Purification of the 110-Kilodalton Glycoprotein Receptor for Mouse Hepatitis Virus (MHV)-A59 from Mouse Liver and Identification of a Nonfunctional, Homologous Protein in MHV-Resistant SJL/J Mice

RICHARD K. WILLIAMS, GUI-SEN JIANG, STUART W. SNYDER,[†] MARK F. FRANA,[‡]
AND KATHRYN V. HOLMES*

Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

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The receptor for mouse hepatitis virus strain A59 (MHV-A59) is a 110- to 120-kilodalton (kDa) glycoprotein which is expressed in MHV-susceptible mouse strains on the membranes of hepatocytes, intestinal epithelial cells, and macrophages. SJL/J mice, which are highly resistant to MHV-A59, were previously shown to lack detectable levels of receptor by using either solid-phase virus receptor assays or binding of a monoclonal anti-receptor antibody (MAb) which blocks infection of MHV-susceptible mouse cells. This MAb was used for affinity purification of the receptor glycoprotein from livers of MHV-susceptible Swiss Webster mice. The MHV receptor and an antigenically related protein of 48 to 58 kDa were copurified and then separated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The first 15 amino acids of the receptor were sequenced, and a synthetic peptide of this amino acid sequence was prepared. Rabbit antiserum made against this peptide bound to the MHV receptor glycoprotein and the 48- to 58-kDa protein from livers of MHV-susceptible BALB/c mice and Swiss Webster mice and from the intestinal brush border of BALB/c mice. In immunoblots of intestinal brush border and hepatocyte membranes of MHV-resistant SJL/J mice, the antibody against the amino terminus of the receptor identified proteins that are 5 to 10 kDa smaller than the MHV receptor and the 48- to 58-kDa related protein from Swiss Webster or BALB/c mice. Thus, SJL/J mice express a protein which shares some sequence homology with the MHV receptor but which lacks virus-binding activity and is not recognized by the blocking anti-receptor MAb. These results suggest that resistance of SJL/J mice to MHV-A59 may be due to absence or mutation of the virus-binding domain in the nonfunctional receptor homolog in SJL/J mice.

Mouse hepatitis virus (MHV) is a murine coronavirus which causes inapparent enteric or respiratory infection, diarrhea, hepatitis, and acute and chronic demyelinating diseases of the central nervous system (33). Classic studies by Bang and co-workers showed that mouse strains differ markedly in their susceptibility to MHV and that mouse strain susceptibility correlates with permissiveness for MHV replication in peritoneal macrophages cultured from the different mouse strains (2). A single recessive gene, *hv-1*, was associated with resistance of C3H mice to MHV-2 (34). A wide variety of mouse strains has been tested for susceptibility to several of the many strains of MHV which differ in virulence and tissue tropism (1, 3, 8-10, 16, 18, 19, 26, 27). SJL/J mice and/or peritoneal macrophages cultured from them are highly resistant to infection with the neurotropic strain MHV-JHM and the hepatotropic strain MHV-A59. This resistance trait has been mapped to a single recessive gene at the *hv-2* locus near the *svp-2* and albino loci on mouse chromosome 7 (15-17, 26). A solid-phase virus-binding assay was used to show that a receptor for MHV-A59 is present on the brush border membranes of enterocytes and hepatocytes from MHV-susceptible BALB/c mice (4). In virus overlay protein blot assays (VOPBA) of mem-

brane proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from intestinal brush border membranes (BBM) and hepatocyte membranes from BALB/c mice, MHV-A59 bound to a single protein with an apparent molecular weight of 110,000 to 120,000. Membrane preparations from SJL/J mice, however, showed no protein with MHV-binding activity (4). These experiments suggested that SJL/J mice were resistant to MHV infection because no virus-binding protein was present on the membranes of the liver and intestinal brush border which are normal target organs for MHV infection.

