CHARACTERIZATION OF THE VIRUS AND MONOCLONAL ANTIBODY BINDING SITES OF THE MOUSE HEPATITIS VIRUS RECEPTOR

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ABSTRACT

Title of thesis: "Characterization of the Virus and Monoclonal Antibody Binding Sites of the Mouse Hepatitis Virus Receptor."

Paul C. Shick, D.D.S., Master of Science, 1995

Thesis directed by: Gabriela S. Dveksler, Ph.D., Assistant Professor, Department of Pathology

The attachment of Mouse Hepatitis Virus (MHV), a coronavirus, to its cellular receptor on the host cell membrane is the first step leading to viral infection. The best characterized receptor for MHV is a 120 kDa glycoprotein encoded by the *biliary glycoprotein (Bgp) 1*[a] gene. Other murine Bgp glycoproteins also serve as receptors for MHV but with less efficiency. Bgps belong to the carcinoembryonic antigen family which, in turn, belongs to the immunoglobulin superfamily. Chimeric glycoproteins and recombinant mutated receptor proteins were generated to identify the sites on the receptor that binds to the Mouse Hepatitis Virus strain A59 (MHV-A59). These proteins were also tested for their ability to bind to the anti-receptor monoclonal antibody CC1 (MAb CC1) which blocks infection of

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by

Paul C. Shick, D.D.S.

Thesis submitted to the Faculty of the Department of Pathology Molecular Pathobiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Master of Science 1995

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INTRODUCTION

Coronaviruses are large (100-150 nm), enveloped, RNA viruses with a characteristic "corona" of large petal-shaped spikes made of oligomers, most likely trimers (Delmas and Laude, 1990), of the 180-200 kDa glycoprotein S (Tyrrell et al., 1968). The spike protein S binds to cell membrane receptors (Holmes, 1989) which probably induces a conformational change in it. This conformational change may then result in exposure of a hydrophobic region of S which results in fusion of the viral envelope with the cell membrane and subsequent entry of the virus into the cell (Daniel et al., 1993). The spike protein is also functional as a protease-activated cell-fusing molecule and is responsible for cell-cell fusion of infected cells (Collins et al., 1982).

Coronaviruses can be divided into at least four distinct antigenic groups. Viruses within each group are host specific and cause readily distinguishable clinical syndromes (Sturman and Holmes, 1983). The murine hepatitis viruses (MHV) belong to antigenic group II and can cause enteric infections, infantile diarrhea, respiratory

infections, acute and chronic demyelinating neurologic diseases, and hepatitis (Wege et al., 1981). There are approximately 25 differents strains and isolates of MHV. Strain A59 (MHV-A59) is a prototype strain of MHV.

MHV are extremely contagious and susceptible mice are killed by a small number of virions when inoculated intranasally (Barthold, 1986). Susceptible strains of mice include BALB/c, C3H, C57BL/6, SWR, and A/J. Adult SJL/J mice are resistant to infection, even when inoculated with large doses of virus (Stohlman and Frelinger, 1978). A monoclonal antibody directed against the MHV-A59 receptor was prepared and named MAb CC1. MAb CC1 blocks infection of cells and recognizes a 58 kDa and a 110-120 kDa membrane glycoprotein of BALB/c mice, but it does not bind to any membrane proteins of SJL/J mice (Williams, 1991; Williams, 1990). Solid phase virus receptor assays to detect MHV-A59 failed to detect virus binding to SJL/J hepatocyte membranes but did detect virus binding to BALB/c hepatocyte membranes (Boyle et al., 1987). Similar results were obtained using radiolabeled MHV virions with BALB/c and SJL/J splenocytes (Holmes et al., 1986). In virus-overlay protein blot assays (VOPBA) of intestinal brush border and liver membrane

preparations, MHV-A59 bound to a BALB/c mouse glycoprotein but did not bind to any of the proteins from SJL/J mice (Boyle et al., 1987).

Screening of a BALB/c liver cDNA library with a nick translated probe generated by reverse transcriptase PCR with primers based on partial amino acid sequence of the receptor glycoprotein yielded a cDNA clone which encoded for the 110 kDa glycoprotein which was called MHVR (Dveksler et al., 1991). Based on amino acid homology and serologic cross-reactivity to human carcinoembryonic antigen (CEA), MHVR was found to be a member of the biliary glycoprotein (BGP) subgroup of the CEA family (Williams et al., 1991). CEA proteins are members of the Immunoglobulin (Ig) superfamily due to the shared sequence and structural homology with Igs. The structure of MHVR as predicted by nucleotide sequence includes four (Ig)-like domains, a transmembrane domain, and an intracytoplasmic domain. Based on alignment, the amino-terminal Ig-like domain (N-domain) of MHVR, which is 108 amino acids long, is structurally similar to the variable domain of Ig molecules and the other three Ig-like domains are structurally similar to the constant regions of Ig molecules (Hammarstrom et al., 1993).

