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Mouse hepatitis virus strain A59 and blocking antireceptor monoclonal antibody bind to the N-terminal domain of cellular receptor

(carcinoembryonic antigen glycoprotein)

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ABSTRACT **Mouse hepatitis virus (MHV) strain A59 uses as cellular receptors members of the carcinoembryonic antigen family in the immunoglobulin superfamily. Recombinant receptor proteins with deletions of whole or partial immunoglobulin domains were used to identify the regions of receptor glycoprotein recognized by virus and by antireceptor monoclonal antibody CC1, which blocks infection of murine cells. Monoclonal antibody CC1 and MHV-A59 virions bound only to recombinant proteins containing the entire first domain of MHV receptor. To determine which of the proteins could serve as functional virus receptors, receptor-negative hamster cells were transfected with recombinant deletion clones and then challenged with MHV-A59 virions. Receptor activity required the entire N-terminal domain with either the second or the fourth domain and the transmembrane and cytoplasmic domains. Recombinant proteins lacking the first domain or its C-terminal portion did not serve as viral receptors. Thus, like other virus receptors in the immunoglobulin superfamily, including CD4, poliovirus receptor, and intercellular adhesion molecule 1, the N-terminal domain of MHV receptor is recognized by the virus and the blocking monoclonal antibody.**

Mouse hepatitis virus (MHV) strain A59 is a coronavirus that infects the respiratory and digestive tracts and nervous system of susceptible strains of mice (1, 2). Antireceptor monoclonal antibody (mAb) CC1 blocks MHV-A59 infection *in vitro* and inhibits virus replication *in vivo* (3, 4). The cellular receptor for MHV-A59 (MHVR) is a murine member of the biliary glycoprotein subfamily in the carcinoembryonic family of glycoproteins in the immunoglobulin superfamily (5, 6). Expression in human or hamster cells of the cDNA (MHVR1) encoding MHVR renders these normally resistant cells susceptible to MHV-A59 infection (6). The extracellular portion of the mature 110- to 120-kDa MHVR glycoprotein contains four immunoglobulin-like domains (6). Membrane-associated posttranslational modification of MHVR is necessary for virus and mAb-CC1-binding activities (7).

We engineered deletion mutants of MHVR to identify the domains essential for MHV and mAb-CC1 binding and for functional MHVR activity. Although mAb-CC1 bound to the first, or N-terminal, domain of MHVR, and MHV-A59 virions bound to proteins containing this region plus 24 amino acids between the first and second domains, functional MHVR activity leading to production of viral antigens after MHV-A59 challenge required both the N-terminal domain and one additional immunoglobulin-like domain.

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MATERIALS AND METHODS

Viruses and Antisera. Propagation of MHV-A59, vaccinia vTF7-3 (a recombinant virus containing the T7 RNA polymerase gene; from Bernard Moss, National Institutes of Health), vaccinia strain WR, and vSC11 (a vaccinia recombinant containing no insert) has been described (8–10). Polyclonal rabbit antibody 655 directed against immunoaffinity-purified MHVR (6), anti-MHVR mAb-CC1 (4), and rabbit serum directed against a 15-amino acid synthetic peptide at the N terminus of mature MHVR (anti-NTR) (3) were preabsorbed against vaccinia vSC11-infected BHK cells. Antiserum against MHV-A59 was from BALB/c mice 4 weeks after oronasal virus inoculation.

Construction of pMHVR1*. To obtain a short construct (pMHVR1*) containing the entire coding sequence of MHVR, clone p10.4, which encodes MHVR starting at amino acid 10 and extending to the first of the two polyadenylylation signals of MHVR1 (6), was ligated to a fragment of MHVR1 that contains 69 bases of 5′ untranslated sequence, bases encoding for the leader peptide, and the first 70 amino acids of the mature protein.

