish peroxidase (HRP)-conjugated sheep anti-mouse immunoglobulin G (Chemicon). Each incubation was for 60 min at 37°C, and the wells were washed four times with PBS after each step. *O*-phenylenediamine substrate (Sigma) was added and after 30 min, 1N H<sub>2</sub>SO<sub>4</sub> was added, and the absorbance at 490 nm was determined. Wells were inoculated in triplicate, and each experiment was performed at least two times. There was a linear relationship between virus input and optical density over a greater than 100-fold range of virus inoculums.

**Infectivity inhibition assays.** The effect of either soluble GAGs, including heparin, heparin sulfate, and CS-A, CS-B, and CS-C, or dextran sulfate on hMPV infectivity was determined by a modification of the hMPV infectivity ELISA. hMPV was pretreated with serial dilutions of GAGs for 30 min at 37°C before the inoculation of HEp-2 cells and the assessment of infectivity as described above. Results are expressed relative to results for virus incubated in medium without GAGs. Nonsulfated dextran was included as a negative control in some experiments. To test the effect of modified heparins, hMPV was incubated with heparin, de-*N*-sulfated heparin, or *N*-acetyl-de-*O*-sulfated heparin at 10 or 500 µg/ml for 1 h at 37°C before the inoculation of HEp-2 cells. The inhibition of hMPV infection by soluble G protein was examined by the preincubation of HEp-2 and LLC-MK2 cells with 150 µg/ml of purified hMPV-G protein for 30 min at 37°C prior to infection with hMPV.

**Construction and expression of recombinant hMPV-G proteins.** A schematic diagram of the G protein constructs used in this study is shown in Fig. 1. RNA was extracted from the culture medium of hMPV subtype B2-infected LLC-MK2 cells collected 10 days postinoculation using the QIAamp viral RNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The extracellular domain of G protein (hMPV-G; nucleotides [nt] 6315 to 6848; GenBank accession no. AY530089) was amplified using PCR and the primers Gfor (5'-GGG GAA TCC GAT CAT GCA ACA TTA AGA AAC ATG-3') and Grev (5'-GGG TCT AGA GCT CCT GCA CCT CYC CGT GCA T-3'). Truncated G fragments hMPV-G1 (nt 6315 to 6605) and hMPV-G2 (nt 6583 to 6848) were amplified using primers Gfor and G1rev (5'-GGG GAA TTC GAA CAG ATC ACC CAG ACA ACC-3') and G2for (5'-GGG TCT AGA

CGC GTT GTC TGG GTG ATC TG-3') and Grev, respectively. Forward and reverse primers incorporated EcoRI and XbaI restriction enzyme sites (underlined in the sequences above), respectively. PCR was performed using 1× PCR buffer (Promega, Madison, WI), 0.75 U of *Taq* DNA polymerase (Promega), 2 mM MgCl<sub>2</sub>, 200 µM deoxynucleoside triphosphates (Promega), 0.2 µM each primer, and 5 µl cDNA. PCR products were cloned into pPICZαA (Invitrogen) downstream of the yeast α-factor signal sequence, resulting in the extracellular secretion of the recombinant protein. G protein was cloned in frame with C-terminal c-Myc and 6-histidine tags to facilitate purification and detection. After transformation into *Pichia pastoris* X33 cells, soluble recombinant proteins were expressed after methanol induction for 3 to 4 days and purified from culture supernatants using Hi-Trap Ni affinity chromatography (Amersham Biosciences, Buckinghamshire, United Kingdom). Two extracellular domain G mutants (G-mut1 and G-mut2) in which positively charged lysine and arginine residues were mutated to alanine (as shown in Fig. 1) were constructed using splice overlap extension PCR. PCR products were cloned into pPICZαA, and substitutions were verified by sequencing prior to expression in *P. pastoris*.

**Heparin-agarose affinity chromatography.** Heparin-agarose affinity chromatography was performed in 50 mM sodium phosphate buffer, pH 7.4 (PB), using 1 ml heparin-agarose columns (Pierce Chemical Corporation, Rockford, IL). Recombinant proteins were dialyzed against PB and passed over the column four times. The column was washed extensively with PB, and bound protein was eluted with a stepwise 0.1 to 0.6 M NaCl gradient. Aliquots of start, fall-through, wash, and elution materials were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

**Western blotting.** Proteins were separated on SDS-12.5% PAGE gels under reducing conditions and transferred to Hybond C+ nitrocellulose (Amersham). Membranes were blocked with 5% skim milk in PBS and incubated with anti-c-Myc MAb, followed by HRP-conjugated sheep anti-mouse immunoglobulin G (1:2,000, vol/vol). The proteins were visualized using enhanced chemiluminescence (ECL).