In this paper, we report purification to homogeneity of the MHV receptor from liver membranes of MHV-susceptible Swiss Webster mice, determination of the amino-terminal amino acid sequence of the isolated protein, and preparation of antibody directed against a synthetic peptide with this amino acid sequence. When this antibody was used in immunoblots of membranes from BALB/c intestine and liver and with purified receptor, it identified the MHV receptor protein and a smaller (48- to 58-kilodalton [kDa]) protein antigenically related to it. Surprisingly, this antibody to the amino-terminal antigenic region of the MHV receptor also recognized two slightly smaller proteins in liver membranes and intestinal BBM from MHV-resistant SJL/J mice. Neither of these SJL/J proteins showed virus-binding activity or was recognized by the anti-receptor monoclonal antibody (MAb) CC1, which blocks infection of mouse cells by MHV-A59. These studies demonstrate that on their liver and intestine membranes SJL/J mice express proteins which

* Corresponding author.

[†] Present address: Program Resources, Inc., at National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21701.

[‡] Present address: Department of Biological Sciences, Salisbury State University, Salisbury, MD 21801.

possess amino-terminal homology with the MHV receptor glycoprotein with BALB/c liver and intestine. These SJL/J proteins lack the recognition sites for binding of virus and the anti-receptor MAb CC1 which are found on the MHV receptor of susceptible mice.

MATERIALS AND METHODS

Cells and viruses. Monolayers of the 17Cl 1 line of spontaneously transformed BALB/c 3T3 cells grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum and 100 U of penicillin G and streptomycin per ml were infected with MHV-A59 at a multiplicity of infection of 0.1 to 0.5 at 37°C in growth medium (28). After 1 h, the inoculum was removed and replaced with fresh growth medium. At 24 to 36 h postinfection the virus-containing medium was removed and clarified by centrifugation for 20 min at $9,425 \times g$ (all centrifugations in this study were at 4°C; g forces are at rotor maximum distance) in an HS-4 rotor (Sorvall Instruments, Du Pont Co., Wilmington, Del.), quick-frozen in 25-ml aliquots, and stored at -70°C.

Preparation of intestinal BBM. Intestinal BBM from BALB/c and SJL/J mice were prepared from adult female mice as previously described (4, 14). Mice were anesthetized and killed, and their small intestines were removed, flushed with ice-cold Dulbecco phosphate-buffered saline (D-PBS; 0.137 M NaCl, 2.7 mM KCl, 8.05 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.47 mM KH_2PO_4 , 0.9 mM CaCl_2 , 0.49 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ [pH 7.2]), sealed in plastic bags, and quickly frozen in liquid nitrogen. Tissues were crushed with a frozen block and homogenized (at 15 ml/g of tissue) in 0.3 M mannitol-2 mM Tris hydrochloride (pH 7.1) by using a motorized homogenizer (Tissuemizer; Tekmar Co., Cincinnati, Ohio) at 70% maximum speed. CaCl_2 was added to a final concentration of 10 mM, and the homogenate was held on ice for 15 min and then centrifuged for 15 min at $3,000 \times g$. The supernatant was centrifuged at $40,000 \times g$ in an SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The resulting BBM pellet was suspended in 10 mM Tris hydrochloride (pH 7.4)-1 mM EDTA and stored at -70°C. Protein concentrations of tissues and fractions were assayed by the Coomassie blue protein assay (5) or with the fluorescamine reagent as specified by the manufacturer (Sigma Chemical Co., St. Louis, Mo.).

Antibodies. Polyclonal anti-receptor antibody was produced in SJL/J mice immunized with protein extracted into 0.25% deoxycholate from intestinal BBM of BALB/c mice (K. V. Holmes, unpublished data). Ascites fluid was induced in these immunized mice by intraperitoneal injection of 1.5×10^6 Sarcoma-180 cells (24) and was removed after 5 days. Hybridomas producing monoclonal anti-receptor antibody were prepared by fusion of spleen cells from mice immunized as described above with SP₂-0 myeloma cells (13). MAbs specific for the MHV receptor were identified by an enzyme-linked immunosorbent assay with the 100- to 120-kDa protein eluted from a preparative SDS-PAGE gel of BALB/c BBM (Holmes, unpublished). One hybridoma, CC1, produced anti-receptor MAb which reacted strongly with the partially purified receptor in the enzyme-linked immunosorbent assay and bound specifically to the 110- to 120-kDa protein in immunoblots of BALB/c BBM but did not recognize any protein in immunoblots of SJL/J BBM. Pretreatment of murine fibroblasts with MAb CC1 prevented binding of MHV-A59 and protected cells from infection with MHV-A59 (Holmes, unpublished). MAb CC1 was subcloned

three times, and purified MAb was produced from serum-free medium for these studies.

