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Expression of the Recombinant Anchorless N-Terminal Domain of Mouse Hepatitis Virus (MHV) Receptor Makes Hamster or Human Cells Susceptible to MHV Infection

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Mouse hepatitis virus (MHV) receptor, the receptor for the murine coronavirus MHV, was expressed in MHV-resistant hamster and human cells as a series of mutant, recombinant glycoproteins with carboxy-terminal deletions lacking the cytoplasmic tail, transmembrane domain, and various amounts of the immunoglobulin constant-region-like domains. The soluble receptor glycoproteins containing the N-terminal virus-binding domain were released into the supernatant medium and inactivated the infectivity of MHV-A59 virions in a concentration-dependent manner. Surprisingly, some of the anchorless glycoproteins were found on the plasma membranes of transfected cells by flow cytometry, and these cells were rendered susceptible to infection with three strains of MHV. Thus, in the cells in which the anchorless, recombinant receptor glycoprotein is synthesized, some of the protein is bound to an unidentified moiety on the plasma membrane, which allows it to serve as a functional virus receptor.

The cellular receptors for the murine coronavirus mouse hepatitis virus (MHV) are encoded by genes in the biliary glycoprotein (Bgp) family in the immunoglobulin (Ig) gene superfamily (19, 20). Two murine genes, *Bgp1* and *Bgp2*, encode multiple glycoprotein isoforms that are generated by alternative splicing of mRNA and have MHV receptor activity (7, 14, 21, 22). The *Bgp1*^{a} gene encodes MHV receptor (MHVR), the first glycoprotein found to have MHV receptor activity (8). Deletion mutagenesis studies of MHVR have shown that the virus binds to the N-terminal Ig-like domain (10). Anti-MHVR monoclonal antibody (MAb) CC1, which blocks MHV infection mediated by MHVR, also binds to the N-terminal domain of MHVR (14, 16). Although a recombinant chimeric protein in which the N-terminal domain of MHVR is linked to the second, third, transmembrane, and cytoplasmic domains of the mouse poliovirus receptor homolog has MHV receptor activity (6), a deletion mutant, Δ(108–381), in which the N-terminal domain alone was linked to the transmembrane and cytoplasmic domains of MHVR did bind virions but had no receptor activity (9).

We generated four mutants of MHVR, named R111, R111S−, R130, and R400, by site-directed mutagenesis (18) or by restriction enzyme digestion followed by self ligation (for R400). The numerals in the designations of the mutant glycoproteins indicate the numbers of amino acids in the open reading frames of the mature proteins (Fig. 1). R111S− differs from R111 in that the N residues in positions 37, 55, and 70 were replaced with Q residues so that R111S− lacks the three potential N-linked glycosylation sites in the N-terminal domain (6). Several amino acids at the carboxy termini of these mutants which are not present in MHVR resulted from shifts in the open reading frame prior to a stop codon (Fig. 1B) (8). Because they lack carboxy-terminal transmembrane domains, the R111, R111S−, R130, and R400 glycoproteins would be expected to be released into the supernatant medium. BHK-21 cells were infected with vaccinia virus vTF7-3 and then transfected with the cDNAs encoding these glycoproteins and the anchored glycoproteins Δ(108–381) and MHVR (10). Proteins of the predicted sizes were detected in the cell lysates by immunoblotting with anti-MHVR MAb-CC1 (Fig. 2). Proteins encoded by the four anchorless mutants were secreted into the supernatant medium, but the anchored proteins encoded by the Δ(108–381) and MHVR cDNAs were not secreted (Fig. 2; R111S− not shown).

For subsequent studies, we used R111 because it is the shortest recombinant protein and has the fewest non-MHVR amino acids at its C terminus. We investigated whether secreted R111 could bind to MHV-A59 virions and neutralize their infectivity. Serial twofold dilutions of supernatant medium from vTF7-3-infected cells transfected with R111 cDNA or empty plasmid (control medium) were incubated for 8 h at room temperature with 10^5 PFU of MHV-A59. The treated virus was allowed to adsorb to L2 cell monolayers in 96-well plates for 1 h at 37°C. Eight hours after virus inoculation, the cells were fixed with cold methanol and the amount of MHV N antigen in the monolayers was detected in an enzyme-linked immunosorbent assay with a MAb directed against the nucleocapsid protein N (kindly provided by J. L. Leibowitz, Texas A&M Health Sciences Center, College Station, Tex.). Figure 3 shows that the soluble R111 glycoprotein inactivated virus infectivity in a concentration-dependent manner, while control medium did not inactivate the virus. Neutralization of virus