**G protein cell binding ELISA.** The binding of G proteins to cells was evaluated using a cell ELISA. Briefly, purified recombinant hMPV G proteins were biotinylated with EZ-link sulfo-NHS-LC-biotin (Pierce). Confluent monolayers of HEp-2 cells, CHO cells, or GAG-deficient CHO-pgsA745 cells in 96-well plates were incubated with serial dilutions of biotinylated G protein at 37°C. After 1 h of incubation, unbound protein was removed by being washed with PB. Cells then were incubated with 1:1,000 (vol/vol) HRP-conjugated streptavidin (Sigma) in 1% skim milk in PB at 37°C for 1 h. *O*-phenylenediamine substrate was added, and the optical density at 490 nm was determined. The optical density of wells without G protein typically were less than 5% of that of wells containing G protein and were subtracted as the background.

The inhibition of G protein binding to cells by soluble competitors was determined by the incubation of HEp-2 cells with 25 µg/ml of biotinylated G protein in the presence of soluble GAGs or modified heparins for 1 h at 37°C. Cells were washed three times with PB and analyzed using the G protein cell binding ELISA. Results are expressed as the percent inhibition relative to that of G protein binding in the absence of GAGs.

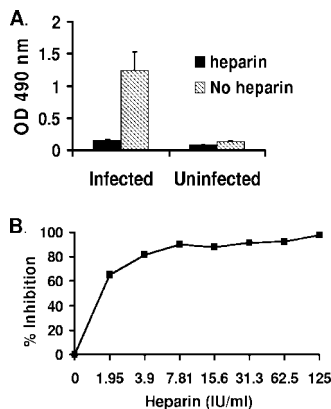


FIG. 2. Effect of heparin on hMPV infectivity. (A) HEp-2 cells were incubated for 2 h with heparin pretreated hMPV. Control cells were incubated with untreated virus (no heparin). Infectivity was assessed by ELISA 48 h postinoculation using a MAb against hMPV matrix protein. Uninfected Hep-2 cells also were treated with heparin or left untreated and analyzed by ELISA. Results shown are for triplicate wells of a representative experiment performed four times. OD 490 nm, optical density at 490 nm. (B) Dose-dependent inhibition of hMPV infectivity by heparin. Results are expressed as percent inhibition relative to values HEp-2 cells infected with untreated virus.

**hMPV infectivity and G protein binding after removal or inhibition of cellular GAGs.** HEp-2 cells were pretreated with various concentrations of heparinase I or heparitinase in PBS containing 1 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 0.1% glucose, and 0.1% bovine serum albumin or with buffer alone for 1 h at 37°C. Cells were washed three times with 199 medium before the analysis of hMPV infectivity or G protein binding as described above.

The contribution of iduronic acid to infectivity and G binding was assessed by the preincubation of HEp-2 cells with serial dilutions of bFGF.

**RESULTS**

**Inhibition of hMPV infectivity by soluble heparin.** The effect of soluble heparin on hMPV infectivity was investigated using a cell infectivity ELISA. hMPV subtype B2 was preincubated with 250 IU/ml heparin for 30 min at 37°C before the inoculation of HEp-2 cells. hMPV infection was assessed 48 h postinoculation and was almost totally blocked by heparin (Fig. 2A). Heparin treatment had no effect on HEp-2 cell growth or viability, indicating that the decreased infection was not due to cell toxicity. Figure 2B shows that heparin decreased hMPV infectivity in a dose-dependent manner, with heparin concentrations as low as 1.95 IU/ml exhibiting more than 60% inhibition.