Construction of Deletion Mutants of MHVR. Deletion constructs of MHVR1* in pBluescript SK(+) linearized with *Nsi* I or *Bam*HI were generated, as described in Fig. 1. Both strands of the mutant cDNAs were sequenced to confirm the reading frame and rule out adventitious mutations. Deletion mutants are designated by Δ; the numbers of amino acids missing from MHVR are shown in parentheses. Primers are in a 5′–3′ orientation: CCGTTCCTCCAAGTCACC, 3′ primer for Δ(108–293) and Δ(50–293); CGGATGTACATGAAATCG, 5′ primer for Δ(108–293) and Δ(108–381); CGGACCATAGATAATGTT, 5′ primer for Δ(206–381); CGGTACAAATCGTGCAATTTC, 5′ primer for Δ(50–381) and Δ(50–293); CCGACACAAGGAGGC, 3′ primer for Δ(206–381), Δ(108–381), and Δ(50–381); GGGCGGCACAGCCTCAATG, 5′ primer for Δ(4–122); CCCGTGGAGGGTGACGAC, 3′ primer for Δ(4–122).

To generate Δ(133–424), MHVR1 digested with *Nar* I was rendered blunt by T4 DNA polymerase incorporation of [α-S]dNTPs. The plasmid was then cut with *Stu* I and incubated with exonuclease III; the single-stranded ends were made blunt with mung bean nuclease. Digested plasmid DNA was self-ligated and transformed.

Abbreviations: MHV, mouse hepatitis virus; MHVR, MHV receptor; mAb, monoclonal antibody; NTR, N terminus of mature MHVR.

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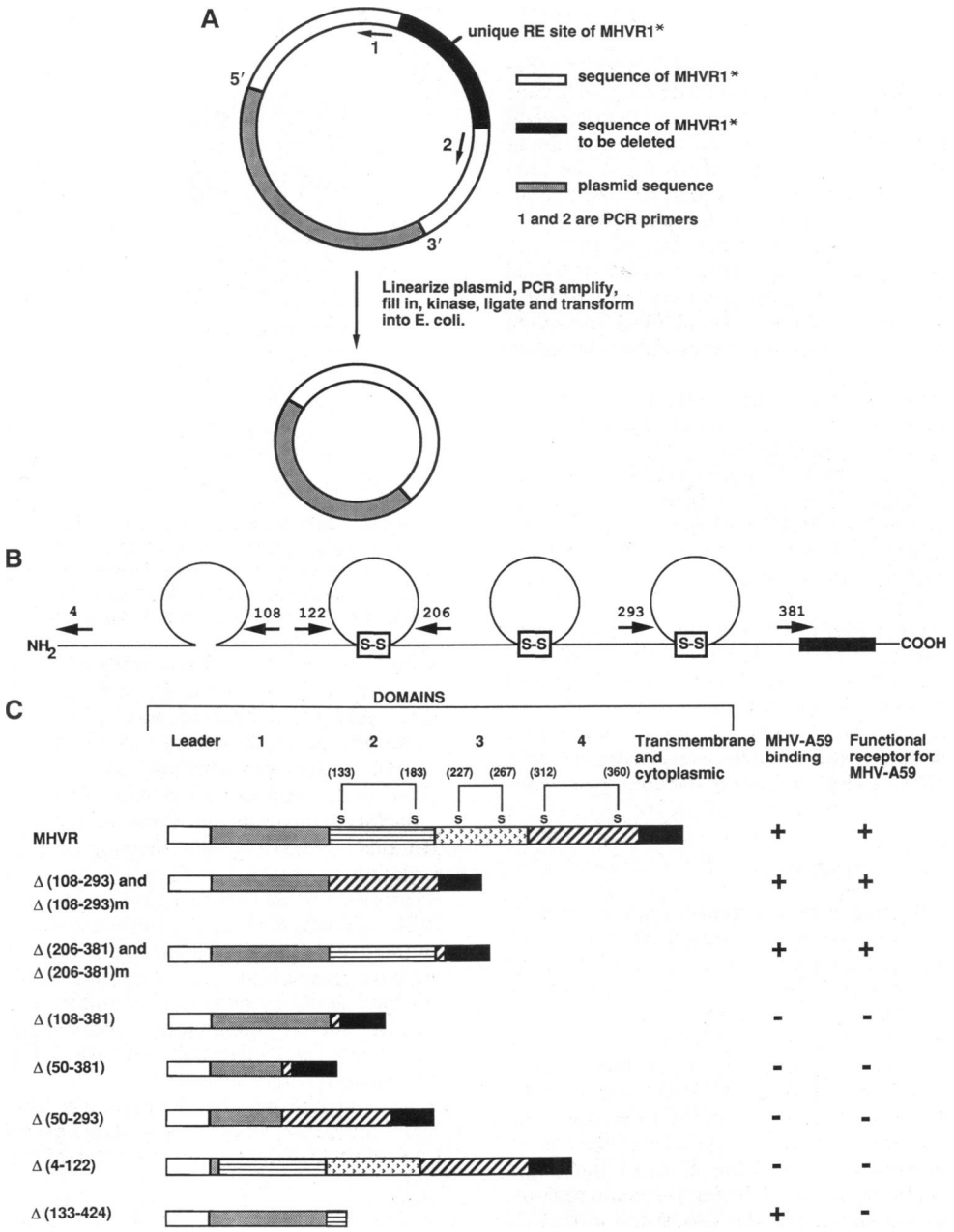