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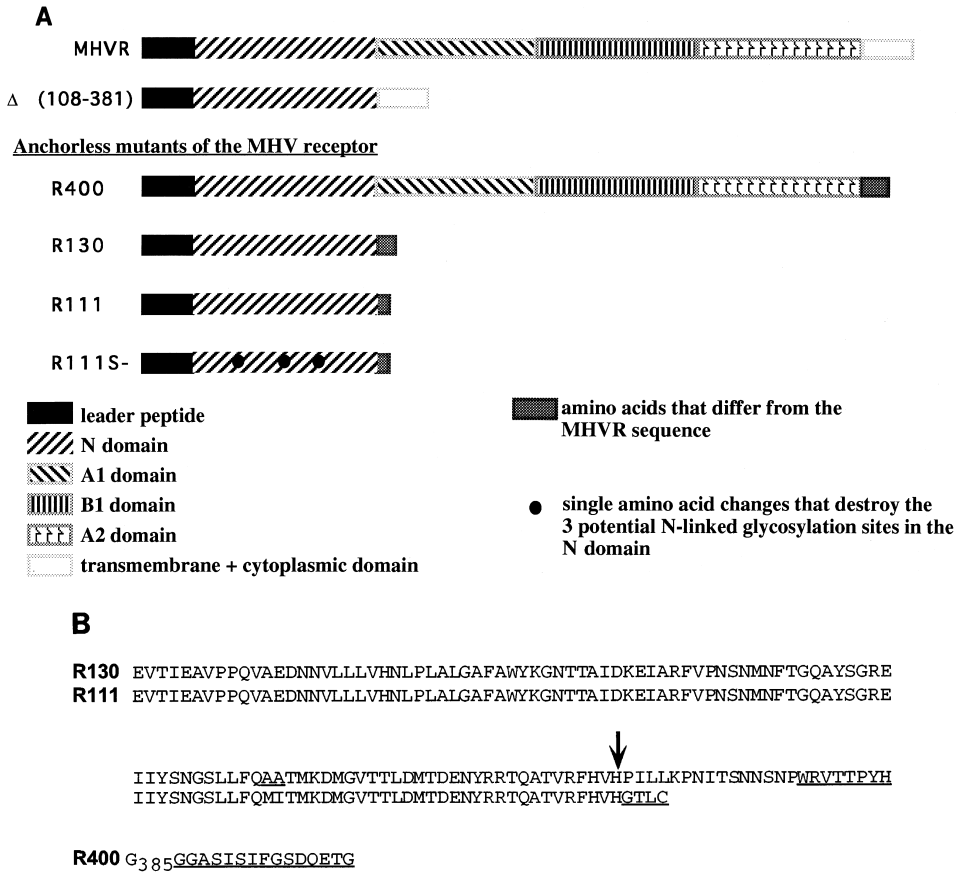

FIG. 1. (A) Schematic representation of MHVR, anchored deletion mutant Δ(108–381), and four anchorless recombinant mutants of MHVR that lack the transmembrane and cytoplasmic domains. (B) Amino acid sequences of mutants R130, R111, and R400. The predicted amino acid sequences of the mature glycoproteins encoded by R130 and R111 cDNAs are shown. The carboxy terminus of R400 starting at amino acid 385 of the mature MHVR glycoprotein is shown. The underlined amino acids in R111, R130, and R400 differ from the sequence of MHVR. The arrow indicates the end of the N-terminal domain of MHVR.

infectivity by other anchorless, recombinant receptors in the Ig superfamily has also been observed for intercellular adhesion molecule 1 for rhinoviruses (3, 12), poliovirus receptor for poliovirus (11), and CD4 for human immunodeficiency virus type 1 (2, 4, 5).