**Binding of hMPV-G glycoprotein to immobilized heparin.** Previous studies with RSV demonstrated that RSV G protein mediates virus attachment to host cells and binds specifically to heparin (10, 27). To examine if hMPV G protein plays a similar role in infectivity by binding to cellular GAGs, we expressed a recombinant hMPV-G protein comprising the extracellular domain of the G protein of hMPV subtype B2 in *P. pastoris*. The recombinant protein was detected by Western blotting using an anti-c-Myc antibody and also was recognized by antisera raised against an hMPV G peptide (23) kindly provided by Y. Li (Manitoba, Canada). Recombinant G protein migrated at an approximate molecular mass of 50 kDa, which is similar to that for the predominant G species detected following the immunoblotting of hMPV-infected cell lysates with the same anti-

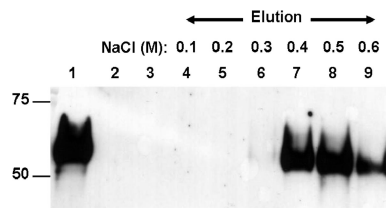


FIG. 3. Western blot analysis following heparin chromatography of recombinant hMPV G. Recombinant proteins were applied to the heparin column four times, the unbound material was collected (fall-through), and the columns were washed with 10 column volumes of PB. The final wash (1 ml) was collected, and bound protein was eluted with a stepwise salt gradient. Fractions were analyzed by SDS-PAGE on a 12.5% gel under reducing conditions, followed by Western blot analysis. The lane designations are as follows: 1, start material; 2, fall-through; 3, final wash; 4 to 9, elution fractions.

body (23). Following the digestion of recombinant G protein with endo- $\alpha$ -N-acetylglucosaminidase H, there was a slight reduction in molecular mass, indicating a low level of N-linked glycosylation (data not shown). Purified recombinant G protein was applied to a heparin agarose column, and after extensive washing the bound protein was eluted using a stepwise salt gradient. The hMPV G protein bound to the heparin column and was detected in the elution fractions (Fig. 3), indicating that hMPV G protein binds to heparin.

We next examined if G protein binds directly to cells by the incubation of HEp-2 with increasing concentrations of biotinylated protein. G protein bound to HEp-2 cells in a saturable manner (Fig. 4). The binding of G protein to cells was inhibited by approximately 70% with an eightfold excess of unlabeled G protein and by 90% with soluble heparin (50 IU/ml), confirming the specificity of G protein binding (not shown).

**Effect of soluble GAGs on hMPV infection and G protein binding.** Since heparin inhibited hMPV infection and G protein binding to HEp-2 cells, we next examined the effect of other soluble GAGs, including porcine intestinal HS, CS-A, CS-B, CS-C, and dextran sulfate. The treatment of hMPV with HS, CS-A, CS-B, CS-C, and dextran sulfate prior to the inoculation of HEp-2 cells inhibited hMPV infection in a dose-dependent manner, while the pretreatment of virus with unsulfated dextran had no effect (Fig. 5).

The effect of competing GAGs on G protein binding to cells also was investigated. HEp-2 cells were incubated with biotinylated G protein in the presence or absence of HS, CS-A, CS-B, CS-C, dextran sulfate, or dextran for 1 h at 37°C. The binding of G protein was inhibited by HS, CS-A, CS-B, CS-C,

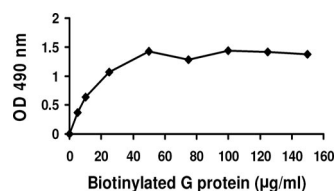


FIG. 4. Binding of G protein to HEp-2 cells. Confluent HEp-2 cell monolayers were incubated with increasing concentrations of biotinylated G protein at 37°C for 1 h in the presence or absence of heparin. After being washed, bound G protein was detected by incubation with streptavidin-HRP. OD 490 nm, optical density at 490 nm.



