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Mouse Hepatitis Virus Receptor Activities of an MHVR/mph Chimera and MHVR Mutants Lacking N-Linked Glycosylation of the N-Terminal Domain

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Mouse hepatitis virus binds to the N-terminal domain of its receptor, MHVR, a murine biliary glycoprotein with four immunoglobulin-like domains (G. S. Dveksler, M. N. Pensiero, C. W. Dieffenbach, C. B. Cardellichio, A. A. Basile, P. E. Elia, and K. V. Holmes, Proc. Natl. Acad. Sci. USA 90:1716–1720, 1993). A recombinant protein with only the anchored N-terminal domain was not a functional receptor, but a recombinant protein with the N-terminal domain of MHVR linked to the second and third immunoglobulin-like domains and anchor from the mouse poliovirus receptor homolog, mph, was a functional receptor for mouse hepatitis virus. The native four-domain MHVR has 16 potential N-linked glycosylation sites, including three on the N-terminal domain. Recombinant proteins lacking each one of these three sites or all three of them were functional receptors. Thus, glycosylation of the N-terminal domain is not required, but a glycoprotein longer than the N-terminal domain is required for virus receptor activity.

Mouse hepatitis virus (MHV)-A59 can initiate infection by interacting with MHVR, a 110- to 120-kDa glycoprotein in the murine biliary glycoprotein (Bgp) subfamily in the carcinoembryonic antigen family (2, 6, 27, 28). This receptor was identified in MHV-A59-susceptible Swiss Webster and BALB/c mouse strains. It consists of a variable-like (V-like) immunoglobulin-type domain followed by three constant immunoglobulin-like domains, a transmembrane segment, and a short intracytoplasmic tail (6). Isoforms of the MHVR glycoprotein derived by alternative splicing of the mRNA can also act as MHV receptors when the recombinant proteins are expressed in BHK cells (5, 29). Natural splice variants have either two or four immunoglobulin domains, with cytoplasmic tails either 10 or 72 amino acids long (5, 16). The Bgps are highly glycosylated; on the four-domain receptor Bgp1[a] (MHVR1) there are 16 potential N-linked glycosylation sites, including 3 in the N-terminal domain, 6 in the second, 5 in the third, and 2 in the fourth domain.

A recombinant glycoprotein with only the N-terminal domain, followed by the transmembrane domain and cytoplasmic tail of MHVR, is recognized in immunoblots by anti-receptor monoclonal antibody (MAb) CC1, which blocks infection but does not confer susceptibility to MHV-A59 when expressed in BHK cells (7, 23). Recombinant glycoproteins containing the N-terminal domain with either the second or the fourth immunoglobulin domain of MHVR between the N-terminal domain and the transmembrane domain bind MAb CC1 in blots, bind virus in a virus overlay protein blot assay (VOPBA), and are functional receptors (7). To investigate the specificity of the additional domain and the cytoplasmic tail needed for MHV receptor function, we analyzed the MHV receptor function of a chimeric protein, designated MHVR/mph, in which the N-terminal domain of MHVR is joined to the second and third immunoglobulin-like domains, transmembrane domain, and cytoplasmic tail of the mouse poliovirus receptor homolog (mph) (18). To engineer the MHVR/mph chimera, a unique *Kpn* restriction site was created in the MHVR cDNA, resulting in the substitution of amino acids P and I at positions 108 and 109 for D and P, respectively, yielding a mutant called $MHVR_{(PI\text{-}DP)}$. The chimeric MHVR/mph glycoprotein thus includes domain 1 up to amino acid position 109 of $MHVR_{(PI\text{-}DP)}$, followed by the amino acid sequence of mph starting at position 141. $MHVR_{(PI\text{-}DP)}$ and MHVR/mph glycoproteins were tested for their abilities to be recognized by MHV-A59 virus in a VOPBA or by the anti-receptor MAb CC1 in immunoblots and for their abilities to act as receptors for MHV-A59 virions. Figure 1A shows that both $MHVR_{(PI\text{-}DP)}$ (110 kDa) and MHVR/mph (70 kDa) were recognized in immunoblots by MAb CC1. To test for MHV-A59 receptor function, BHK cells transfected with cDNA encoding each recombinant glycoprotein under the T7 promoter were infected with vaccinia virus vTF7-3 (9), incubated at 37°C for 1 h, treated with 40 μg of 1-β-D-arabinofuranosylcytosine (Sigma Chemical Co., St. Louis, Mo.) per ml to inhibit vaccinia virus replication, incubated for an additional 2 h, and then inoculated with MHV-A59 (multiplicity of infection, 3 PFU per cell) for 1 h at 37°C. Additional medium (3 ml) was added, and the cultures were incubated for 16 h at 37°C, fixed at −20°C with acetone, and then immunolabeled to detect infected cells in which MHV-A59 antigens had been synthesized. Expression of the recombinant mph alone did not make BHK cells susceptible to MHV-A59 (data not shown). In contrast, both $MHVR_{(PI\text{-}DP)}$ and MHVR/mph were functional receptors for MHV-A59 when expressed in BHK cells with the vaccinia virus T7 system (Fig. 2). These experiments show that the N-terminal domain of MHVR is sufficient to confer the specificity for MHV-A59 receptor activity on the chimeric protein. The failure of the anchored N-terminal domain alone to serve as a functional receptor suggests that the longer mph-MHVR chimeric protein provides some structural feature required for virus penetration. The constant immunoglobulin domains of mph may serve as spacers to raise the MHVR N-terminal domain high enough above the glycocalyx to permit virus binding, as postulated for ICAM-1 receptors for human rhinovirus (24). Alternatively, the presence of the constant-like domains may affect the structure or the flexibility of the glycoprotein, altering its virus receptor activity (24, 26). Per-

