Several Members of the Mouse Carcinoembryonic Antigen-Related Glycoprotein Family Are Functional Receptors for the Coronavirus Mouse Hepatitis Virus-A59

GABRIELA S. DVEKSLER,[1] CARL W. DIEFFENBACH,[1]† CHRISTINE B. CARDELLICHIO,[1]
KIMBERLY McCUAIG,[2] MICHAEL N. PENSIERO,[1]‡ GUI-SEN JIANG,[1]
NICOLE BEAUCHEMIN,[2] AND KATHRYN V. HOLMES[1]*

*Department of Pathology, Uniformed Services University of the Health Sciences,
Bethesda, Maryland 20814-4799,[1] and McGill Cancer Centre,
McGill University, Montreal, Canada H3G 1Y6[2]*

Mouse hepatitis virus-A59 (MHV-A59), a murine coronavirus, can utilize as a cellular receptor MHVR, a murine glycoprotein in the biliary glycoprotein (BGP) subfamily of the carcinoembryonic antigen (CEA) family in the immunoglobulin superfamily (G. S. Dveksler, M. N. Pensiero, C. B. Cardellichio, R. K. Williams, G.-S. Jiang, K. V. Holmes, and C. W. Dieffenbach, J. Virol. 65:6881–6891, 1991). Several different BGP isoforms are expressed in tissues of different mouse strains, and we have explored which of these glycoproteins can serve as functional receptors for MHV-A59. cDNA cloning, RNA-mediated polymerase chain reaction analysis, and Western immunoblotting with a monoclonal antibody, CC1, specific for the N-terminal domain of MHVR showed that the inbred mouse strains BALB/c, C3H, and C57BL/6 expressed transcripts and proteins of the MHVR isoform and/or its splice variants but not the mmCGM$_2$ isoform. In contrast, adult SJL/J mice, which are resistant to infection with MHV-A59, express transcripts and proteins only of the mmCGM$_2$-related isoforms, not MHVR. These data are compatible with the hypothesis that the MHVR and mmCGM$_2$ glycoproteins may be encoded by different alleles of the same gene. We studied binding of anti-MHVR antibodies or MHV-A59 virions to proteins encoded by transcripts of MHVR and mmCGM$_2$ and two splice variants of MHVR, one containing two immunoglobulin-like domains [MHVR(2d)] and the other with four domains as in MHVR but with a longer cytoplasmic domain [MHVR(4d)$_L$]. We found that the three isoforms tested could serve as functional receptors for MHV-A59, although only isoforms that include the N-terminal domain of MHVR were recognized by monoclonal antibody CC1 in immunoblots or by MHV-A59 virions in virus overlay protein blot assays. Thus, in addition to MHVR, both the two-domain isoforms, mmCGM$_2$ and MHVR(2d), and the MHVR(4d)$_L$ isoform served as functional virus receptors for MHV-A59. This is the first report of multiple related glycoprotein isoforms that can serve as functional receptors for a single enveloped virus.

Murine coronaviruses cause a variety of syndromes in susceptible strains of mice, including inapparent enteric infection, respiratory infection, hepatitis, acute and chronic demyelinating disease, and wasting in nude mice (4, 32). Our laboratory demonstrated that the A59 strain of the coronavirus mouse hepatitis virus (MHV-A59) recognizes a receptor determinant that is expressed on membranes from cells and tissues of MHV-A59-susceptible BALB/c mice but not on membranes from cells and tissues of MHV-A59-resistant adult SJL/J mice or on brush border membranes (BBM) from nonmurine species that are resistant to MHV-A59 infection (9, 10). Monoclonal antibody (MAb) CC1, which is directed against the N-terminal domain of this receptor (12), blocks virus attachment to murine fibroblasts, prevents infection of several murine cell lines, and partially protects neonatal BALB/c mice against infection with MHV-A59 (22, 34).

From a BALB/c liver cDNA library, we cloned a cDNA, MHVR1, that encodes a cell membrane receptor for MHV-

A59, called MHVR (11). MHVR is a 110- to 120-kDa member of the biliary glycoprotein (BGP) subgroup of the carcinoembryonic antigen (CEA)-related glycoprotein (CGM) family in the immunoglobulin (Ig) superfamily (6, 11, 33). Human (21), rat (2), and mouse (31) BGPs have been shown to function in vitro as cell adhesion proteins, and the rat homolog was reported to have ecto-ATPase activity (14). Transfection of MHVR1 into MHV-resistant hamster or human cells made them susceptible to infection with MHV-A59 (11). MAb CC1 recognized a 110-kDa glycoprotein encoded by the MHVR1 cDNA clone and blocked MHV-A59 infection of BHK-21 cells transfected with MHVR1 (11, 34, 35). These experiments demonstrated that MHVR is a receptor for MHV-A59 in cells and tissues of BALB/c mice. In humans and rodents, many different CGM isoforms are expressed at different developmental stages (3, 7). The human homolog of MHVR is one of at least 12 isoforms of BGP that have been identified by molecular cloning and immunochemistry. These variants apparently result from complex alternative splicing patterns of a single BGP gene (3). In mice, nine isoforms of BGP have been identified by immunoblotting, immunoprecipitation, cloning, sequencing, and expression of cDNAs (11, 16). This report addresses the question of whether the CGM encoded by MHVR1 is the only isoform of the murine CGMs that can serve as a

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* Corresponding author.
† Present address: Division of AIDS, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.
‡ Present address: Virology Division, Genetic Therapy, Inc., Gaithersburg, MD 20878.

receptor for MHV-A59 or whether different murine CGMs are also functional receptors.