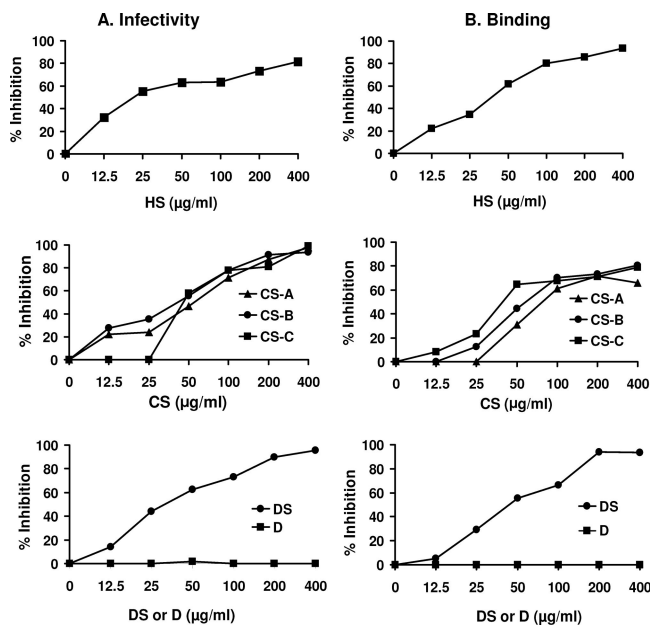


FIG. 5. Effect of HS, CS-A, CS-B, CS-C, dextran sulfate (DS), and dextran (D) on hMPV infectivity and G protein binding. (A) hMPV was pretreated with various concentrations of GAGs prior to the inoculation of HEp-2 cells. Infectivity was assessed at 48 h. (B) Biotinylated G protein was added to HEp-2 cell monolayers in the presence or absence of soluble GAGs, and the binding of G protein was determined after 1 h. Results are expressed as the percent inhibition relative to that of untreated hMPV or G protein binding in the absence of GAGs.

and dextran sulfate in a dose-dependent manner, with 70 to 80% maximal inhibition observed. In contrast, dextran did not inhibit G protein binding (Fig. 5).

**Treatment of cells with GAG lyases reduces infection and G protein binding.** To confirm that cell surface GAGs are involved in hMPV infection, we examined the effect of the removal of cellular GAGs by treatment with heparinase I, heparitinase, or chondroitinase ABC. Heparinase I cleaves highly sulfated domains of both heparin and HS, while heparitinase specifically cleaves HS within poorly sulfated domains (13, 22, 29). HEp-2 cells were treated with various concentrations of GAG lyases prior to the cells being inoculated with hMPV. The treatment of HEp-2 cells with heparinase I inhibited infection by 90% (Fig. 6A), while heparitinase inhibited infection by 97% (Fig. 6A). As heparin is not found on the surface of cells, this effect must be due to the digestion of HS on the cell surface by both enzymes. The pretreatment of HEp-2 cells with chondroitinase ABC also inhibited virus infection, with the highest concentration used in this study (250 mIU/ml) causing 56% inhibition of infection (Fig. 6A). These results provide further evidence for the role of cellular GAGs in hMPV infection.

To extend these findings, G protein binding to GAG lyase-treated cells was examined. All enzymatic treatments inhibited the binding of G protein to HEp-2 cells, with the highest concentrations (5 mIU/ml) of heparinase I and heparitinase inhibiting binding by 70 to 80% (Fig. 6). Chondroitinase ABC treatment also inhibited G protein binding to cells in a concentration-dependent manner (Fig. 6A). These results are sim-

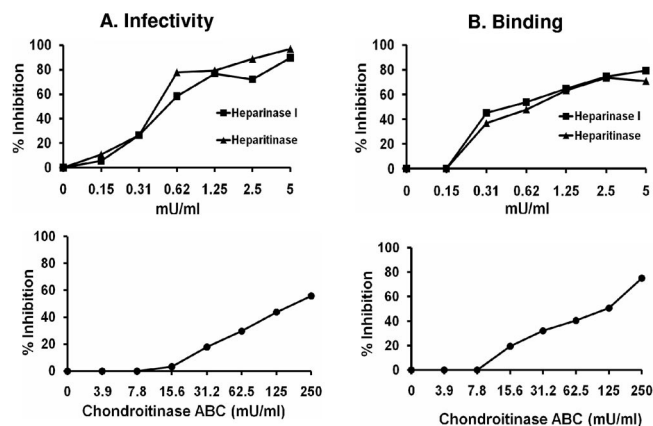


FIG. 6. Effect of the GAG lyase treatment of HEp-2 cells on virus infectivity and G protein binding. HEp-2 cells were treated with heparinase I, heparitinase, or chondroitinase ABC at 37°C for 1 h. Cells were washed three times with serum-free medium before infection with hMPV (A) or the addition of biotinylated G protein (B). hMPV infectivity and the binding of G protein was determined by ELISA. Results are expressed as the percent inhibition of infection or binding relative to that of untreated cells.

ilar to the effects of lyases on hMPV infectivity and confirm that cellular GAGs play a role in hMPV infectivity, which may be mediated by an effect on G protein.