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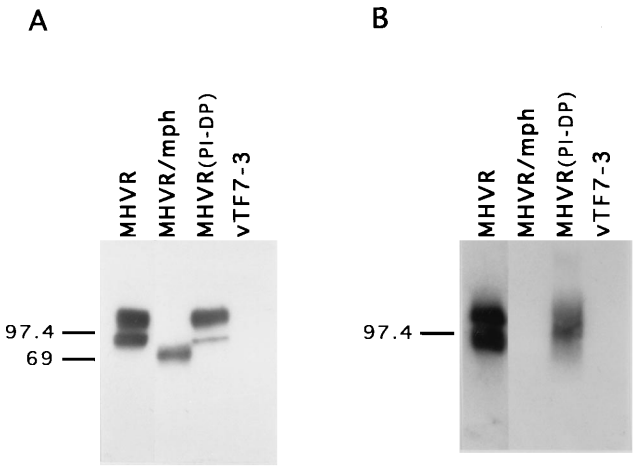

FIG. 1. Recognition of MHVR/mph chimera and mutant $MHVR_{(PI-DP)}$ glycoproteins by anti-receptor MAb CC1 and MHV-A59 virions. BHK cells were infected with vaccinia virus vTF7-3, and at 2 h after virus inoculation, plasmid DNAs containing the indicated cDNA under the T7 polymerase promoter were transfected into the cells, using lipofectAMINE (Life Technologies, Gaithersburg, Md.). The cells were lysed at 24 h postinfection, and the recombinant proteins were analyzed by immunoblotting with MAb CC1 (A) and by VOPBA for MHV-A59 binding activity (B) as previously described (2, 5). Molecular mass standards (in kilodaltons) are indicated.

haps the mph portion of the chimera interacts with a molecule such as CD44, an accessory factor required for poliovirus penetration in human cells.

Although the MHVR/mph chimera on hamster cell membranes was a functional MHV receptor, the glycoprotein did not bind virus in VOPBA (Fig. 1B). We previously used deletion mutants of MHVR to show that the binding determinants for both the MHV-A59 spike glycoprotein and the MAb CC1 are on the N-terminal domain of MHVR (7). Although the recombinant deletion mutant containing the N-terminal domain, transmembrane, and cytoplasmic tail MHVR is not recognized by the virus in a VOPBA, a secreted protein containing the N-terminal domain and 24 amino acids of the second domain is recognized (7). These results show that to retain VOPBA activity, the N-terminal domain of MHVR must be expressed in the presence of the first 24 amino acids or all of a constant-like immunoglobulin domain such as domain 2 or 4 of MHVR.