Anti-NTR serum was produced against a synthetic peptide representing the first 15 amino acids of the receptor glycoprotein. This peptide (called NTR) was synthesized in a peptide synthesizer (Applied Biosystems, Inc., Foster City, Calif.) at the Uniformed Services University of the Health Sciences. The NTR peptide was coupled to keyhole limpet hemocyanin by mixing peptide, keyhole limpet hemocyanin, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in the ratio of 1:1:1 (wt/wt/wt) as described by Richardson et al. (23). Two rabbits were immunized once with 0.25 mg of NTR-keyhole limpet hemocyanin complex in complete Freund adjuvant and twice in incomplete Freund adjuvant at 2-week intervals. The rabbits were test bled at 2-week intervals. Affinity-purified anti-peptide antibody was purified as follows from the anti-NTR antiserum. The NTR peptide (7 mg) was coupled via EDAC (100 mg) to 7 ml of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, Calif.) that had been coupled to L-lysine (20 mg) to produce NTR-LYS-Affi-Gel. Anti-NTR immunoglobulin G, prepared by ammonium sulfate fractionation from 20 ml of serum, was adsorbed to the NTR-LYS-Affi-Gel overnight at 4°C. Unbound antibody was washed away with 20 ml of D-PBS followed by 5 ml each of 0.1 M citrate (pH 6.0) and 0.1 M citrate (pH 4.5). Affinity-purified antibody was eluted with 0.1 M citrate (pH 2.2) and immediately neutralized with 2 M Tris base, dialyzed against D-PBS, and concentrated to 1.0 ml with a Centricon-30 microconcentrator (Amicon Corp., Danvers, Mass.).

Detection of the MHV receptor glycoprotein. Virus-binding activity of proteins separated by SDS-PAGE and then blotted to nitrocellulose was detected by the VOPBA (4). Intestinal BBM, liver membranes, or subcellular fractions were boiled for 5 min in 0.0625 M Tris hydrochloride (pH 6.8)-2% SDS-5% 2-mercaptoethanol-10% glycerol-0.0001% bromophenol blue and analyzed by SDS-PAGE with 8% separating and 5% stacking gels. After electrophoresis, the gels were soaked for 15 min in transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% [vol/vol] methanol). Proteins were transferred to nitrocellulose, as described by Towbin et al. (31), for 1 h at 100 V with 4 cm between electrodes. After transfer, unoccupied sites on the nitrocellulose were blocked for 1 h in B3 buffer (0.15 M NaCl, 0.05 M Tris hydrochloride, 1 mM EDTA, 0.05% Tween-20, 0.1% bovine serum albumin [Sigma; fraction V]) with an additional 2% bovine serum albumin. The blot was then rocked for 1 h in virus supernatant (25 ml/75 cm²) supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.4). The blot was washed extensively in B3 buffer and incubated for 1 h with a 1:100 dilution of goat antiserum directed against the S protein of MHV-A59. The blot was washed extensively again with B3 and incubated for 1 h with 10^5 cpm of ¹²⁵I-labeled staphylococcal protein A (Du Pont, NEN Research Products, Boston, Mass; specific activity, 5 to 10 $\mu\text{Ci}/\mu\text{g}$) per ml. The blot was washed, dried, and autoradiographed on XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) at -70°C for 1 to 5 days. Receptor protein was also detected by immunoblotting with polyclonal antibodies and MAbs directed against the MHV receptor from BALB/c BBM. Relative amounts of receptor were determined by scanning autoradiographs on a GS 300 densitometer (Hoefer Scientific Instruments, San Francisco, Calif.) equipped with an Altex automatic integrator (Shimadzu Scientific Instruments, Columbia, Md.). Receptor units in the liver membranes and purified fractions were determined

directly from the peak area integrated from the scans of the 110-kDa receptor protein on the autoradiograph.