**Role of heparin sulfate and iduronic acid in hMPV infection.** The linkage of heparin sulfate groups previously has been demonstrated to be important in RSV infection (15, 29). To determine the roles of *N*-sulfated and *O*-sulfated GAGs in hMPV infection, the effect of chemically modified heparin with *N*-sulfate groups replaced by *N*-acetyl groups or heparin in which *O*-sulfated groups had been removed was examined. Virus was preincubated with chemically modified heparins or unmodified heparin prior to the inoculation of HEp-2 cells. Virus infectivity was assessed 48 h postinoculation using the infectivity ELISA. As shown in Fig. 7A, de-*O*-sulfate heparin largely retained its ability to inhibit infection, while inhibition by de-*N*-heparin was markedly reduced. Similar results were obtained when the effect of modified heparins on G protein binding was examined (Fig. 7B). These results suggest that the *N*-sulfated domains of heparin play a more pivotal role in mediating hMPV-cellular GAG interactions than *O*-sulfated domains.

Cellular GAGs containing iduronic acid, including heparin, HS, and CS-B, previously have been shown to inhibit RSV infection (13). To determine the role of iduronic acid in hMPV infection, we pretreated HEp-2 cells with bFGF, which binds specifically to GAGs containing iduronic acid (13, 29, 32), prior to inoculation with virus. Infection by hMPV and the binding of G protein was inhibited by bFGF in a dose-dependent manner (Fig. 7).

**hMPV-G protein binding on cells lacking GAGs.** To confirm that the interaction of G protein and cells is mediated via cellular GAGs, the binding of biotinylated G protein to CHO cells was compared to binding to mutant CHO-pgsA745 cells, which lack xylosyltransferase activity and therefore are deficient in cellular GAGs (8). G protein bound to wild-type CHO

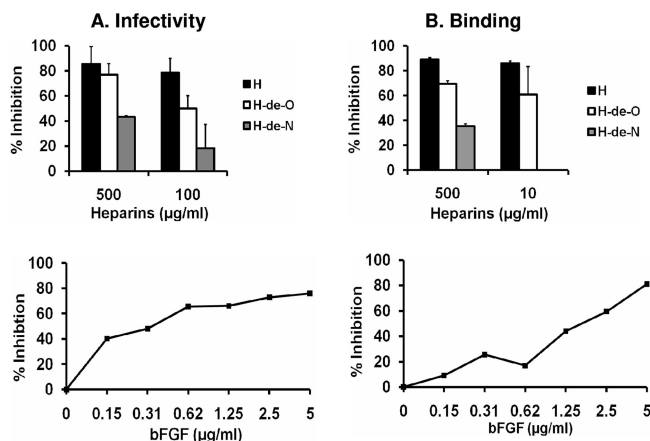


FIG. 7. Effect of chemically modified heparins and bFGF on hMPV infection and G protein binding to HEp-2 cells. (A) hMPV was incubated with 10 or 500 µg/ml heparin (H), chemically modified heparins (de-O-sulfate [H-de-O] or de-N-sulfate [H-de-N]), or bFGF (0–5 µg/ml) for 30 min at 37°C prior to the inoculation of HEp-2 cells. Infectivity was examined 48 h postinoculation. (B) Biotinylated G protein was mixed with 10 or 500 µg/ml of heparin, chemically modified heparins, or bFGF (0 to 5 µg/ml) and incubated with HEp-2 cells. Binding was detected using streptavidin-HRP.

cells in a dose-dependent manner, while no binding was detected on GAG-deficient cells (Fig. 8).

**Functional domains in hMPV G protein involved in GAG interactions.** An analysis of the amino acid sequence of hMPV G protein revealed two adjacent regions of positively charged amino acids that were considered potential heparin binding sites (<sup>149</sup>EKKKTRA<sup>155</sup> and <sup>159</sup>QRRGKGKE<sup>166</sup>). Two truncated G fragments (hMPV-G1 and hMPV-G2) were cloned and expressed in *P. pastoris* (Fig. 1), and binding to heparin was determined. The hMPV-G1 fragment, which lacks the putative heparin binding sites, did not bind to heparin, and all protein was detected in the fall-through (Fig. 9A). In contrast, the hMPV-G2 fragment, which contains both positively charged regions, bound to heparin and was eluted with a salt gradient (Fig. 9A).