Many isoforms of BGP have been identified (McCuaig et al., 1993) and these result from alternative splicing of a single *Bgp* gene (Barnett, 1989). Multiple splice variants of a single *Bgp* gene can be coexpressed in a single cell. MHVR is encoded by the *Bgp1*^{a} gene. The splice variants of MHVR consist of the four domain isoform referred to as MHVR, a splice variant which joins domains 1 and 4 thus eliminating domains 2 and 3, and both of the above with an extra 62 amino acids in the cytoplasmic domain i.e., 72 amino acids rather than 10. These are referred to as MHVR(4d), MHVR(2d), MHVR(4d)$_{L}$, and MHVR(2d)$_{L}$ (McCuaig et al., 1992). When transfected into MHV-A59 resistant baby hamster kidney (BHK) cells, each of the four splice variants serve as functional receptors for MHV-A59 (Dveksler et al., 1993a). Dveksler et al. (1993b) showed, through binding studies with several splice variants of MHVR and MHVR molecules with deletions in the different domains, that MHV-A59 virions and MAb CC1 bind to different sites on the N-domain of MHVR. MAb CC1 binding to MHVR prevents subsequent binding of virus (Dveksler et al., 1993b). MHVR from which any single Ig-like domain other than the N-domain was deleted still bound MHV-A59 and MAb CC1 and did serve as a

functional receptor for the virus. An anchored construct of the N-domain alone did not serve as a functional receptor, but when a chimeric protein composed of the N-domain of MHVR linked to the second and third Ig-like domains and anchor of the mouse poliovirus receptor homolog was constructed and expressed in BHK cells, it did serve as a functional receptor (Dveksler et al., 1995).

Subsequently, four BGP cDNAs were isolated from the SJL/J strain of inbred mice and the CD1 strain of outbred mice: mmCGM2(4d), mmCGM2(4d)$_L$, mmCGM2(2d), and mmCGM2(2d)$_L$. These cDNAs are splice variants of the *Bgp1*^{b} gene. mmCGM2 reveals remarkable similarity with MHVR with the largest number of differences in the N-domain. There are 29 amino acid differences between MHVR and mmCGM2 in the 108 amino acids of the N-domain and 5 amino acid substitutions in domains 2-4 (Dveksler et al., 1993a). These mmCGM2 glycoproteins are 3-5 kDa smaller than MHVR and they do not bind to MHV-A59 or MAb CC1 in VOPBAs or immunoblots (Williams et al., 1990). They do serve as weak MHV receptors when transfected into BHK cells; therefore, the site for virus and MAb CC1 binding are different. Cell lines derived from SJL/J mice are resistant to infection

with MHV-A59 but when transfected with MHVR or mmCGM2 cDNAs, they become susceptible even though mmCGM2 is normally expressed in these cells as shown by RNA-PCR. SJL/J mice are the only inbred mouse strain so far characterized to express mmCGM2 and its splice variants; yet, they do not express MHVR and its variants. CD1 outbred mice express both MHVR and mmCGM2 variants (Dveksler et al., 1993a). This strongly suggests that *Bgp1*^{a} and *Bgp1*^{b} are not isoforms of each other but rather alleles of the same gene.

The *Bgp2* gene has recently been cloned (Nédellec et al., 1994). Its protein product consists of two Ig-like domains, a transmembrane domain, and an intracytoplasmic domain. Amino acid sequence comparison demonstrates that there is homology between the *Bgp2* encoded protein and proteins encoded by the *Bgp1*^{a} and *Bgp1*^{b} genes. *Bgp2* gene products are expressed in both BALB/c and SJL/J mice. Bgp2 serves as a receptor for MHV-A59 when transfected into BHK cells; however, the number of observed infected cells was much lower when compared to MHVR transfected cells. MAb CC1 does not protect Bgp2 transfected cells from virus infection (Nédellec et al., 1994).