FIG. 1. Schematic representation of the MHVR deletion mutants and the strategy used in their construction. (*A*) MHVR1* in pBluescript SK(+) was amplified with the corresponding set of primers with AmpliTaq or Vent DNA polymerase. The cycling conditions were 94°C for 2 min, 3°C below the melting temperature of the primers, and 72°C for 4 min. Amplification products were electrophoresed on a 1% agarose gel, purified, and treated with T4 DNA polymerase with 2.5 mM dTTP and dATP to create appropriate overhangs. After phenol extraction and ethanol precipitation, samples were incubated with polynucleotide kinase and 1 mM ATP and self-ligated in 50-μl reaction with T4 DNA ligase. The recombinant protein Δ(133–424) was constructed as indicated. RE, restriction enzyme. (*B*) Numbers above arrows indicate position of the last amino acid as defined by the 3' end of primer. S-S, cysteines. (*C*) Numbers in parentheses indicate position of cysteines; m indicates point mutations in cDNA clones, as indicated in text.

Generation of Vaccinia Recombinant vac-Δ(108–381). Deletion mutant Δ(108–381) was excised from Bluescript, blunt-ended, and subcloned into the vaccinia shuttle vector pSC11 to give pSC11-Δ(108–381). Its orientation and sequence were confirmed. CV-1 cells infected with vaccinia were transfected with DNA from pSC11-Δ(108–381), and vac-Δ(108–381) was obtained and plaque purified by published methods (10).

Expression of cDNA Clones in Hamster (BHK) Cells. For transient and stable expression, the cDNAs of recombinant proteins were subcloned behind the Rous sarcoma virus promoter and transfected into BHK cells by electroporation (6). To obtain a stable BHK cell line expressing Δ(108–293), cells were transfected with pRSVneo mixed with Δ(108–293) cDNA. Colonies resistant to Geneticin were selected and screened by panning with antireceptor mAb-CC1, as described (6), and by surface immunofluorescence with antireceptor antibody 655.

MHV-A59 Infection of BHK Cells Expressing vac-Δ(108–381). BHK cells on coverslips were inoculated with vac-Δ(108–381) at multiplicity of infection of 0.1, 1.0, or 10 plaque-forming units per cell, incubated at 37°C for 3 hr, and challenged with MHV-A59 at a multiplicity of infection of 10 plaque-forming units per cell. Cells inoculated with vac-MHVR or vSC11 before challenge with MHV-A59 were used as positive and negative controls, respectively (7). Eight or 17 hr after MHV-A59 inoculation, intracellular MHV-A59 anti-

gens were detected by immunofluorescence with anti-MHV-A59.

Virus and Antibody-Binding Activities of Transiently Expressed Recombinant Deletion Mutants of MHVR. BHK cells were infected with vTF7-3 at a multiplicity of infection of 10 plaque-forming units per cell. At 1–1.5 hr after virus inoculation, 10 μg of plasmid DNA was transfected into the cells using lipofectin. At 24 hr after transfection, the cell monolayers were lysed with 0.5 ml of disruption buffer [2.3% SDS/10% (vol/vol) glycerol/5% 2-mercaptoethanol/62.5 mM Tris·HCl, pH 6.8]. Recombinant proteins were analyzed by immunoblotting with either polyclonal antibody 655 (6), anti-NTR (3), or with mAb-CC1 (4), and MHV-A59-binding activity of recombinant proteins was detected by the virus-overlay protein-blot assay (12).