To determine whether N-linked glycosylation of the N-terminal domain of MHVR is essential for virus or MAb CC1 binding or for receptor function, we introduced point mutations that eliminated each of the three N-linked glycosylation sites on the N-terminal domain, either separately or in combinations, by replacing asparagine with glutamine residues at positions 37, 55, and 70. The mutant MHVR glycoproteins expressed in the vaccinia virus T7 system migrated at about 110 to 97 kDa in sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fig. 3). The faster migrating of the double bands detected in Fig. 1 and 3 are probably glycoproteins that have been incompletely processed as observed with other recombinant glycoproteins expressed with the T7 vaccinia virus system (19). MHVR mutants lacking any one of the three glycosylation sites or both the first and second sites were recognized by MAb CC1 in immunoblots (Fig. 3) and by MHV-A59 virions in VOPBA (data not shown). The MHVR mutant that lacks all three of the glycosylation sites was recognized by MAb CC1 (Fig. 3) but not by MHV-A59 in VOPBA (data not shown). Thus, N-linked oligosaccharides in the N-terminal domain of MHVR are not required for recognition by MAb CC1, but glycosylation of at least one of the three sites in the N-terminal domain is needed for recognition by MHV-A59 virions in VOPBA.

We previously demonstrated that 50-kDa MHVR proteins obtained from tunicamycin-treated cells or from in vitro translation without microsomal membranes were not recognized by MAb CC1 in immunoblots or by MHV-A59 virions in VOPBA (19), although both MAb CC1 and MHV-A59 recognize the N-terminal domain of MHVR (7). The failure of virus to bind to tunicamycin-treated MHVR in a VOPBA may be explained by the requirement for at least one of the three N-linked glycosylation sites in the N-terminal domain of MHVR. In addition, glycosylation in any of the other domains or other posttranslational modifications of MHVR may be required for virus receptor function and/or for binding of virus in a VOPBA.

BHK cells transiently transfected with cDNAs encoding the glycosylation mutants of MHVR were challenged with MHV-A59, and the virus receptor function of the mutant proteins was determined by detection of viral antigens in the cytoplasm. Figure 4 shows that expression of each of the five mutants made BHK cells susceptible to virus infection and that none of