Williams et al. (34, 35) showed that although MHV-A59-resistant adult SJL/J mice do not express a CGM that binds MAb CC1 or MHV-A59 virions in immunoblots or virus overlay protein blot assays (VOPBAs), their intestinal BBM and liver membranes express a related glycoprotein that is recognized by antibody directed against the N-terminal peptide of MHVR and by antibodies to human CGMs. We postulated that adult SJL/J mice were resistant to MHV-A59 because their MHVR homolog lacked the virus-binding determinant of MHVR (9, 35). We have now characterized the murine CGMs expressed in BALB/c, SJL/J, and CD-1 liver and intestine cells, developed probes specific for mRNAs encoding several different murine CGMs, analyzed the expression of these mRNAs and the proteins that they encode in tissues of BALB/c, SJL/J, C3H, and C57BL/6 mice, and tested three additional murine CGMs for the ability to bind MHV-A59 or MAb CC1 and to serve as functional receptors for MHV-A59 when transfected into hamster cells. We found that several naturally occurring splice variants of MHVR1 and the two-domain homolog (mmCGM$_2$) expressed in CD-1 and SJL/J mice can function as receptors for MHV-A59. The observation that BALB/c mice expressed only MHVR1 mRNA and its splice variants, while SJL/J mice expressed only mRNA for mmCGM$_2$ and its splice variants, suggests that MHVR and mmCGM$_2$ may be encoded by different alleles of the same gene.

MATERIALS AND METHODS

Viruses and cells. MHV-A59 was propagated in the 17 Cl 1 line of spontaneously transformed BALB/c 3T3 cells and plaque assayed in L2 cells (28). Vaccinia virus strain WR was obtained from B. Moss (National Institutes of Health, Bethesda, Md.) and propagated in CV-1 cells. CV-1 cells and TK$^-$ cells were propagated as described previously (20). A recombinant vaccinia virus carrying MHVR1 cDNA was propagated as previously described (19). The LTA line of C3H mouse fibroblasts and the S15 line of LTA cells stably transfected with the two-domain mmCGM$_2$, containing a short cytoplasmic domain expressed under the adenovirus major late promoter of plasmid p91023B, were propagated as previously described (31).

An SJL/J embryo fibroblast cell line was established from decapitated 9-day embryos. Tissues were minced, triturated, and incubated in trypsin-EDTA for 15 min at 37°C. The cells were filtered through sterile cheesecloth, washed, and plated in T-75 flasks at a density of 10^5 viable cells per cm^2. The cultures were passaged at a dilution of 1:2 or 1:4 until crisis at passages 5 to 7. During crisis, the flasks were refed twice weekly and cells were passaged only when confluent. Within 3 weeks after crisis, the culture returned to vigorous growth, establishing a cell line. SJL/J embryo fibroblasts and the BHK-21 line of baby hamster kidney fibroblasts were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics (GIBCO Laboratories, Grand Island, N.Y.).

Northern (RNA) analysis. Ten micrograms of total RNA was separated in a 1.2% agarose gel (29) and transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.). The membrane was hybridized at 42°C with ^{32}P-labeled probes indicated in the figure legends. After hybridization, the filter was washed at 60°C at a final stringency of 0.1% SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO$_4$, and 1 mM EDTA, pH 7.7)–0.1% sodium dodecyl sulfate (SDS). For

some experiments, poly(A)$^+$ RNA was isolated with the polyATtract mRNA isolation system (Promega).

RNA-mediated PCR (RNAPCR) and digestion and sequencing of PCR products. Four micrograms of total RNA or 0.5 μg of poly(A)$^+$ RNA was reverse transcribed with 250 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, Md.), 0.5 μg of antisense primer, and 0.5 mM concentrations of each of the four deoxynucleoside triphosphates at 37°C for 60 min in a 25-μl reaction mixture. The reaction was terminated by inactivation of the enzyme at 95°C. The cDNA product was then amplified with 0.5 μg of each primer and 0.65 mM concentrations of mixture each of the four deoxynucleoside triphosphates in a 100-μl reaction mixture with Vent DNA polymerase (New England Biolabs, Beverly, Mass.). Controls for contamination and for residual genomic DNA were always included. For analysis of the polymerase chain reaction (PCR) products, 10% of the reaction was electrophoresed on a 2% agarose gel, transferred to a membrane, and hybridized with the appropriate ^{32}P-labeled oligonucleotide. The probes were washed at a final stringency of 5°C below their calculated melting temperatures in 5× SSPE–0.1% SDS or with 3 M tetramethylammonium chloride buffer (3 M tetramethylammonium chloride, 50 mM Tris-Cl [pH 8.0], 2 mM EDTA, 0.1% SDS) at the calculated melting temperature according to their lengths (36). Sequencing of the two strands of the subcloned PCR products was performed with a model 373A DNA sequencing system (Applied Biosystems, Foster City, Calif.). For digestion of the PCR products, the remaining 90 μl of the PCR mixture was diluted to 2 ml in water and concentrated to 50 μl with a Centricon-100 microconcentrator (Amicon, Beverly, Mass.). Half of the recovered PCR product was digested with AccI and the other half was digested with DraII for 3 h, after which the digestion products were separated on a 4% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, Maine), Southern blotted (24), and hybridized to a pool of oligonucleotide probes. The oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer; their sequences (in 5'-to-3' orientation) were as follows:

1, GGTCGACTGGGGCTTCTCATTGATAAG

2, CCAGGTTGCTGAAGACAAC

3, CGATTTCATGTACACTCG

4, TGAATTTCACGGGGCAAG

5, AGACTACAACAGGGCCTG

6, GCTGGCATCGTGATTGGA

7, GTTCTTCTACTTGTTCACAATCT

8, GAAATTGCACGATTTGTACCG

9, ACACTACGGCTATAGACAAAG

10, CGGCAGAGAGATAATATACAG

11, TTCCTGCTCTTCCAAATGATCACC

12, CCATGAAGGATATGGGAGTCTAC

Transfection of cDNAs and challenge with MHV-A59. Transfections were done with the following cDNA clones: MHVR1 (GenBank number M77196 [11]), a clone isolated from BALB/c liver cDNA (GenBank number X67279) which encodes a glycoprotein with four Ig-like domains on the external side of the plasma membrane and a short (10-amino-

acid) cytoplasmic domain; MHVR(2d), isolated from BALB/c colon cDNA, the two-domain splice variant of MHVR1 with a short cytoplasmic domain; MHVR1(4d)$_L$, isolated from CD-1 mouse colon cDNA, which encodes a four-domain glycoprotein with a cytoplasmic tail longer than that in MHVR; and mmCGM$_2$ (GenBank number X53084), isolated from CD-1 mouse colon cDNA and from SJL/J mouse colon cDNA, a two-domain glycoprotein with the same short cytoplasmic domain as in MHVR (31). The cytoplasmic domain of MHVR(4d)$_L$ differs from that of MHVR in that the last 3 amino acids of the MHVR sequence are substituted in MHVR(4d)$_L$ to D, Q, and R and MHVR(4d)$_L$ contains 62 additional amino acids at the carboxyl terminus (17).

All cDNAs were subcloned in the HindIII-EagI sites of pRSVneo for transient expression studies in tissue culture cells. Transfections into hamster cells (BHK) were performed by electroporation (Life Technologies), and transfections into SJL/J fibroblasts were performed with electroporation or with DOTAP as instructed by the manufacturer (Boehringer Mannheim, Indianapolis, Ind.). Seventy-two hours after transfection with pRSVneo containing the cDNAs or no transfection, the cells were incubated with MHV-A59 (4 × 10^6 PFU) for 1 h at 37°C. The inoculum was removed, and cells were incubated at 37°C for an additional 6.5 h, examined for cytopathic effects by phase-contrast microscopy, and fixed in cold acetone for immunofluorescence analysis of viral antigens as previously described (11).

Generation of a vaccinia virus recombinant. The cDNA clone of mmCGM$_2$ was excised with EcoRI, blunt ended, and subcloned into the SmaI site of the vaccinia virus shuttle vector pSC11 (20) to generate pSC11-mmCGM$_2$, and its correct orientation was confirmed by asymmetric restriction digestion. pSC11-mmCGM$_2$ was used to transfect CV-1 cells already infected with vaccinia virus, and a thymidine kinase-vaccinia virus recombinant was isolated (20). The recombinant was subjected to three rounds of plaque purification in the presence of 25 µg of bromodeoxyuridine per ml. The resultant vaccinia virus recombinant was named vac-CGM2. For analysis of the vac-CGM2-generated protein, BHK cells grown on 60-mm-diameter plates were infected with vac-CGM2 at a multiplicity of infection of 10 PFU per cell and incubated at 37°C for 24 h. Cells were then lysed in 0.5 ml of 2.3% SDS–10% glycerol–5% β-mercaptoethanol–62.5 mM Tris-Cl (pH 6.8), and proteins were analyzed on SDS-polyacrylamide gels.

Antisera. Two antibodies directed against murine CGMs were used in these studies. Anti-MHVR MAb CC1, prepared from SJL/J mice immunized with a deoxycholate extract of BALB/c BBM, blocks infection of 17 Cl 1 and L2 mouse fibroblasts with MHV-A59 and detects the N-terminal domain of MHVR and its two-domain splice variant, MHVR, from liver and intestine tissues of BALB/c mice but not from SJL/J tissues (34, 35). Polyclonal anti-MHVR antibody 655 was raised against MHVR and its two-domain splice variant, MHVR(2d), which were immunoaffinity purified with MAb CC1 from Swiss Webster mouse liver cells.

Immunoblot analysis, VOPBAs, and immunofluorescence. Cell lysates prepared as described above were used for analysis of antibody- and virus-binding activities of CGMs. Forty microliters of the cell lysates (approximately equivalent to 1.6 × 10^5 cells) was used for immunoblot and VOPBA analysis. The lysates were separated on an SDS–10% polyacrylamide gel, transferred to nitrocellulose sheets, and after blocking, incubated with either a 1:500 dilution of rabbit polyclonal anti-MHVR preadsorbed with vaccinia virus-
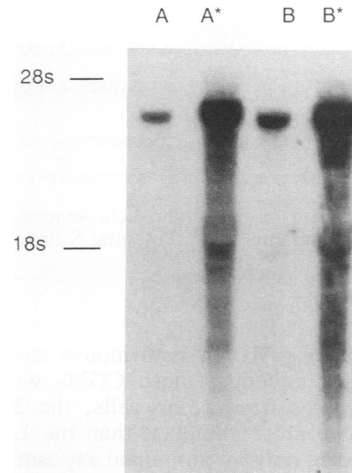