To further characterize the heparin binding region(s) in hMPV, alanine substitutions of selected residues in the two positively charged clusters were performed. G-mut1, with alanine substitutions in the first cluster (K151A, K153A, and R155A), showed a substantial but not complete loss of binding

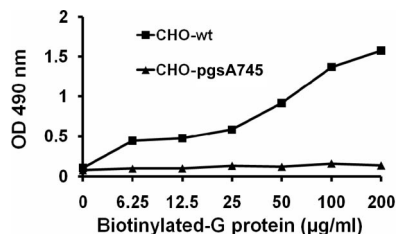


FIG. 8. Binding of hMPV-G protein to wild-type CHO (CHO-wt) and mutant CHO-pgsA745 cells. The cells were incubated with increasing concentrations of biotinylated G protein at 37°C for 1 h in the presence or absence of heparin. After being washed, bound G protein was detected by incubation with streptavidin-HRP. OD 490 nm, optical density at 490 nm.

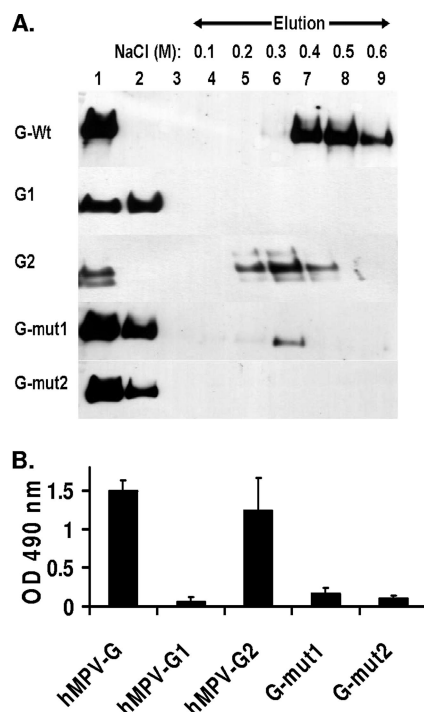


FIG. 9. Binding of hMPV-G (G-wt), hMPV-G1 (G1), hMPV-G2 (G2), G-mut1, and G-mut2 to heparin and HEp-2 cells. (A) Proteins were passed over heparin columns, the unbound material was collected (fall through), and the columns were washed with PB. Bound protein was eluted with a stepwise salt gradient. Fractions were analyzed by SDS–12.5% PAGE and Western blotting. The lane designations are as follows: 1, start material; 2, fallthrough; 3, final wash; 4 to 9, elution fractions. (B) HEp-2 cells were incubated with biotinylated hMPV-G, hMPV-G1, hMPV-G2, G-mut1, or G-mut2 proteins at 37°C for 1 h. Bound G protein was detected by incubation with streptavidin-HRP. OD 490 nm, optical density at 490 nm.

to heparin, with some residual protein detected in the eluate (Fig. 9A). In contrast, substitutions in the second cluster (G-mut2; R161A, K163A, and K165A) abolished all heparin binding (Fig. 9A). These results indicate that the second cluster (<sup>159</sup>QRRGKGKE<sup>166</sup>) on G protein is essential for binding.

hMPV-G, hMPV-G1, hMPV-G2, G-mut1, or G-mut2 also was tested for the ability to bind to HEp-2 cells (Fig. 9B). The results were similar to those obtained by heparin affinity chromatography, with only those proteins retaining the positively charged regions (hMPV-G and hMPV-G2) binding to cells. Neither of the alanine substitution mutants had cell binding activity. The small amount of G-mut1 binding detected by heparin affinity chromatography could not be detected in the cell binding assay.

**Inhibition of hMPV infection by G protein.** To confirm the role of G protein and GAGs in hMPV infection, we examined the effect of treating cells with G protein prior to inoculation. Recombinant hMPV-G protein inhibited virus infection of HEp-2 and LLC-MK2 cells by 80 and 68%, respectively (data not shown).

**DISCUSSION**

Virus attachment and penetration of host cells are essential steps for efficient infection by most viruses, and these are

frequently mediated via interactions with cellular GAGs (28). In this study, the role of GAGs in hMPV infectivity was confirmed by the inhibition of virus infection by heparin. This finding is in agreement with those of Wyde et al. (35), who demonstrated that hMPV infection was inhibited by heparin and also by a sulfated sialyl lipid, NMSO<sub>3</sub>. A number of viruses, including RSV, herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, varicella zoster virus, enterovirus, dengue virus, yellow fever virus, and coronaviruses, also utilize cell surface glycans to facilitate virion attachment and/or fusion (1, 10, 11, 17, 20, 35, 37). This is frequently mediated by electrostatic interactions between positively charged virion proteins and negatively charged cell surface glycans.