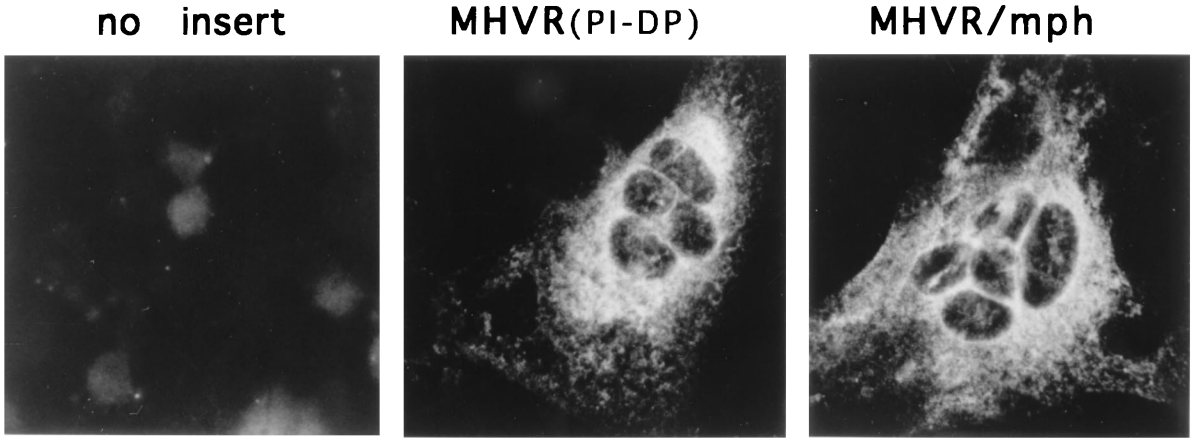

FIG. 2. Immunofluorescence of MHV-A59 antigens in the cytoplasm of BHK cells expressing recombinant MHVR/mph and $MHVR_{PI-DP)}$ glycoproteins. BHK cells expressing these glycoproteins as described in the legend to Fig. 1 were inoculated with MHV-A59 at 3 h after transfection. MHV antigens in the cells were detected 16 h after MHV-A59 inoculation by indirect immunofluorescence with mouse anti-MHV antibody and rhodamine-labeled goat anti-mouse immunoglobulin G.

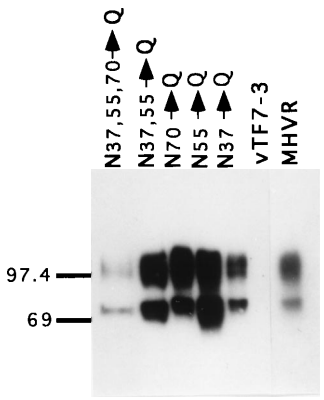

FIG. 3. Effects of removal of the three potential N-linked glycosylation sites in the N-terminal domain of MHVR in the binding of the anti-receptor MAb CC1. Point mutations that delete one or more potential N-linked glycosylation sites in the N-terminal domain of MHVR are indicated by the position(s) and nature of the amino acid substituted. The lane labeled vTF7-3 corresponds to the cell lysate obtained from cells infected with vTF7-3 and transfected with a plasmid containing no insert. Samples were immunoblotted with anti-receptor MAb CC1. Molecular mass standards (in kilodaltons) are indicated.

the three N-linked sugars in the N-terminal domain of MHVR was required for MHV receptor activity. Interestingly, the mutant glycoprotein that lacks all three glycosylation sites in the N-terminal domain was a functional receptor, although it was negative for virus binding in VOPBA. The assay for virus infectivity depends on virus binding to undenatured MHVR glycoproteins on living cell membranes rather than binding of virus to SDS-treated proteins as in the VOPBA. Clearly, the virus receptor assay is a more sensitive indication of the virus binding activity of a receptor protein than is the VOPBA. Therefore, some proteins that have receptor activity are negative by VOPBA, as shown for the $mmCGM_2$ (also called $Bgp1^b$) glycoprotein expressed in SJL/J mice (5, 30).

Viruses in unrelated groups have evolved to use glycoproteins in the immunoglobulin superfamily as receptors, including ICAM-1, CD4, and PVR, which are receptors for the major human rhinovirus group, human immunodeficiency virus (HIV), and poliovirus, respectively (10, 14, 17). HIV, rhinoviruses, polioviruses, and MHV all bind to the N-terminal variable-like immunoglobulin domains of their receptors (1, 3, 8, 12, 13, 15, 20). N-linked glycosylation of the N-terminal domains of CD4, ICAM-1, and PVR is not required for recognition by the virus attachment proteins of HIV, human rhinoviruses, and polioviruses, respectively (1, 15, 31). However, glycosylation of other domains in ICAM-1 may be required for proper folding, intracellular transport, and/or subsequent posttranslational modifications (13, 15). Immunoglobulin-like proteins are highly glycosylated, and the extent of glycosylation can modulate their interactions with their natural ligands. For example, elimination of N-linked glycosylation sites in the third domain of ICAM-1 by mutation enhances its