FIG. 1. Northern blot analysis of MHVR-related transcripts from adult BALB/c and SJL/J colon tissues. Total RNA (10 µg per lane) isolated from colon tissues of BALB/c mice (lanes A and A∗) and SJL/J mice (lanes B and B∗) was probed with the insert of MHVR1. MHVR-related transcripts were detected by autoradiography when the film was exposed for 24 h (lanes A and B); less abundant transcripts were also detected when the film was exposed for 7 days (lanes A∗ and B∗).

infected BHK cells or a 1:50 dilution of MAb CC1, and then incubated with rabbit anti-mouse antibody. The blots were then incubated with 10^5 cpm of ^{125}I-labeled staphylococcal protein A (Du Pont, NEN Research Products), washed, and exposed to film. MHV-A59-binding activities of proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted to nitrocellulose were detected by the VOPBA as previously described (9). MHV-A59 antigens in acetone-fixed infected cells were detected by immunofluorescence with anti-MHV-A59 serum from convalescent mice.

Receptor blockade experiments. Coverslip cultures of cells stably transfected with the murine CGM2 cDNA clone were pretreated with anti-MHVR MAb CC1, a 1:5 dilution of supernatant medium from the hybridoma culture, for 1 h prior to challenge with MHV-A59. After the virus inoculum was removed, cultures were incubated without MAb CC1 until 7 to 9 h after MHV inoculation, when the cells were fixed in cold acetone. Development of MHV-A59 antigens in the cytoplasm was detected by immunofluorescence. Controls included cells pretreated with medium alone or with an equivalent amount of a MAb directed against an irrelevant antigen (cholera toxin) of the same IgG1 isotype as MAb CC1. The numbers of cells positive for virus antigen in the presence or absence of MAb CC1 were compared to determine whether this antibody blocked infection of the cells with MHV-A59 (11).

RESULTS

Identification of mRNAs encoding murine CGMs in BALB/c and SJL/J tissues. Williams et al. (35) reported that the two SJL/J glycoproteins homologous to MHVR and its 58-kDa isoform showed a slightly lower molecular size (3 to 5 kDa) in SDS-PAGE than did the BALB/c proteins. To determine whether the apparently smaller sizes of the SJL/J proteins were due to a large deletion in the coding region of the SJL/J gene, we compared Northern blots of RNA from BALB/c

N-DOMAIN

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MHVR        EVTIEAVPPQVAEDNNVLLLVHNLPLALGAFAWYKGNTTAIDKEI
                                                 ::::::
mmCGM2      EVTIEAVPPQVAEDNNVLLLVHNLPLALGAFAWYKGNPVSTNAEI


ARFVPNSNMNFTGQAYSGREIIYSNGSLLFQMITMKDMGVTTLDMTDENYRRTQATVRFHVH
::  ::: ::: : :      ::     : :: : : :  ::      :  :    :
VHFVTGTNKTTTGPAHSGRETVYSNGSLLIQRVTVKDTGVYTIEMTDENFRRTEATVQFHVH
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FIG. 2. Comparison of the amino acid sequences of the first domains of MHVR and mmCGM₂. Potential N-linked glycosylation sites are underlined.

and SJL/J colon (Fig. 1). In both mouse strains, 3.4- and 1.7-kb transcripts encoding these CGMs were found. In these RNA samples from colon cells, the 3.4-kb mRNA species was much more abundant than the 1.7-kb species, which was detected only by prolonged exposure of the blots. Multiple additional faint bands of related transcripts were observed in mRNAs from both mouse strains. Thus, no deletion in the transcripts of SJL/J glycoproteins was identified by this technique.

cDNA cloning of additional murine CGM isoforms. To obtain cDNA clones encoding additional CGM isoforms of MHVR, we used RNAPCR with oligonucleotides based on the MHVR1 sequence (11) to amplify cDNA derived from SJL/J and BALB/c colon cells. The nucleotide sequence of the open reading frame of the BALB/c clone, MHVR(2d), is identical to the sequence of BGP1c obtained from CD-1 mouse colon cDNA (16), and the sequence of the open reading frame of the SJL/J clone was identical to that of mmCGM₂, also obtained from CD-1 colon cDNA (31). The clone [MHVR(2d)] that we obtained from BALB/c colon cDNA contained the leader, domains 1 and 4, the transmembrane sequence, and the shorter cytoplasmic domain of MHVR. The only sequence of MHVR(2d) that is not found in MHVR1 is the result of the splicing event that joins domains 1 and 4 together. The first base of the proline codon at position 108 of MHVR and the last two bases of the glutamic acid codon at position 288 of MHVR form a new triplet that encodes glutamine, which corresponds to amino acid 109 of MHVR(2d). The nucleotide and amino acid sequences of MHVR(2d) and mmCGM₂ were very similar. The largest region of sequence differences between these two clones was in the N-terminal domain (Fig. 2). These nucleotide differences permit distinction between MHVR and mmCGM₂ transcripts with probes targeted to this region. Amino acid sequence differences in this region showed one less potential N-linked glycosylation site in the SJL/J than in the BALB/c proteins, which could account for their smaller molecular weight. Two additional amino acid changes were found between MHVR(2d) and mmCGM₂ in the last Ig-like domain.