Expression of Mutant Receptor Glycoproteins on the Plasma Membrane. To determine whether MHVR mutants were transported to the cell surface, BHK cells were infected with vTF7-3 and transfected with the mutant MHVR clones in pBluescript. Twelve hours later, the cells were fixed in 2% cold paraformaldehyde, and MHVR or mutant proteins on the plasma membranes were detected with antireceptor antibody 655, followed by rhodamine-labeled goat anti-rabbit IgG.

Detection of Functional MHV-A59-Receptor Activity of Mutant MHVRs. At 48 hr after transfection with the MHVR mutants subcloned in pRSVneo, BHK cells were challenged with MHV-A59 (4×10^6 plaque-forming units) for 1 hr or mock infected. At 7.5 hr after inoculation, the cells were fixed in acetone. Intracellular viral antigens were detected with anti-MHV-A59 followed by rhodamine-labeled goat anti-mouse IgG (6).

RESULTS

Construction of MHVR Deletion Mutant cDNAs. A series of deletion mutants of MHVR was engineered by PCR amplification of the cDNA encoding MHVR (Fig. 1). Because the efficiency of amplification decreases as the length of the amplified region increases, we first engineered a shorter template from MHVR1, designated MHVR1*, by removing 1.3 kb of 3′ untranslated sequence (6). Entire immunoglobulin-like domains were deleted from MHVR1*, whereas the distance between adjacent domains of MHVR was conserved. To conserve the secondary structure of the immunoglobulin-like domains, we joined the domains flanking a deletion at proline residues found in the inter-domain regions. For more detailed mapping of the MHV-A59 and mAb-CC1 binding sites within domain 1, we analyzed a deletion within the first domain, a recombinant protein expressing the N-terminal 132 amino acids, and four point mutations generated by incorporation errors of *Taq* DNA polymerase. For all recombinants except Δ(133–424), a short segment of domain 4, the transmembrane and intracytoplasmic domains were retained to anchor the glycoproteins in the membrane, so that virus receptor activity could be assayed. Fig. 1*C* illustrates the deletion mutants relative to the MHVR glycoprotein.

Analysis of Recombinant Deletion Mutants of MHVR. The recombinant proteins transiently expressed in BHK cells with the vTF7-3 expression system were immunoblotted with polyclonal antireceptor antibody 655, with anti-NTR (Fig. 2 *A* and *B*), and with mAb-CC1 (Fig. 3). They were also analyzed for their ability to bind to MHV-A59 virions in a virus-overlay protein-blot assay (Fig. 4). Sizes of the mutant proteins were those predicted from their sequences. mAb-CC1 and virions bound only to the mutant proteins that included the entire first immunoglobulin-like domain of MHVR (Fig. 3). The virus bound strongly to all recombinant proteins that encoded the first 132 amino acids of MHVR and did not recognize the proteins lacking the entire first domain

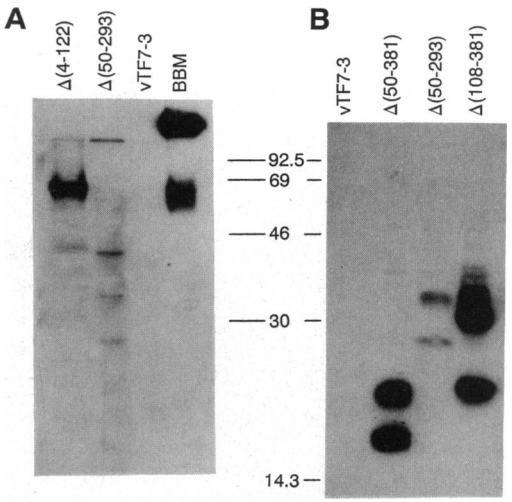

FIG. 2. Identification of proteins encoded by specific deletion mutants with antireceptor antibodies. Molecular weights for the MHVR deletion mutants were determined by immunoblot analysis with polyclonal antireceptor 655 (*A*) or anti-NTR (*B*), as described, and M_r ($\times 10^{-3}$) standards. BBM, BALB/c mice.

or lacking the last 57 amino acids of the first domain [Δ(50–381) and Δ(50–293)] (Fig. 4). Neither mAb-CC1 binding nor MHV-A59 virus binding was affected by the four point mutations in Δ(108–293)m, which has Val-85 → Ile and Asn-94 → Asp substitutions, and Δ(206–381)m, which has Thr-6 → Ala and Thr-30 → Ala substitutions.