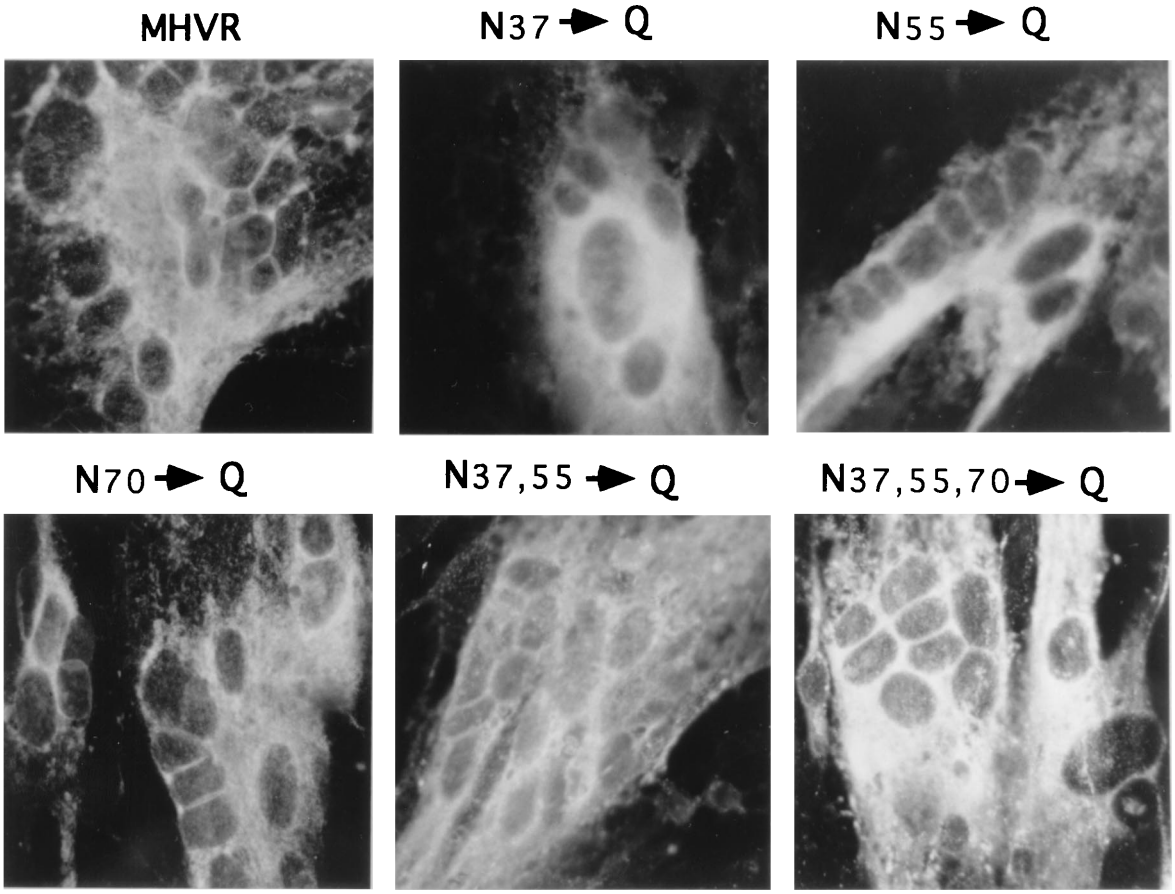

FIG. 4. Effects of removal of the three potential N-linked glycosylation sites in the N-terminal domain of MHVR in its virus receptor activity. BHK cells grown on coverslips were transfected with the indicated cDNAs cloned into pcDNA3 (Invitrogen, San Diego, Calif.). The cells were inoculated with MHV-A59 at 50 h posttransfection, and virus antigens were detected 16 h after MHV-A59 inoculation by immunofluorescence, as indicated in the legend to Fig. 2.

binding to purified Mac-1 (4). Similarly, alteration of N-linked glycosylation of CD44 may affect its avidity for hyaluronic acid (11).

The virus specificity of the virus receptors in the immunoglobulin family resides in their N-terminal domains. Substitution of the second and/or subsequent domains of PVR by those of ICAM-1 or CD4 or substitution of the C-terminal domains of MHVR by those of mph yields chimeric glycoproteins that are functional receptors only for the virus that binds to the N-terminal domain (21, 22, 25). The three-dimensional structures of the N-terminal domains of these receptors, together with the amino acid sequences at or near their virus-binding sites, apparently confer virus specificity as well as specificity for the binding of the various cellular ligands of these immunoglobulin-related glycoproteins.

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