Comparison of CEA-related mRNAs from BALB/c and SJL/J tissues by RNAPCR. While adult outbred CD-1 mice expressed several murine CGM transcripts, including transcripts of both MHVR and mmCGM₂ and their spliced variants, immunoblotting of liver and intestine BBM with MAb CC1 or polyclonal antisera suggested that adult BALB/c expressed MHVR-related glycoproteins, while SJL/J mice expressed a different set of MHVR-related glycoproteins (35). We used a more sensitive assay to determine whether the two inbred mouse strains expressed both MHVR and mmCGM₂ transcripts. We synthesized two oligonucleotides within the N-terminal domain which hybridized specifically under stringent washing conditions to
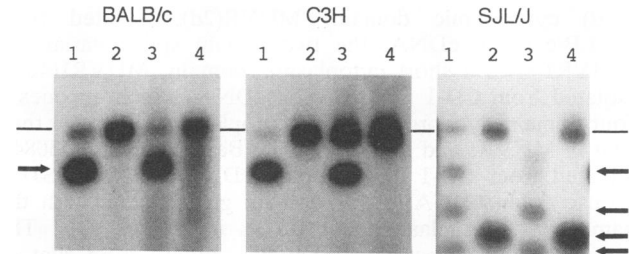

FIG. 3. Identification of restriction fragment length polymorphisms in MHVR-related transcripts analyzed by RNAPCR of RNA from BALB/c, C3H, and SJL/J colon and liver tissues. cDNA obtained from liver (lanes 1 and 2) or colon (lanes 3 and 4) tissues of these mouse strains was amplified with primers 2 and 3, which map within the N-terminal domain of MHVR and mmCGM₂. The resulting 297-bp products were digested with AccI (lanes 1 and 3) or DraII (lanes 2 and 4), electrophoresed on a 2% agarose gel, blotted to Nytran, and probed with a pool of ³²P-labeled oligonucleotides (oligonucleotides 4, 5, and 7 through 12) that recognize sequences in the N-terminal domain of MHVR and mmCGM₂. For MHVR1, the AccI digestion products should be 225 and 72 bp. Since no DraII recognition sites are present in the sequence between the primers, the 297-bp product would not be expected to be cleaved by DraII. On the basis of the nucleotide sequence of mmCGM₂, digestion of the PCR product with AccI would result in 89-, 82-, 72-, and 54-bp fragments, and digestion with DraII would result in 143- and 154-bp fragments. Bars indicate the undigested 297-bp PCR products, and arrows indicate the bands obtained after digestion of the PCR products with restriction enzymes.

either mmCGM₂ or MHVR1 but not to both (oligonucleotides 5 and 4, respectively). RNA isolated from the colon, small intestine, and liver of the two mouse strains was amplified with primers 2 and 1. PCR products from the SJL/J tissues did not hybridize to the oligonucleotide specific for MHVR1, while PCR products from BALB/c tissues, as expected, showed strong hybridization to this probe under the same washing conditions (data not shown). The PCR products obtained from BALB/c tissues did not hybridize to the mmCGM₂-specific probe, which hybridized strongly to the PCR products from SJL/J tissues.

To analyze further the difference in expression of the CEA family members in these two mouse strains and to extend the study to the C3H and C57BL/6 strains of mice, we analyzed the fragments of the N-terminal domains of the CGMs obtained by digestion of RNAPCR products with restriction enzymes. RNA prepared from liver and colon tissues of the four strains of mice was reverse transcribed, and the resulting cDNA was amplified with primers 2 and 3. The 297-bp PCR products obtained were digested with either AccI or DraII. Figure 3 shows a Southern blot of the fragments obtained upon restriction enzyme digestion of the PCR products from BALB/c, C3H, and SJL/J mice strains hybridized to a pool of oligonucleotide probes (probes 4, 5, and 7 through 12). The digestion patterns from DNA prepared from C57BL/6 cDNA were identical to those observed for BALB/c and C3H mice (data not shown). The 225-bp MHVR1 AccI fragment was detected in both liver and colon tissues. The 72-bp fragments from AccI digestion of BALB/c, C3H, and C57BL/6 PCR products were visualized in the ethidium bromide-stained gels but were not detected in the autoradiograph because of the absence of an oligonucleotide probe in the pool that will hybridize to this region. None of the PCR products from these three strains of mice was digested with DraII. The data show that these three
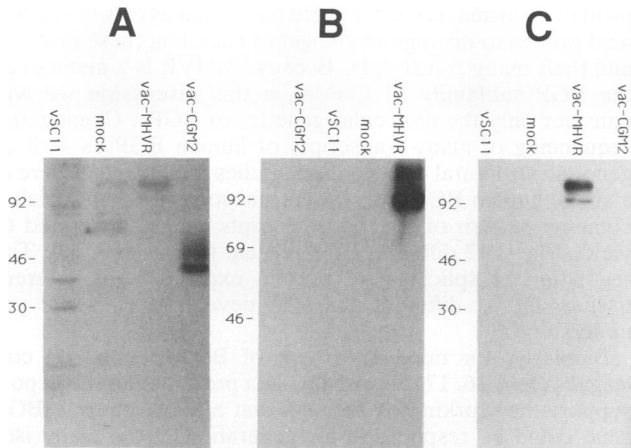
FIG. 4. Abilities of the mmCGM$_2$- and MHVR-encoded proteins to bind antibodies directed against the MHV-A59 receptor or MHV-A59 virions. Membranes from BHK cells infected with a recombinant vaccinia virus expressing MHVR1 (vac-MHVR) or mmCGM$_2$ (vac-CGM$_2$) were analyzed on Western immunoblots with rabbit polyclonal anti-MHVR antibody 655 (A) and with anti-MHVR MAb CC1 (B) and in VOPBAs with MHV-A59 virions (C). Controls were mock-infected BHK cells and BHK cells infected with a vaccinia virus vector without any murine cDNAs (vSC11). Sizes are indicated in kilodaltons.