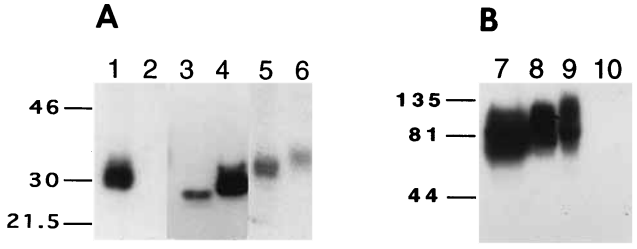

FIG. 2. Immunoblot analysis with MAb-CC1. BHK-21 cells were infected with vaccinia virus vTF7-3 and transfected with Bluescript SK(+) containing the cDNAs for Δ(108–381) (lanes 1 and 2), R111 (lanes 3 and 4), R130 (lanes 5 and 6), R400 (lanes 7 and 8), and MHVR (lanes 9 and 10) under the control of the T7 promoter. Twenty-four hours posttransfection, the media were collected, concentrated, and filtered and the cells were lysed. Forty microliters of concentrated medium (lanes 2, 4, 6, 8, and 10) or cell lysate (lanes 1, 3, 5, 7, and 9) was analyzed in either sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (A) or SDS–8% polyacrylamide gels (B) and transferred to nitrocellulose. The proteins were detected with anti-MHVR MAb-CC1, followed by rabbit anti-mouse IgG and ^{125}I-staphylococcal protein A. Molecular mass standards are indicated in kilodaltons on the left of each panel.

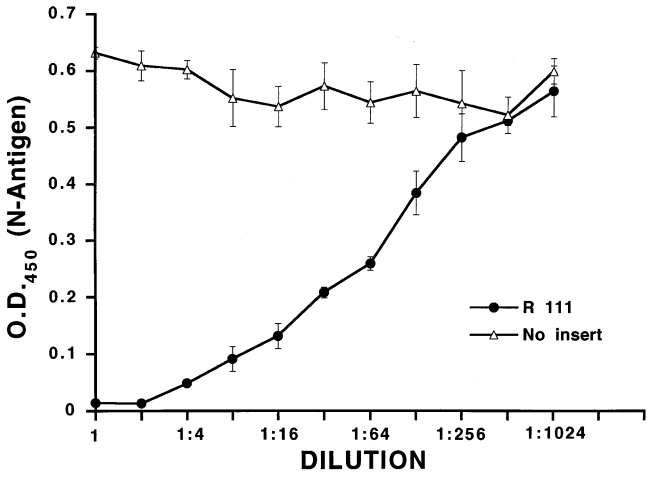

FIG. 3. Neutralization of MHV-A59 with anchorless, recombinant R111 glycoprotein. MHV-A59 was incubated for 8 h at 25°C with serial twofold dilutions of either filtered, 5× concentrated medium from cultures expressing anchorless, recombinant R111 or medium from control cultures transfected with an empty plasmid (no insert). After the incubation period, the virus was allowed to adsorb to L2 cells. After 8 h, the cells were fixed, and the MHV N antigen was detected with an anti-N MAb. Error bars indicate the standard deviation from six replicate assays. O.D.$_{450}$, optical density at 450 nm.