Previous studies with RSV strongly suggest that RSV G protein participates in cell glycan recognition, as RSV G protein binds specifically to immobilized heparin (10, 20). Although RSV and hMPV G proteins do not have a high level of amino acid homology, we examined whether hMPV G protein plays a similar role. The ectodomain of hMPV G protein (subtype B2) was cloned and expressed, and it bound to and was eluted from a heparin affinity column. We subsequently demonstrated the binding of hMPV G protein to HEp-2 cells. Binding was dose dependent, saturable, and specific, and it was inhibited by unlabeled ligand and soluble heparin.

The surface of most cells is coated with several GAGs, including HS, CS-A, CS-B, and CS-C. Hallak et al. (13) examined GAG interactions with RSV and determined that heparin, HS, and CS-B, but not CS-A, CS-C, or hyaluronic acid, inhibited infection. Interestingly, bovine kidney HS had no effect on RSV infection, whereas HS from bovine and porcine intestinal mucosa markedly inhibited RSV infection. We characterized the role of different GAGs in hMPV infection and determined that both the infection of HEp-2 cells and G protein binding is inhibited by porcine intestinal HS, CS-A, CS-B, and CS-C. These findings were supported by results from experiments with GAG lyases, including heparinase I, which cleaves highly sulfated polysaccharide chains at the 1-4 linkage between hexosamines and *O*-sulfated iduronic acid of heparin and HS, and heparitinase, which cleaves poorly sulfated domains of HS exclusively. Chondroitinase ABC treatment of cells (which cleaves CS-A, CS-B, and CS-C) also inhibited infection and G protein binding to cells.

The inhibition of hMPV by a broad range of GAGs contrasts with that of RSV, where only cell surface glycans containing iduronic acid (HS and CS-B) but not GAGs lacking iduronic acid (CS-A, CA-C, and hyaluronic acid) inhibit infection (13). Other viruses, including dengue and yellow fever, also have been shown to bind to cells by utilizing a limited repertoire of cell surface GAGs (11). As for RSV (13), however, the specific requirement of iduronic acid remains for hMPV infection, as the pretreatment of cells with bFGF inhibited infectivity and G protein binding.

Dextran sulfate is a heavily sulfated and negatively charged glucose polysaccharide. Dextran sulfate markedly inhibited hMPV infection and the G protein binding of HEp-2 cells, while unsulfated dextran had no effect. This result indicates that sulfate groups are important for hMPV infectivity and G protein binding to cells. Likewise, dextran sulfate but not dextran has been shown to efficiently inhibit RSV infection (15). To further characterize sulfation requirements, we used chem-

ically modified heparins with either *N*-sulfate groups replaced by *N*-acetyl groups or with *O*-sulfate groups removed. De-*N*-sulfate heparin showed a reduced ability to inhibit infection, while de-*O*-heparin largely retained its inhibitory activity. Similar results were obtained when the effect of chemically modified heparin on G protein binding to cells was examined. These results suggest that *N*-sulfate groups of heparin play more of a role in hMPV infectivity than *O*-sulfate groups, which is similar to the sulfation requirements of RSV (14, 15, 29).

To finally confirm the role of cellular GAGs, we found that G protein bound to wild-type CHO cells, while no binding was detected to GAG-deficient CHO-pgsA745 cells. This result, in conjunction with the findings that infectivity and G protein binding are inhibited by soluble GAGs or by the enzymatic removal of cellular GAGs, conclusively shows that cell surface GAGs and G protein interactions play a key role in hMPV infectivity.