Surface Expression and Virus Receptor Activities of Deletion Mutants of MHVR. We investigated the processing and targeting to the cell surface of the mutant glycoproteins. Expression of mutant proteins on the plasma membrane of BHK cells was detected by surface immunofluorescence with antibody 655 (Fig. 5). All but two of the recombinant proteins were detected on the cell membrane: Δ(50–381), which does not bind antibody 655, and recombinant Δ(133–424), which lacks an anchor domain and is secreted. In control cells that were mock-infected or infected with vTF7-3 alone, no MHV-A59 antigens were detected.

To determine whether the mutant glycoproteins function as MHV receptors, all deletion mutants except Δ(133–424) were transiently transfected into receptor-negative BHK cells and

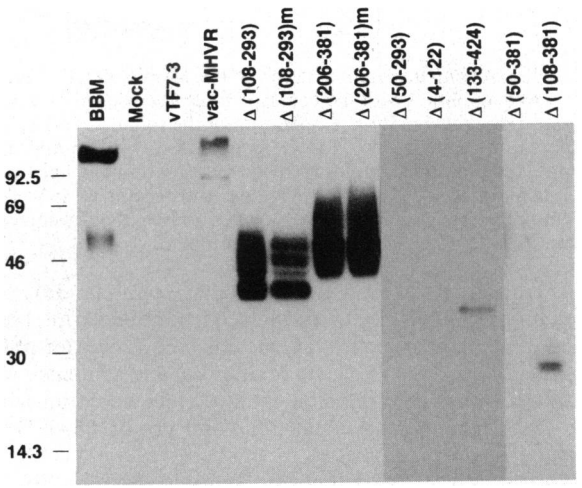

FIG. 3. Recognition of mutated receptor proteins by anti-receptor mAb-CC1. Fifty microliters of cell lysate was analyzed by SDS/12% PAGE. Proteins were detected by immunoblot with mAb-CC1 followed by rabbit antimouse serum and ^{125}I-labeled staphylococcal–protein A. BBM, BALB/c mice.

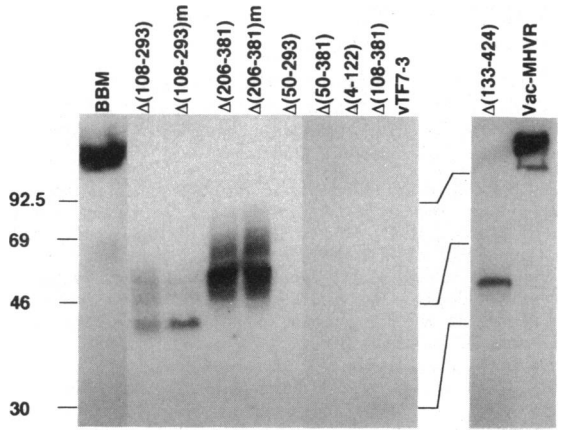

FIG. 4. Virus-binding activity of the deletion mutants, as determined by virus-overlay protein-blot assay. Intestinal brush-border membranes from BALB/c mice (BBM) and MHVR expressed in vaccinia (Vac-MHVR) were used as positive controls.

challenged with MHV-A59. Virus receptor activity was assessed by whether MHV antigens developed in the cytoplasm of at least three cells in an 18-mm^2 coverslip 8 hr after MHV inoculation in at least four independent experiments. All clones that included the entire first domain plus either domain 2 or 4 and the anchor sequences rendered BHK cells susceptible to MHV-A59 infection, and four point mutations in the first domain did not alter receptor activity (Fig. 6). Mutant proteins Δ(50–381), Δ(50–293), and Δ(4–122), in which part or all of the first domain was deleted, had no virus-receptor activity. Because transfection efficiency varied between experiments, the receptor activities of different recombinant proteins could not be compared quantitatively.

High levels of expression of mutant Δ(108–381) were achieved with a vaccinia vector. BHK cells infected with vac-Δ(108–381) were superinfected with MHV-A59 at three different multiplicity of infection. The deletion mutant Δ(108–381) containing only the first domain did not permit infection.