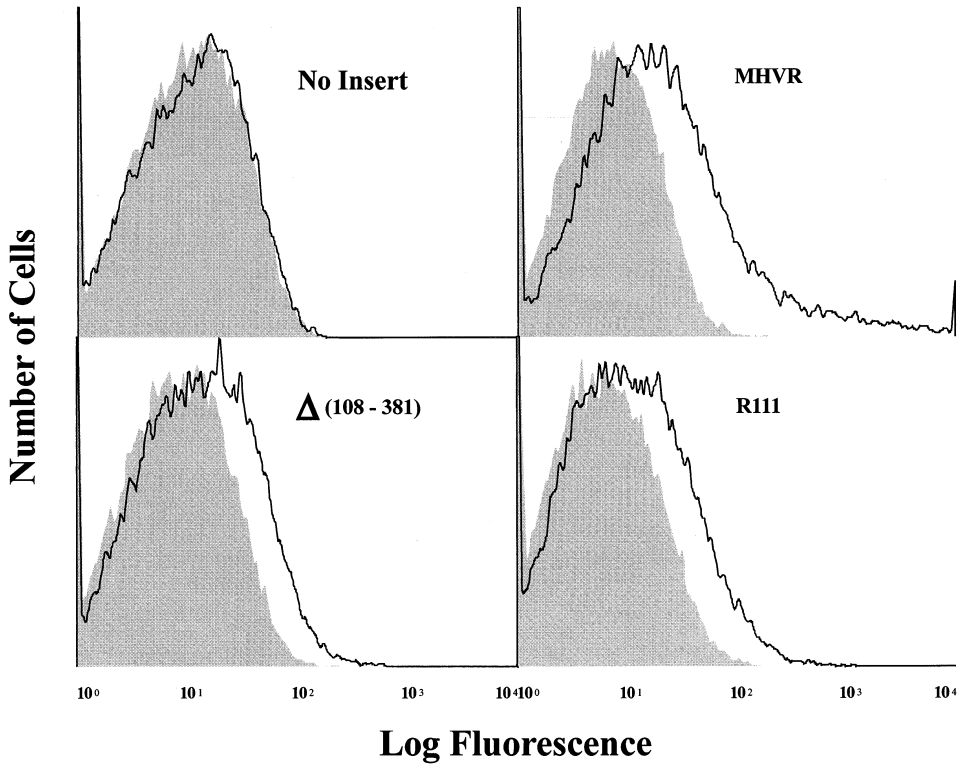

FIG. 4. Flow cytometric analysis of BHK-21 transiently transfected with MHVR or anchored or anchorless N-terminal domain-containing mutants of MHVR. BHK-21 cells transfected with a plasmid containing no insert, MHVR, Δ(108–381), or anchorless N-terminal-domain mutant R111 were fixed with 2% paraformaldehyde and then incubated with a control murine IgG1 antibody (shaded area) or with anti-MHVR MAb CC1 (solid line). Bound antibodies were detected with affinity-purified rabbit anti-mouse IgG R-phycoerythrin conjugate in a fluorescence-activated cell sorter. Data are representative of two repeated experiments.

Surprisingly, even though R111 does not encode a transmembrane domain, it was detected on the plasma membranes of transfected hamster cells by flow cytometry with anti-MHVR MAb-CC1 (Fig. 4). The presence of the anchorless R111 glycoprotein on the cell membrane made the transfected cells susceptible to infection with three strains of MHV (Fig. 5). Similar results were observed in human RD cells, HeLa cells, and hamster cells transfected with cDNAs encoding R111, R130, or R400 (Fig. 6 and data not shown). The percentage of cells infected with MHV observed in the three cell lines expressing anchorless MHVR mutants was significantly lower than that observed in cell lines expressing the full-length anchored MHVR glycoprotein (data not shown). MAb CC1 pretreatment of hamster cells expressing the anchorless recombinant R111 glycoprotein prevented MHV infection (Fig. 5). In marked contrast, even prolonged incubation of nontransfected hamster cells with soluble anchorless R111 glycoprotein, with or without Lipofectamine reagent, did not make the cells susceptible to infection (Fig. 5). These results suggest that in order to function as an MHV receptor, the anchorless R111 must be synthesized in the cell.

These studies show that a recombinant soluble MHVR glycoprotein as small as the N-terminal domain alone is able to confer susceptibility upon the hamster or human cells that express it and that no other Ig-like domains or transmembrane or cytoplasmic domain is required for the initiation of virus infection. None of the four recombinant glycoproteins studied had a sufficient number of hydrophobic C-terminal amino acids to serve as a membrane anchor. We postulate that some of the anchorless MHVR glycoproteins produced in the transfected