Purification of the receptor glycoprotein. Frozen Swiss Webster mouse liver (100 g; Rockland Inc., Gilbertsville, Pa.) was thawed and quickly homogenized for 5 min (Tissuemizer; 70% maximum speed) in 200 ml of ice-cold D-PBS without calcium or magnesium. The homogenate was centrifuged for 10 min at $1,000 \times g$ in a Sorvall RT6000B centrifuge (H1000B rotor), and the pellet containing the nuclear fraction was discarded. The supernatant was centrifuged at $39,000 \times g$ to produce a crude microsomal pellet (P2), which was suspended in 600 ml of cold D-PBS and centrifuged again at $39,000 \times g$. The washed P2 pellet was diluted to 1,000 ml with ice-cold D-PBS containing 1% Nonidet P-40 detergent, 0.1% aprotinin, and 0.1% phenylmethylsulfonyl fluoride, and membrane proteins were solubilized by stirring this solution for 30 min at 4°C. Undissolved material was removed by centrifuging for 60 min at $39,000 \times g$. The solubilized membrane proteins were added directly to 3 ml of MAb CC1 coupled to Affi-Gel 10 (2 mg/ml) and stirred for 5 h at 4°C in a flask equipped with a suspended stir bar. The affinity absorbent was collected in a 10-ml column, washed with 30 ml of TBS buffer (10 mM Tris [pH 7.4], 150 mM NaCl)–1% Nonidet P-40. The column was preeluted with TBS–1% NP-40–1 M NaSCN. The receptor was eluted with 6 M NaSCN–1% Nonidet P-40, and 1-ml fractions were collected. The released receptor was analyzed in all fractions by immunoblots and silver staining of SDS-PAGE gels. Receptor-containing fractions that eluted with 6 M NaSCN were pooled, dialyzed for 1 h against TBS, and concentrated to 0.5 ml by using Centricon-30 microconcentrators. Concentrated receptor was stored at -70°C until used for sequencing on a polyvinylidene difluoride (PVDF) membrane or further purified by SDS-PAGE. Preparative SDS-PAGE was used to complete the purification of the receptor. The receptor was labeled with ^{125}I by using chloramine-T (12) and analyzed by electrophoresis in two dimensions (22). The first dimension was an isoelectric focusing gel with a 2.5 to 7.5 pH gradient, and the second dimension was an SDS-PAGE with an 8% separating gel.

Amino acid sequencing. Concentrated, pooled receptor (6 M NaSCN eluate) from the CC1-Affi-gel column was run in two lanes of a 0.75-mm-thick SDS-PAGE gel (12% acrylamide). To prevent N-terminal blocking, the receptor sample in sample treatment mix was not boiled but was heated to 55°C for 15 min, separating gels were cast 2 days before running, and 0.1 mM sodium thioglycolate was added to the top reservoir buffer to scavenge unreacted polymerizing agent (11). After electrophoresis, the gel was transferred to a PVDF membrane (Immobilon P; Millipore Corp., Bedford, Mass.) at 100 V for 4 h for sequencing (21). PVDF blots were stained for 5 min with 0.1% amido black in 50% methanol and destained for 5 min in 7.5% acetic acid–50% methanol. The stained 110-kDa receptor protein was excised with a scalpel blade for sequencing. The receptor was also sequenced on the affinity-purified receptor protein that had been electroeluted from preparative SDS-PAGE gel slices in a model 422 electroeluter (Bio-Rad). Electroeluted receptor was concentrated in a Centricon-30 and precipitated with trichloroacetic acid at a final concentration of 10%. The precipitated protein was washed twice with ice-cold acetone, dried, and directly sequenced. Receptor sequences were determined (Harvard University Microchemistry Facility or University of California, Davis, Protein Structure Laboratory) by using Edman degradation in Applied Biosystems 1470 sequencers with on-line high-pressure liquid chroma-

FIG. 1. Purification of the MHV receptor glycoprotein. (A) Silver-stained SDS-PAGE. Lanes: 1, Nonidet P-40-solubilized membrane proteins from Swiss Webster mouse liver; 2, MHV receptor eluted from MAb CC1 column with 6 M NaSCN; 3, affinity-purified 110-kDa receptor protein eluted from a preparative gel; 4, 48- to 58-kDa protein eluted from a preparative gel. (B) Polyclonal anti-receptor mouse ascites fluid immunoblot of Nonidet P-40-solubilized liver membrane proteins (lane 1) or MAb CC1 affinity column-purified receptor (6 M NaSCN fraction) (lane 2).

tography detection of phenylthiohydantoin-derivatized amino acids.