FIG. 5. Abilities of MHVR$_1$ splice variants and mmCGM$_2$ to act as functional receptors for MHV-A59. BHK cells were transiently transfected with cDNA clones encoding MHVR(2d) (a), MHVR(4d)$_L$ (b), and mmCGM$_2$ (c). Forty-eight hours posttransfection, the cells were challenged with MHV-A59, and the presence of viral antigens was detected 7.5 h later with mouse anti-MHV serum and rhodamine-labeled goat anti-mouse IgG.

strains of mice expressed mRNA encoding the N-terminal domain of MHVR1 but not the corresponding domain of its homolog, mmCGM$_2$. In contrast, PCR of colon and liver cDNA of SJL/J mice did not yield any MHVR1 products but did amplify mmCGM$_2$ transcripts which were cleaved by DraII.

Virus- and MAb CC1-binding activities of MHVR and mmCGM$_2$. To determine the virus- and antibody-binding activities of the two-domain form of mmCGM$_2$, we overexpressed the protein encoded by the mmCGM$_2$ cDNA by using a vaccinia virus vector (vac-CGM2) and analyzed its ability to bind virus, MAb CC1, and rabbit anti-MHVR. The 55-kDa mmCGM$_2$ glycoprotein was recognized only by polyclonal anti-MHVR, not by the virus or MAb CC1 (Fig. 4). In contrast, the 58-kDa glycoprotein encoded by MHVR(2d) and expressed transiently in BHK cells with a vaccinia virus T7 system reacted with the MAb CC1, the polyclonal anti-MHVR antibody, and the virus (data not shown).

Abilities of MHVR1 splice variants and mmCGM$_2$ to act as functional receptors for MHV-A59. The abilities of an SJL/J-derived CGM, mmCGM$_2$, and two splice variants of MHVR1, MHVR(4d)$_L$ and MHVR(2d), to act as virus receptors when transiently transfected into receptor-negative, MHV-A59-resistant BHK cells were explored. Transfection of BHK cells with mmCGM$_2$, MHVR(2d), or MHVR(4d)$_L$ in pRSVneo made the cells susceptible to MHV-A59 infection (Fig. 5), while BHK cells transfected only with pRSVneo remained resistant (11). This result for mmCGM$_2$ was surprising in view of the absence of MAb CC1 binding and virus binding in immunoblots and VOPBAs of proteins from SJL/J tissues that express mmCGM$_2$ glycoproteins (35) and in view of the resistance to MHV-A59 and MHV-JHM infection of adult SJL/J mice and peritoneal macrophages from these animals (13, 23, 27). We therefore prepared a continuous line of SJL/J embryo fibroblasts and tested its ability to support MHV-A59 infection and the ability of the murine CGMs in
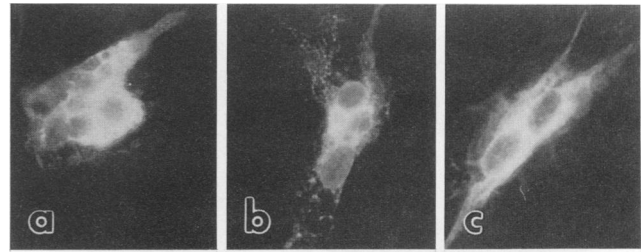
its membranes to bind MHV-A59, MAb CC1, or anti-MHVR. Two-domain and four-domain transcripts of mmCGM$_2$ were detected in the SJL/J cell line by RNAPCR using primers 2 and 6 (data not shown). At passage 3, the SJL/J cells were completely resistant to infection with MHV-A59, as shown by immunofluorescence with an antiviral antibody at 7.5 h after virus challenge (Fig. 6).

To determine whether the observed resistance of the SJL/J cell line to infection with MHV-A59 was due to an SJL-specific posttranslational modification of the MHVR homolog(s), we introduced MHVR1 cDNA into SJL/J cells in two ways: we transiently transfected the cells with MHVR1 cDNA in pRSVneo, and we infected the cells at passage 3 with vac-MHVR (19). We then studied the expression of MHVR on the plasma membrane of SJL/J cells and its ability to act as a functional receptor for MHV-A59. The results showed that in transfected or vac-MHVR-infected SJL/J cells, MHVR could be expressed on the plasma membrane in a form that made the cells susceptible to infection (Fig. 6c). Thus, SJL/J cells were able to process the MHVR glycoprotein normally so that it could serve as a functional virus receptor.