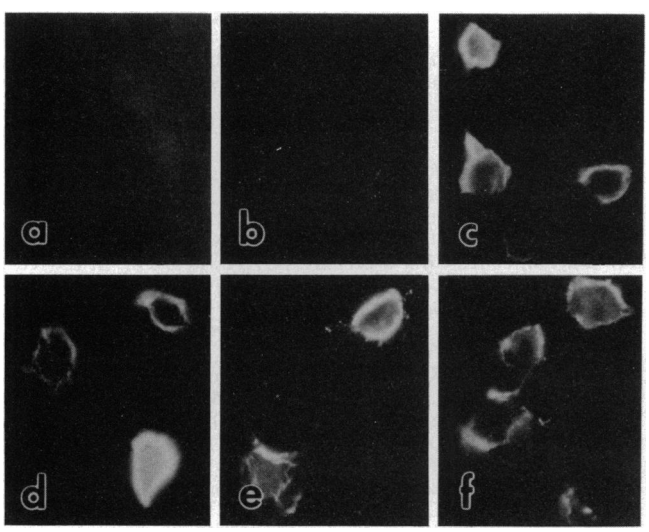

FIG. 5. Detection of mutant receptor glycoproteins at the plasma membrane. BHK cells were infected with vTF7-3 transfected with the cDNA coding for the deletion mutants and fixed in paraformaldehyde. Mutated receptor proteins were detected with polyclonal antireceptor antibody 655 followed by rhodamine-labeled goat anti-rabbit serum. Mock-infected BHK cells (*a*), cells infected with vTF7-3 (*b*), and cells infected with vTF7-3 followed by transfection with cDNA clones MHVR1 (*c*), Δ(108–381) (*d*), Δ(50–293) (*e*), or Δ(4–122) (*f*) are shown.

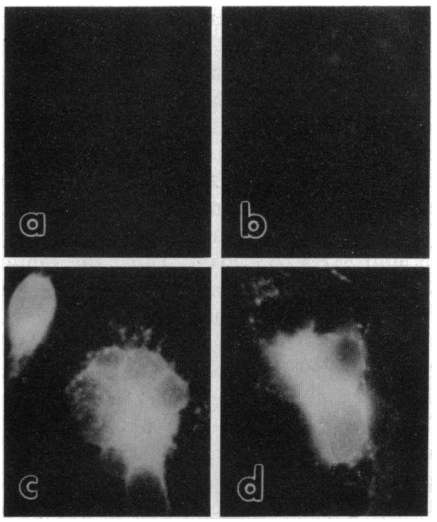

FIG. 6. Detection of viral antigens in cells transfected with mutated MHVR1 cDNA clones and challenged with MHV-A59. BHK cells were transfected with Δ(4–122) (*a*), Δ(108–381) (*b*), Δ(108–293) (*c*), and Δ(206–381) (*d*). At 7.5 hr after inoculation, MHV-A59 antigens were detected with anti-MHV serum followed by rhodamine-labeled anti-mouse IgG.

Two naturally occurring 58- to 55-kDa glycoproteins were cloned from mouse intestine (13, 14) and are naturally occurring splice variants of MHVR and of a four-domain MHVR homolog with the N-terminal domain of *Mus musculus* carcinoembryonic antigen gene family member 2 (mmCGM$_2$). In both variants, domains 2 and 3 were deleted, so that domains 1 and 4 were joined at amino acid 108 (13). We prepared a BHK cell line stably transfected with Δ(108–293), which is identical to MHVR(2d), the naturally occurring 58-kDa isoform of MHVR, except that it lacks five amino acids (E, P, V, T, and Q; amino acids 288–292 of MHVR) in the junction between the first and fourth domains. When challenged with MHV-A59, Δ(108–293) showed extensive cell fusion (Fig. 6). This stably transfected cell line will be useful in further study of receptor–virus interactions and virus penetration.

DISCUSSION

The cellular receptors for human immunodeficiency virus, poliovirus, the major rhinovirus group, and MHV-A59 are members of the immunoglobulin superfamily of glycoproteins that have different numbers of immunoglobulin-like domains (6, 15–21). The virus-binding sites on each of the first three of these receptors were identified by construction of chimeric or mutant receptors and analysis of virus-binding and receptor activities (22–26). Identification of the essential amino acids involved in virus–receptor interaction has been controversial—in part because deletions may distort secondary structure of the molecules (24, 27). Therefore, to map the domains of MHVR responsible for interaction with MHV-A59 or antireceptor mAb-CC1, we constructed a series of deletion mutants by PCR so that the transmembrane and cytoplasmic domains were retained, whereas entire immunoglobulin domains were deleted, and the number of amino acid residues between the remaining domains was conserved. Immunoblot analysis and immunofluorescence of hamster cells transiently transfected with the recombinants showed that they encoded proteins of the predicted molecular masses. All but two of the recombinant proteins were expressed on the plasma membrane. Δ(133–424) was secreted because its transmembrane and cytoplasmic domains were deleted, and Δ(50–381) was not recognized by the detecting antibody.