Assay for protection of cells from virus infection. Purified anti-NTR antibody was tested for the ability to protect cells from virus infection by preincubating 17Cl 1 cells for 1 h in a 96-well plate with twofold serial dilutions of anti-NTR antiserum starting at an initial dilution of 1:50. As a positive control for cell protection, cells in control wells were preincubated with twofold serial dilutions of MAb CC1 starting at 1 to 2 μg per well. Antibody was aspirated, and MHV-A59 (10^5 PFU per well) was added to each well. Cell monolayers were stained with crystal violet at 18 to 36 h and monitored by light microscopy for virus-induced cell lysis and fusion.

RESULTS

Purification of the receptor glycoprotein. Silver staining of proteins on SDS-PAGE gels was used to monitor the purification of the receptor glycoprotein. The liver membrane extract showed more than 30 major proteins on silver-stained SDS-PAGE gels. Virus-binding activity did not line up with any of the major proteins in this starting material (Fig. 1A), confirming previous studies showing that the receptor glycoprotein is present in relatively low abundance on membranes of mouse liver (4). Our preliminary studies showed that the receptor glycoprotein was released only partially from the column by NaSCN concentrations below 6 M. In the 6 M NaSCN-eluted fractions, the MHV receptor was readily visible as the major silver-stained protein, showing the enrichment of the receptor by the affinity purification step. As expected, a smaller, receptor-related protein of 48 to 58 kDa was also eluted in the 6 M NaSCN fraction. Immunoblots of BALB/c BBM and hepatocyte membrane proteins showed that MAb CC1 recognized both the MHV receptor glycoprotein and the 48- to 58-kDa protein. To further purify the MHV receptor protein, the 6 M NaSCN eluate was run on a preparative SDS-PAGE gel, the 110-kDa protein was excised, and the receptor was eluted. After this step, the MHV receptor glycoprotein was found to be a single protein by silver staining. The identity of this protein with the receptor was shown by immunoblotting of the

FIG. 2. (A) MAb CC1 immunoblot of purified receptor protein fractions from Swiss Webster mouse liver. Lanes: 1, Nonidet P-40-solubilized liver membrane protein; 2, MAb CC1 affinity-purified receptor (6 M NaSCN fraction); 3, affinity-purified 110-kDa receptor eluted from preparative SDS-PAGE; 4, affinity-purified 48- to 58-kDa protein eluted from preparative SDS-PAGE. (B) MHV-A59 was used in the VOPBA to detect receptor. Lanes: 1, MAb CC1 affinity-purified receptor (6 M NaSCN fraction); 2, affinity-purified, SDS-PAGE-eluted 110-kDa receptor; 3, affinity-purified, SDS-PAGE-eluted 48- to 58-kDa protein.

purified protein with polyclonal anti-receptor antibody (Fig. 1B) and with the blocking MAb CC1 (Fig. 2A) and by VOPBA with MHV-A59 (Fig. 2B).

Purification of the receptor was quantitated by densitometry of the 110-kDa protein in immunoblots by using polyclonal anti-receptor antibody and the VOPBA assay. The original liver membrane extract contained 5.4×10^5 arbitrary units of receptor per 5.25×10^3 mg of protein as determined by the density of the autoradiograph. Nearly all (91%) of this activity, but only 9.5% of the total protein, was bound to the affinity column. The column was eluted with 1 M NaSCN, which removed nonspecifically bound protein but no detectable receptor activity. Elution with 6 M NaSCN yielded 1×10^4 receptor units, and purification by preparative SDS-PAGE yielded 5.5×10^3 units of receptor activity in 5 μ g of protein. Thus, the final purification of the receptor was approximately 10,000 fold.

To confirm that the MHV receptor glycoprotein was purified to homogeneity, the SDS-PAGE-purified receptor was labeled with 125 I and run on a two-dimensional gel (Fig. 3). The labeled receptor was a single large spot on the two-dimensional gel at its characteristically low pI of <4.5. VOPBA of two-dimensional gels of receptor from BALB/c BBM and from the unlabeled purified receptor showed an identical pI and molecular weight (data not shown).