We then investigated whether SJL/J cells infected with vac-CGM2 (Fig. 6b) or transfected with mmCGM$_2$, MHVR(4d)$_L$, and MHVR(2d) (data not shown) in pRSVneo became susceptible to MHV-A59 infection. Surprisingly, we found that the SJL/J cells became susceptible to infection with MHV-A59 after transfection with mmCGM$_2$, which encodes one of the same CGMs expressed in SJL/J liver and
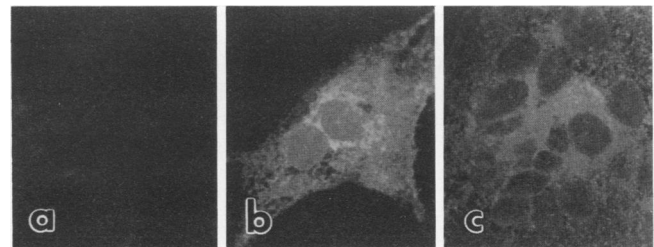

FIG. 6. Abilities of MHVR$_1$ and mmCGM$_2$ to act as receptors for MHV-A59 in an SJL/J embryo fibroblast cell line. SJL/J cells were infected with a vaccinia virus containing no insert (vSC11) (a), mmCGM$_2$ (vac-CGM2) (b), or MHVR$_1$ (vac-MHVR) (c). After MHV-A59 challenge, the cells were fixed with acetone and examined for the presence of viral antigens by immunofluorescence as described for Fig. 5.
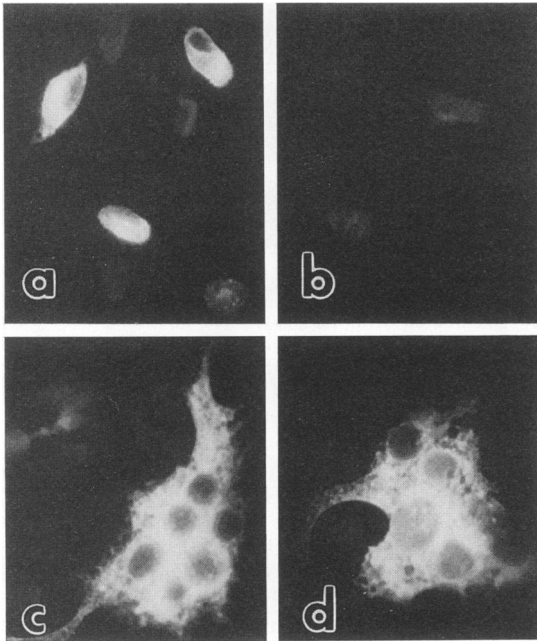
FIG. 7. Protection from virus infection by pretreatment with MAb CC1. LTA cells and S15 cells (LTA cells stably transfected with mmCGM$_2$) were challenged with MHV-A59 (a and c, respectively) or pretreated with MAb CC1 prior to virus challenge (b and d, respectively). After 7.5 h of infection, the cells were fixed and the presence of viral antigens was examined by immunofluorescence as described for Fig. 5.

intestine cells, as well as with all the MHVR variants. Thus MHVR, MHVR(2d), MHVR(4d)$_L$, and mmCGM$_2$ can serve as functional receptors for MHV-A59 when expressed in SJL/J cells. These data demonstrate that SJL/J fibroblasts process these glycoproteins in a manner appropriate to permit their use as receptors for MHV-A59.

We next tested whether cells expressing mmCGM$_2$ could be protected from MHV-A59 infection by pretreatment with anti-MHVR MAb CC1 in a receptor blockade experiment. The mouse LTA cell line stably transfected with mmCGM$_2$ was used for this purpose, and untransfected LTA cells were used as a control. The cells were pretreated with MAb CC1 or a control MAb of the same IgG1 isotype for 1 h, the antibody was removed, and the cells were challenged with MHV-A59. Immunofluorescence was used to detect synthesis of viral antigens in susceptible cells. A small fraction of control LTA cells, whether pretreated or not pretreated with the control MAb, was susceptible to MHV infection. This infection was completely blocked by pretreatment with MAb CC1. In contrast, LTA cells expressing mmCGM$_2$ were highly susceptible to MHV-A59 and formed large syncytia, and MAb CC1 did not prevent virus infection or fusion of these cells (Fig. 7).

DISCUSSION

Molecular genetics of the murine BGPs. The molecular genetics of the CGMs of humans, rats, and mice has been challenging to study because of the large number of very closely related transcripts and proteins that may be expressed in a single cell or tissue. Until quite recently, there were no adequate markers such as MAbs to differentiate the

protein isoforms, nor were there functional assays or nucleic acid probes to distinguish the genes encoding these proteins and their many transcripts. Because MHVR is a member of the BGP subfamily of CGMs, in this discussion we will consider only the molecular genetics of BGPs. Cloning and sequencing of many transcripts of human BGP as well as genomic structural organization studies confirm that there is a single human BGP gene on chromosome 19 from which a complex pattern of spliced transcripts can be generated to yield at least 12 different BGP protein isoforms (3, 30). The regulation of splicing and protein expression in different tissues and at different times in development is not yet understood.

Similarly, for mice, the work of Beauchemin and colleagues (6, 7, 16, 17, 31) and the data presented in this report support the working hypothesis that a single murine BGP gene could be responsible for generation of the many isoforms of glycoproteins related to MHVR that are found in many murine tissues and that MHVR and mmCGM$_2$ may be different alleles of this gene. Turbide et al. (31) and McCuaig et al. (16, 17) isolated cDNA clones for various isoforms of MHVR (mmCGM$_1$) and mmCGM$_2$ from colon tissue of outbred CD-1 mice, whereas we found only MHVR transcripts in liver, colon, and small intestine tissues of inbred BALB/c, C3H, and C57BL/6 mice, which all permit replication of MHV-A59, and only mmCGM$_2$ transcripts in these tissues of adult SJL/J mice, which are resistant to infection with MHV-A59. Interestingly, intestinal BBM and hepatocyte membranes from F$_1$ progeny from BALB/c × SJL/J matings showed about 40% of the MHV-A59-binding activity of the BALB/c parent, while the SJL/J mice showed no virus-binding activity in VOPBAs or solid-phase virus binding assays using undenatured membrane proteins (9). This result is close to the 50% predicted if the receptor-related glycoproteins were expressed as two codominant alleles of a single gene. With these data, we cannot rule out the possibility that the various isoforms observed are products of alternative splicing in different strains of mice. However, extensive cDNA cloning and PCR amplification of adult CD-1 colon cDNA suggest that the N-terminal domain of MHVR is always spliced to the same domains, i.e., 2, 3 and 4, or 4 only, and never to the corresponding domains from mmCGM$_2$, while the transmembrane region and the short or long cytoplasmic domains are identical for MHVR and mmCGM$_2$ (16).