All recombinant proteins that contained the entire first domain bound mAb-CC1, but proteins lacking domain 1 did not. Therefore, the first domain of MHVR is the site of mAb-CC1 antibody binding. Similar results were found for binding of MHV-A59 virus. Although in a virus-overlay protein-blot assay MHV-A59 virus bound very poorly to Δ(108–381), which includes only the first domain (data not shown), the secreted Δ(133–424) protein, which contains domain 1 plus 24 amino acids of the junction between the first and second domains, bound MHV-A59 well. Thus, the binding site for MHV-A59 is in the first 132 amino acids of MHVR, which includes domain 1. Point mutations at amino acids 6, 30, 85, and 94 did not affect virus or mAb binding to MHVR proteins.

To further map the regions within domain 1 responsible for mAb-CC1 or virus binding, we analyzed deletions Δ(50–381) and Δ(50–293). The results indicate that the residues between amino acids 50 and 108 are critical for virus binding. Either the binding site is in this region or a deletion of this region causes conformational changes in domain 1 that obscure the binding site(s) for virus or mAb-CC1. Preliminary data from our laboratory suggests that the mAb-CC1-binding site and the virus-binding site are not identical (13).

The natural functions of most carcinoembryonic-related glycoproteins are not understood, but several have been shown to function as cell-adhesion molecules. The specific heterotypic interaction between carcinoembryonic family members W272 and nonspecific cross-reacting antigen (NCA) that leads to cell adhesion has been shown to depend upon binding of W272 to the N-terminal domain of NCA (28). Thus, as for other immunoglobulin superfamily members in which the natural ligands are known (27), in the carcinoembryonic family of glycoproteins both a natural ligand and the virus bind to the N-terminal domain (28).

Recombinant Δ(133–424), which contains the first 132 amino acids corresponding to the 108 amino acids of the first domain plus the 24 amino acids between the first and second domains of MHVR, mediated virus binding. However, the recombinant protein expressing the first domain only [Δ(108–381)] did not act as a functional receptor when transfected into cultured cells. When Δ(108–381) was subcloned into vaccinia, it also failed to act as a receptor, even though high levels of the mutant glycoprotein were expressed on the plasma membrane (7). Interestingly, although domains 2 and 4 differ considerably in amino acid sequence, either one can assist domain 1 in leading to virus entry. The simplest hypothesis to explain these data is that the short length of the Δ(108–381) protein on the cell surface could limit its accessibility to the virus. A second immunoglobulin domain might serve as a spacer to make the first domain accessible to the virus. Alternatively, a second immunoglobulin domain might interact with the first domain to achieve the conformation of the receptor required for virus entry.

It is interesting that at least two immunoglobulin-like domains are also required for functional virus-receptor activity of several other virus receptors in the immunoglobulin superfamily, including ICAM-1, and CD4 (27, 29). In contrast, for poliovirus receptor, a construct with less than two domains can function as a virus receptor (30, 31). X-ray crystallography of CD4 shows structural interactions between domains 1 and 2, which may stabilize the conformation of domain 1 needed for virus binding (11, 32). EM study of solubilized ICAM-1 suggests that, like CD4, the first two domains of ICAM-1 may form an elongated rod-like structure (27). All or part of a second domain of these receptors and of MHVR may be required for virus entry subsequent to virus binding. Such a second-step reaction of the virus receptor may be an important general mechanism for virus entry because this behavior has now been identified for rhinovirus and for two unrelated enveloped viruses, human immunodeficiency virus and MHV-A59.

Drs. Dveksler and Pensiero contributed equally to this paper and should be considered co-first authors. We thank Hanh Nguyen for technical assistance. This work was supported by U.S. Public Health Service Grants AI25231 and AI26075.

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