The second most abundant component in the silver-stained gel of the 6 M NaSCN eluate was the 48- to 58-kDa receptor-related protein (Fig. 1A). This protein was strongly reactive with MAb CC1 but was only weakly reactive with MHV-A59 in the VOPBA. MAb CC1 bound to the 48- to 58-kDa protein in the 6 M NaSCN receptor pool and to the 48- to 58-kDa protein that had been further purified by preparative SDS-PAGE (Fig. 2A). MHV binding to the 48- to 58-kDa protein was at a low level in both the 6 M NaSCN pool and the preparative PAGE-isolated protein (Fig. 2B). This 48- to 58-kDa protein may be either a smaller protein related to the receptor or a fragment of the receptor that was produced during receptor isolation.

Quantitation of the VOPBA. The amount of receptor required to produce a signal by VOPBA technique was

FIG. 3. Two-dimensional gel analysis of 125 I-labeled preparative gel-eluted, affinity-purified MHV receptor. The first dimension was a 2.5 to 7.5 pH isoelectric focusing gel. A and B refer to acidic and basic ends of the isoelectric focusing gel, and pH positions along the isoelectric focusing gel are indicated at the top. The second dimension was SDS-PAGE.

determined by adding increasing amounts of receptor to wells of an SDS-PAGE gel. The smallest amount of receptor visible in a 3-day film exposure was 0.125 ng (Fig. 4). This amount corresponds to about 6×10^8 molecules of the MHV receptor. Approximately 10 μ g of intestinal BBM was required to produce a signal comparable to 5 ng of the purified receptor from liver. With increasing amounts of the affinity-purified receptor, increasing amounts of signal were seen. The signal level measured by scanning the autoradiograph in Fig. 4 showed an approximately linear relation between protein density and the log of the number of nanograms of affinity-purified receptor (Fig. 5).

Sequencing of the receptor glycoprotein and analysis of the amino-terminal end of the receptor in tissues from different mouse strains. Microsequencing was performed on the affinity-purified MHV receptor from Swiss Webster mouse liver. The sequence from amino acids 1 through 15 was obtained

FIG. 4. VOPBA of affinity-purified receptor. Lanes: 1, 10 μ g of BALB/c mouse intestinal BBM; 2 to 6, affinity-purified receptor (lane 2, 10 ng; lane 3, 5 ng; lane 4, 0.5 ng; lane 5, 0.25 ng; lane 6, 0.125 ng).

FIG. 5. Quantitation of film density in 110-kDa receptor from the VOPBA in Fig. 4.

from approximately 6 to 10 pmol of purified MHV receptor. The sequence was confirmed on separate sequencing runs. A peptide, NH₂-Glu-Val-Thr-Ile-Glu-Ala-Val-Pro-Pro-Gln-Val-Ala-Glu-Asp-Asn-COOH, corresponding to the first 15 amino acids of the receptor, was synthesized, coupled to keyhole limpet hemocyanin, and used to raise antipeptide sera in two rabbits. This antiserum against the amino-terminal sequence from the receptor (anti-NTR) was tested for reactivity against the affinity-purified MHV receptor (Fig. 6B). Anti-NTR reacted against the MHV receptor and against the smaller receptor-related protein at 48 to 58 kDa. The binding of the anti-NTR was specific and could be blocked by the addition of excess free synthetic peptide (Fig. 6D). These results show that the receptor and the smaller 48- to 58-kDa protein share a common epitope at the N terminus.

The anti-NTR antibody was tested for the ability to protect 17Cl 1 cells from infection with MHV-A59. In contrast to the cell protection observed with MAb CC1, no reduction of cell lysis or fusion was observed with any concentration of anti-NTR antibody. Lack of cell protection with the anti-NTR antibody suggests that the virus-binding domain of the receptor is not topologically close to the first 15 amino acids.