The binding of proteins to cellular GAGs is frequently mediated by electrostatic interactions. For example, we have shown by molecular modeling and site-directed mutagenesis that a heparin binding domain in the complement regulatory protein factor H resides in a cluster of positively charged residues (12). The G protein of RSV contains a lysine-rich region (21); peptides from this region bind to heparin, and the pretreatment of cells with these peptides inhibits infectivity (10). Cardin and Weintraub (7) have proposed the consensus motifs (-X-B-B-X-B-X-) and (-X-B-B-B-X-X-B-X-), where B is the probability of a basic residue and X is a hydrophobic residue, that mediate GAG recognition. However, not all heparin binding proteins, including factor H, exhibit this recognition motif, suggesting that the orientation and spacing of amino acids and the local absence of a negative charge also are important. Margalit et al. (24) identified the importance of spatial arrangement and showed that a 20-Å interval between basic amino acids is characteristic of heparin binding proteins. The analysis of the hMPV G protein sequence (subgroup B2) revealed two adjacent positively charged regions that were considered potential heparin binding sites (<sup>149</sup>EKKKTRA<sup>155</sup> and <sup>159</sup>QRRGKKE<sup>166</sup>). To determine the role of these regions in hMPV-GAG interactions, two truncated G fragments (hMPV-G1 and hMPV-G2) were cloned and expressed in yeast. hMPV-G1, which lacks these putative heparin binding sites, did not bind to a heparin affinity column. In contrast, hMPV-G2, which contains both positively charged regions, bound to and was eluted from the column. We subsequently introduced alanine substitutions for the basic amino acids in each cluster, and the mutant proteins were expressed and analyzed for heparin binding. G-mut1 with substitutions in the first cluster (K151A, K153A, and R155A) showed a substantial but not complete loss of binding to heparin. In contrast, G-mut2, containing alanine substitutions within the second cluster (R161A, K163A, and K165A), had a complete loss of binding to heparin. These results indicate that the interaction of viral G protein with heparin is mediated via these clusters of positively charged residues. The identification of the specific cellular receptor for hMPV is under further investigation. It is not currently clear whether the binding of hMPV to GAGs is specific or related to nonspecific electrostatic inter-



actions, or whether it represents the first step in a multivalent receptor process.

Some viruses, such as Sindbis, foot and mouth disease, Ross River, and tick-borne encephalitis viruses, appear to utilize HS as an attachment receptor, but they do so only after cell culture passage (6, 16, 19, 36). Culture-adapted Sindbis virus binds heparin and grows to a higher titer in cell lines but is no longer pathogenic in mice. The sequence analysis of culture-adapted Sindbis and Ross River viruses reveals basic amino acid substitutions that likely create heparin binding sites and expand the host range of the viruses (6, 16, 19). Our results with the GAG dependency of hMPV infection are not, however, a result of virus adaptation during tissue culture passage. We have directly amplified and sequenced hMPV G from nasopharyngeal samples and confirmed that the identified positively charged domains of G protein are present in these nonpassaged isolates. Moreover, the primary isolation of hMPV from clinical samples also is sensitive to heparin (data not shown).

The requirement of RSV and hMPV G protein for viral infectivity has been the subject of some investigation. In the case of RSV, Teng et al. (31) found that the effect of deleting G protein was cell specific. Recombinant RSV lacking G protein infected Vero cells with an efficiency similar to that of wild-type virus, but it replicated poorly in HEp-2 cells. Moreover, in the respiratory tract of mice, the replication of virus lacking G protein was very highly restricted, indicating that G protein is necessary for RSV infection in vivo. Teng et al. (30, 31) also demonstrated that a mutant virus, in which the linear heparin binding domain identified by Feldman et al. (10) was deleted, replicated efficiently. However, additional heparin binding domains in RSV G protein have since been identified by Shields et al. (27), and these authors suggested that Teng's G mutant virus could bind to GAGs via additional heparin binding domains in G protein. For hMPV, a G protein deletion mutant could replicate in cell culture but was markedly attenuated in vivo and was, indeed, proposed as an attenuated vaccine candidate (3). Although we have shown that both hMPV infectivity and G protein binding are mediated by interactions with GAGs, this does not necessarily indicate that the mechanism of the inhibition of infectivity is related solely to a reduction in G protein binding. In the case of RSV, the F protein also plays a role in mediating infection through a GAG-dependent mechanism (9, 29). Recombinant hMPV F protein derived from transfected CHO cells also binds heparin (data not shown), indicating that this protein also facilitates virus infectivity via an interaction with cellular GAGs. Nevertheless, our finding that infection was substantially inhibited by soluble G protein strongly supports a role for this protein in mediating hMPV infection. The inability of G protein to inhibit hMPV infectivity to the level observed with competing GAGs suggests that the more complete inhibition also reflects GAG interactions with other virion proteins, most probably F protein.

In summary, these experiments provide strong evidence for an important role of cellular GAGs in mediating hMPV infection. Further studies are under way to determine GAG binding domains in G protein from other hMPV lineages and F protein and to characterize the structural requirements for these interactions.

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