Studies on recombinant domain-specific deletions of MHVR showed clearly that MAb CC1 recognized the first domain of MHVR (12). Since MAb CC1 does not recognize SJL/J tissues that express mmCGM$_2$ isoforms (35) or BHK-21 cells transfected with mmCGM$_2$ cDNA, it is now clear that MAb CC1 is specific for the N-terminal domain of MHVR and its splice variants. Sequence differences between the N-terminal domains of MHVR and mmCGM$_2$ probably account for this difference in MAb CC1 binding. Similarly, the immunoblots with polyclonal anti-MHVR antibody 655 performed in this study, together with the nucleotide sequence data for mmCGM$_2$, indicate that this antibody recognizes at least one epitope common to mmCGM$_2$ and MHVR which could be in either domain 1 or domain 4.

Ability of multiple isoforms of MHVR from BALB/c mice to serve as functional receptors for MHV-A59. The glycoproteins MHVR and MHVR(2d) from BALB/c mice or CD-1 mice were recognized by MAb CC1, as expected since both of these glycoproteins have the N-terminal domain that contains the binding site of the antibody (12). At least three isoforms of MHVR [MHVR, MHVR(4d)$_L$, and MHVR(2d)],

when expressed in BHK cells, can serve as functional receptors for MHV-A59. Studies with recombinant chimeric proteins of other virus receptors in the Ig superfamily, including CD4 and ICAM-1, suggest that the cytoplasmic domain of the protein does not play an essential role in virus receptor functions (8, 15, 25). Nevertheless, it remains to be determined whether the different intracytoplasmic domains of the MHVR isoforms affect the cellular functions of these glycoproteins.

Adult SJL/J mice are much more resistant to MHV strains than are other mouse strains such as BALB/c. For example, the median infectious dose of MHV-S that leads to seroconversion of adult SJL/J mice following intranasal inoculation is more than 1,000 times that required for seroconversion of BALB/c mice (5); 80% of adult SJL/J mice survive intracerebral challenge with 1,000 50% lethal doses of MHV-JHM assayed in suckling Swiss Webster mice, and the survivors do not show any virus antigens or infectious virus in any organ for 8 days postinoculation (27). In addition, peritoneal macrophages derived from SJL/J mice are resistant to infection with MHV-A59 (13, 23, 26, 27) and SJL/J tissues fail to bind MHV-A59 virions in VOPBAs (9, 35). It therefore appeared likely that the SJL/J homologs of MHVR glycoproteins would not be functional receptors for MHV (35). Hence, it was surprising that expression in BHK cells of cloned mmCGM$_2$, one of the isoforms of SJL/J mice, rendered these cells susceptible to infection with MHV-A59. This observation suggested either that the posttranslational processing of mmCGM$_2$, MHVR, MHVR(4d)$_L$, and MHVR (2d) in SJL/J cells might in some manner prevent the glycoproteins from serving as functional receptors for MHV-A59, that some other host protein or factor could be blocking the ability of these glycoproteins to serve as virus receptors, or that interaction of MHV-A59 with cell surface receptors was not the step determining the resistance to MHV-A59 of SJL/J cells. However, we found that expression of mmCGM$_2$ or MHVR isoforms in the SJL/J embryo fibroblast cell line allowed processing of the glycoproteins in these SJL/J cells in such a way that they could function as virus receptors. Thus, no host-dependent posttranslational processing event or receptor-blocking factor was detected in SJL/J cells. Further quantitative studies on virus binding and penetration in these SJL/J cells will be needed to determine how they restrict the functioning of MHVR or mmCGM$_2$ as a virus receptor.

The studies described in this report have investigated the ability of various splice variants of two different, probably allelic, murine BGPs to serve as receptors for MHV-A59. The observed biological differences in susceptibility to MHV-A59 among BALB/c, C3H, C57BL/6, and SJL/J mice may be explained by variation in the quantities and distribution of the isoforms on the cell surface as well as the relative efficiency with which each isoform can serve as a receptor. Even relatively small differences in the efficacy of a virus receptor could result in very large biological differences in the multiple cycle infections that are required to cause disease in animals (1).

To our knowledge, the MHV receptor is the first receptor glycoprotein for an enveloped virus that has been shown to have multiple functional variants expressed in the tissues of the natural host of the virus, although two different transcripts of the poliovirus receptor (which, like MHVR, is a member of the Ig superfamily) can function as virus receptors (18). For the murine coronaviruses, expression in the same host of multiple alternative glycoprotein receptors may play an important role in determining the wide variation in the tissue tropism, virulence, and pathogenesis of different MHV strains in murine hosts of different strains and ages.

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