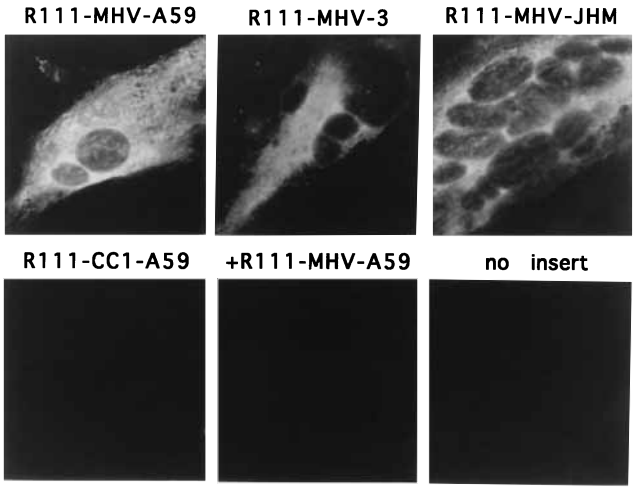

FIG. 5. Detection of MHV antigens in hamster cells transfected with a plasmid containing the R111 cDNA and then challenged with three strains of MHV. The top row shows BHK-21 cells grown on coverslips that were transfected with a plasmid containing the cDNA encoding the recombinant, anchorless R111 glycoprotein under the control of the cytomegalovirus promoter. Transfected cells were inoculated with MHV-A59, MHV-3, or MHV-JHM, incubated for 16 h, and then fixed with cold acetone. The panel labeled "R111-CC1-A59" shows BHK-21 cells transfected with R111 cDNA and pretreated with anti-MHVR MAb-CC1 prior to and during incubation with MHV-A59. "+R111-MHV-A59" indicates BHK-21 cells pretreated for 8 h with concentrated medium containing R111 glycoprotein and then inoculated with MHV-A59. "No insert" indicates cells transfected with a plasmid with no insert and then challenged with MHV-A59. The development of viral antigens in the cytoplasm was detected by immunofluorescence with anti-MHV convalescent-phase serum and rhodamine-labeled goat anti-mouse IgG.

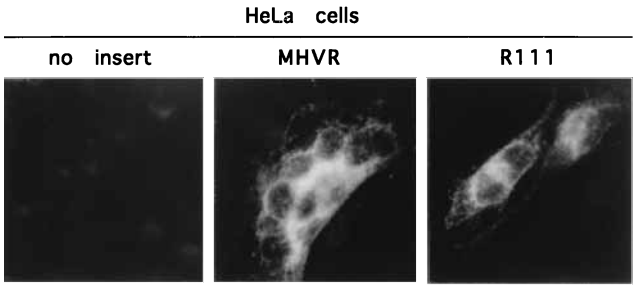

FIG. 6. Detection of MHV antigens in human cells transfected with plasmid containing no insert or MHVR or R111 cDNAs and then challenged with MHV-A59. Transfected HeLa cells were inoculated with MHV-A59, and viral antigens in the cytoplasm of the cells were detected by immunofluorescence as described in the legend for Fig. 5.

cells remain associated with a carrier molecule on the plasma membrane and that this cell-bound but anchorless MHVR serves to bind the virus to the cell and initiate infection. The nature of the cellular carrier for soluble MHVR is not yet known. Perhaps it is a common glycoprotein chaperone protein that becomes associated with the nascent, anchorless MHVR glycoproteins in the rough endoplasmic reticulum or Golgi apparatus and helps to transport them to the plasma membrane. Expression of chaperone proteins bound to soluble Igs has been demonstrated previously (13). Alternatively, since Bgps have been shown by chemical cross-linking experiments to be expressed on the cell membrane as non-covalently linked dimers (14a), it is possible that the cellular carrier for soluble recombinant MHVR is a cellular Bgp or a Bgp-related protein that is anchored in the cell membrane.

The observation that anchorless, soluble MHVR glycoproteins can serve as functional receptors for MHV by using a carrier molecule to achieve expression on the plasma membrane suggests a novel mechanism by which some viruses could enter cells. If a virion can bind to a specific anchorless receptor molecule that is associated with the plasma membrane through a heterodimeric interaction with an anchored carrier molecule, and if the carrier can provide the steps needed for virus penetration, then infection could result. An example of such naturally occurring anchorless receptor glycoproteins for some MHV strains might be the pregnancy-specific glycoproteins (PSGs), which like Bgps are members of the carcinoembryonic antigen family expressed in humans and rodents (1, 15, 17). Unlike Bgps, PSGs do not contain membrane anchor sequences, but we postulate that they might be expressed in small amounts on the cell membrane if they can form heteromeric association with an anchored molecule such as Bgp.

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ADDENDUM

While the manuscript was being reviewed, Chen et al. published a paper showing that expression in COS-7 cells of a recombinant PSG that is normally expressed in murine brain can make the cells susceptible to MHV infection (3a). We suggest that this anchorless PSG virus receptor may be associated with the cell membrane by the same mechanism used by the anchorless, soluble MHVR glycoproteins that we show above can serve as MHV receptors.

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