In immunoblots of intestinal BBM from BALB/c mice, anti-NTR bound to the MHV receptor and to the smaller 48- to 58-kDa receptor-related protein (Fig. 6B). As in previous studies (4), the SJL/J BBM and liver membranes did not bind to MHV-A59 (Fig. 6C). In liver membranes of BALB/c mice, immunoblots with MAb CC1 showed specific binding to the receptor and to the receptor-related protein at about 48 kDa and nonspecific binding to a 55-kDa protein. This 55-kDa protein was found to be heavy chain of mouse immunoglobulin G that remained in the liver membrane fraction, since this 55-kDa protein was observed when the blot was probed with only rabbit anti-mouse immunoglobulin G antibody followed by ¹²⁵I-labeled staphylococcal protein A (data not shown). MAb CC1 did not specifically bind to any proteins in the SJL/J liver membranes or BBM, and immunoglobulin G heavy chain was the only protein seen in the MAb CC1 immunoblot (Fig. 6A). In the SJL/J intestinal BBM, however, the anti-NTR antibody showed specific binding to a protein of 104 kDa (Fig. 6B, lane 4) with comparable intensity to the MHV receptor of BALB/c BBM and to an

FIG. 6. Immunoblot and VOPBA analysis of affinity-purified MHV receptor from Swiss Webster mouse liver and BALB/c and SJL/J mouse liver and intestinal membranes. (A) MAb CC1 immunoblot. Lanes: 1, 3 ng of affinity-purified receptor; 2, 100 μ g of BALB/c mouse liver membrane P2 fraction; 3, 100 μ g of SJL/J mouse liver membrane P2 fraction; 4, 100 μ g of BALB/c mouse intestinal BBM; 5, 100 μ g of SJL/J mouse intestinal BBM. (B through D) samples identical to panel A were run in the same lanes and probed with antibody to NTR peptide (panel B), by VOPBA with MHV-A59 (panel C), or with anti-NTR that had been preadsorbed with 100 μ g of free NTR peptide per ml (panel D).

approximately 50-kDa protein. Anti-NTR antibody occasionally detected a faint protein near 150 kDa in both BALB/c and SJL/J intestine, but this protein was not detected by MAb CC1 or by MHV in the VOPBA. In SJL/J liver, anti-NTR antibody detected proteins of approximately 102 and 43 kDa. The molecular weights of these SJL/J proteins were determined by averaging measurements from several gels. These results suggest that intestines and livers of SJL/J mice express proteins homologous to the MHV receptor and to the smaller protein that is antigenically related to it. On immunoblots with anti-NTR, SJL/J membranes gave a strikingly similar pattern to BALB/c membranes, although the SJL/J anti-NTR-reactive proteins were approximately 5 to 10 kDa smaller than the comparable proteins from liver and intestinal membranes from BALB/c mice.

DISCUSSION

The 110-kDa glycoprotein purified by affinity chromatography from livers of Swiss Webster mice is the same glycoprotein as the MHV receptor in BALB/c mice. This is demonstrated by the observations that both proteins are of about the same molecular weight, have a pI of <4.5, react with MHV virions, are specifically recognized in immunoblots with polyclonal anti-receptor antibody and anti-receptor MAb CC1, both of which block infection of cells with MHV-A59, and have antibody directed against a synthetic peptide with the amino acid sequence of the Swiss Webster glycoprotein. The MHV receptor is expressed on the mem-

branes of murine cell lines and primary macrophage cultures which are susceptible to MHV infection and is absent from cell lines from other species which lack the MHV receptor (S. Compton, unpublished data). In susceptible mouse strains, the MHV receptor is found on membranes of the intestine and liver, which are major targets for MHV-induced lesions (4).

MAB CC1, which was used for the affinity purification of the MHV receptor glycoprotein, has been shown to block infection of L2 or 17Cl 1 mouse fibroblasts by MHV-A59 (Holmes, unpublished). These observations suggest that the MAB binds to the native receptor glycoprotein at a site which is near enough to prevent virus binding by steric hindrance. Binding between the receptor glycoprotein and the MAB was very firm, as shown by the requirement for 6 M sodium thiocyanate to release the glycoprotein from the affinity column. This agrees with estimates of antibody-receptor binding made by other methods (S. Snyder, unpublished data).

Anti-receptor MAB CC1 reacted in immunoblots with a 55-kDa protein from BALB/c mouse intestine and a 48-kDa protein from BALB/c mouse liver, and a 48- to 58-kDa protein was copurified from Swiss Webster mouse liver with the MHV receptor. This 48- to 58-kDa glycoprotein appears to be closely related to the MHV receptor, since they share both the amino-terminal antigenic domain and the MAB CC1-binding domain. However, unlike the MHV receptor glycoprotein, the 48- to 58-kDa protein binds MHV-A59 virions poorly compared with its binding to MAB CC1. Therefore, the domain of the receptor which binds the S (E2) glycoprotein of MHV-A59 is not identical to the domain which binds MAB CC1, although these two domains must be near enough to permit blocking of virus by this antibody. Possibly the 48- to 58-kDa proteins are naturally occurring proteolytic cleavage fragments of the MHV receptor. Alternatively, the smaller MAB CC1-binding proteins could be expressed from different genes in a family of related genes, or from the same gene as the MHV receptor, by using alternate splicing of mRNAs, which is translated into a smaller protein with diminished virus-binding properties.

The viral protein which recognizes the MHV receptor glycoprotein is the S (or E2) glycoprotein that forms the spikes on coronavirus virions. In addition to the S glycoprotein, some coronaviruses, such as bovine coronavirus and human coronavirus-OC43, express a hemagglutinin esterase glycoprotein (HE) which is homologous to the glycoprotein of influenza C virus (20). The HE glycoprotein binds to nine O-acetylated sialic acid residues on cell membranes (32). Whether this binding can lead to coronavirus infectivity is not yet known. Several strains of MHV, such as MHV-DVIM and MHV-JHM, express HE glycoprotein (25, 29, 30). However, in cells infected with MHV-A59, little or no HE glycoprotein is made (20, 25). The HE gene of MHV-A59 lacks an initiator methionine, and cells infected with MHV-A59 do not make an mRNA which would be translated to make the HE glycoprotein. In mouse cell lines, the infectivity of MHV-A59 appears to be mediated solely by interaction of the spike glycoprotein S, since pretreatment of cells with anti-receptor MAB CC1 blocks virus binding and prevents virus infection (Holmes, unpublished). Therefore, interaction of the MHV receptor glycoprotein with the S glycoprotein of virions appears to be sufficient for initiation of virus infections.

The development of antibody directed against the amino-terminal antigenic region of the MHV receptor from Swiss Webster mouse liver permitted us to ask whether SJL/J mice

fail to express a protein homologous to the MHV receptor, or whether they express a homologous protein which does not bind MHV virions or MAB CC1 but may carry out the normal cellular function of the glycoprotein. Our results showed that SJL/J mice expressed a homologous protein which shares the amino-terminal antigenic region with the MHV receptor from Swiss Webster mouse liver. SJL/J mouse intestinal BBM and liver membranes expressed proteins homologous to both the MHV receptor and 48- to 58-kDa related protein of BALB/c BBM and liver membranes, but the SJL/J proteins are smaller than their MHV-binding BALB/c counterparts by approximately 5 to 10 kDa. The differences in molecular mass, virus binding, and MAB CC1 binding activities could result from different posttranslational processing, from deletion or mutation in the virus-binding or MAB CC1-binding domains of the SJL/J gene(s), or from alternative splicing in liver and intestinal epithelial cells of SJL/J mice, resulting in deletion of the exon(s) required for expression of the virus-binding or MAB CC1-binding domain. Absence of the virus-binding site on the receptor-homologous proteins from SJL/J mouse liver and intestine may play a major role in determining the resistance of SJL/J mice to MHV-A59.

Although the MHV receptor has been purified to homogeneity, its cellular function has not yet been identified. The tissue distribution of the MHV receptor, enriched in intestine and liver membrane, and its pI are similar to those of two previously isolated membrane proteins at about 100 to 120 kDa in the mouse, LAMP-1 and LAMP-2 (7). However, the sequence of the first 15 amino acids of the receptor is not contained in LAMP-1 (6), and MABs against LAMP-1 and LAMP-2 (donated by J. T. August, Johns Hopkins University, Baltimore, Md.) did not recognize the affinity-purified MHV receptor glycoprotein (data not shown). Additional sequence information will assist in comparison of the MHV receptor with other cellular glycoproteins. Cloning of cDNA for the MHV receptor glycoprotein is under way in our laboratory, using MAB CC1 and NTR antibodies to identify clones of λ gt11 which express proteins related to the MHV receptor. Sequencing of the MHV receptor clones will help to determine the relationships between the MHV receptor, the antigenically cross-reacting 48- to 58-kDa proteins of BALB/c mouse liver and intestine, and the homologous proteins of SJL/J mouse liver and intestine.

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