More recently, a new member of the murine CEA family, mmCGM3 (*cea10*), was cloned (Keck et al., 1995). mmCGM3 has two N-domains and three Ig-like carboxy-terminal domains with the N-domains being the most different from MHVR, mmCGM2 and bgp2. Of the 108 amino acids in the N-domain, 43 of these are different between mmCGM3 and MHVR (Fig 1). Transmembrane and intracytoplasmic domains are lacking in the mmCGM3 protein (Keck et al., 1995). The N-domain of mmCGM3 in a chimera with MHVR does not bind MHV-A59 nor do membrane anchored chimeras serve as functional receptors for the virus (J. Lu et al., unpublished results).

Rat *Bgps* show homology to the murine *Bgps* (Lin and Guidotti, 1989). There are 54 amino acid differences in the N-domain of rat bgp when compared to MHVR and 46 amino acid differences between the N-domains of rat bgp and mmCGM3. Rat bgps do not bind MHV-A59 or MAb CC1 nor do they serve as functional receptors for MHV-A59 when transfected into MHV-resistant BHK cells.

BGPs can act as cell adhesion molecules (Hammarstrom et al., 1993). Besides BGPs, other molecules associated with cell-cell interactions within the Ig superfamily have been shown to serve as virus receptors: CD4 for the human

immunodeficiency virus-1 (HIV-1), poliovirus receptor (PVR) for poliovirus, and intercellular adhesion molecule-1 (ICAM-1) for the major group of human rhinoviruses (White and Littman, 1989). All of these receptors have N-terminal domains which are homologous to Ig variable domains. It is to the N-terminal domain of each of these receptors that their respective viruses bind (Arthos et al., 1989; Freistadt et al., 1991; Lineberger et al., 1991; McClelland et al., 1991; Register et al., 1991). The virus binding site on ICAM-1 is at the BC and FG loops, on PVR at the C'C"D and DE regions (Bernhardt et al., 1994), and on CD4 at the C'C"D region (Moebius et al., 1992).

The goal of this work is two fold. First, to determine whether *Bgp1*^{a} and *Bgp1*^{b} are allelic variants of the same gene, and second, to determine which amino acids are involved in MHVR-virus binding and MHVR-MAb CC1 binding.

Figure 1. Comparison of the amino acid sequence of the N-domains of MHVR, mmCGM2, mmCGM3, and rat bgp. Only amino acids that differ from the MHVR sequence are shown for mmCGM2, mmCGM3 and rat bgp.

N-domain

```
                    10          20          30          40          50
 MHVR    EVTIEAVPPQVAEDNNVLLLVHNLPLALGAFAWYKGNTTAIDKEIARFVPNS
mmCGM2                                          PVSTNA  VH  TGT
mmCGM3  Q  V    L RTA            QT RV Y     SG GHN  G   TSI
Rat bgp Q  VD    N V ESS    T    QEFQV Y   VT  GLNS    YIRS

        60          70          80          90          100
 NMNFTGQAYSGREIIYSNGSLLFQMITMKDMGVTTLDMTDENYRRTQATVRFHVHP
  KTT  P H    TV       I RV V  T  Y IE     F   E   Q
  RSKL L H    T       F  SV KN E  Y  Y L Q FEI PIS
  TSQ EP     VT       F  NVNKT E PY  SVI KQFNPI TS Q R
```

MATERIALS AND METHODS

Amplification of genomic DNA by PCR: 500 ng of mouse genomic DNA (purchased from Jackson Laboratory, Bar Harbor, ME), were mixed with 0.3 µg each of sense primer #1 and antisense primer #2 from Table 1 [oligonucleotide primers were synthesized using an Applied Biosystems DNA Synthesizer (Applied Biosystems Inc., Foster City, CA) at USUHS], 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus), 5 µl of 10x AmpliTaq buffer (500 mM KCl, 100 nM Tris HCl pH 9.0, 1% Triton X-100, 15 nM $MgCl_2$)(Perkin Elmer Cetus), and 5 µl of 10 mM each dXTPs (Invitrogen), and water in a 50 µl reaction. The reactions were overlaid with mineral oil and heated at 94°C for 4 min. followed by 27 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 30 sec in the Gene Amp PCR System 9600 (Perkin Elmer Cetus, Norwalk, CT).

Three-step mutagenesis PCR: Plasmid DNA to be used as template was linearized with *Not*I (New England Biolabs, Beverly, MA). 1 ng of linearized template was combined with 0.5 µg each of sense and antisense primers (listed in Table 1), 2 µl of 10 mM each dXTPs, 10 µl of 10x Vent buffer (1x:

Table 1. Sequences of the oligonucleotides employed. Sequences are listed in 5′ to 3′ orientation.

OLIGONUCLEOTIDE SEQUENCES USED FOR MUTAGENESIS PCRs

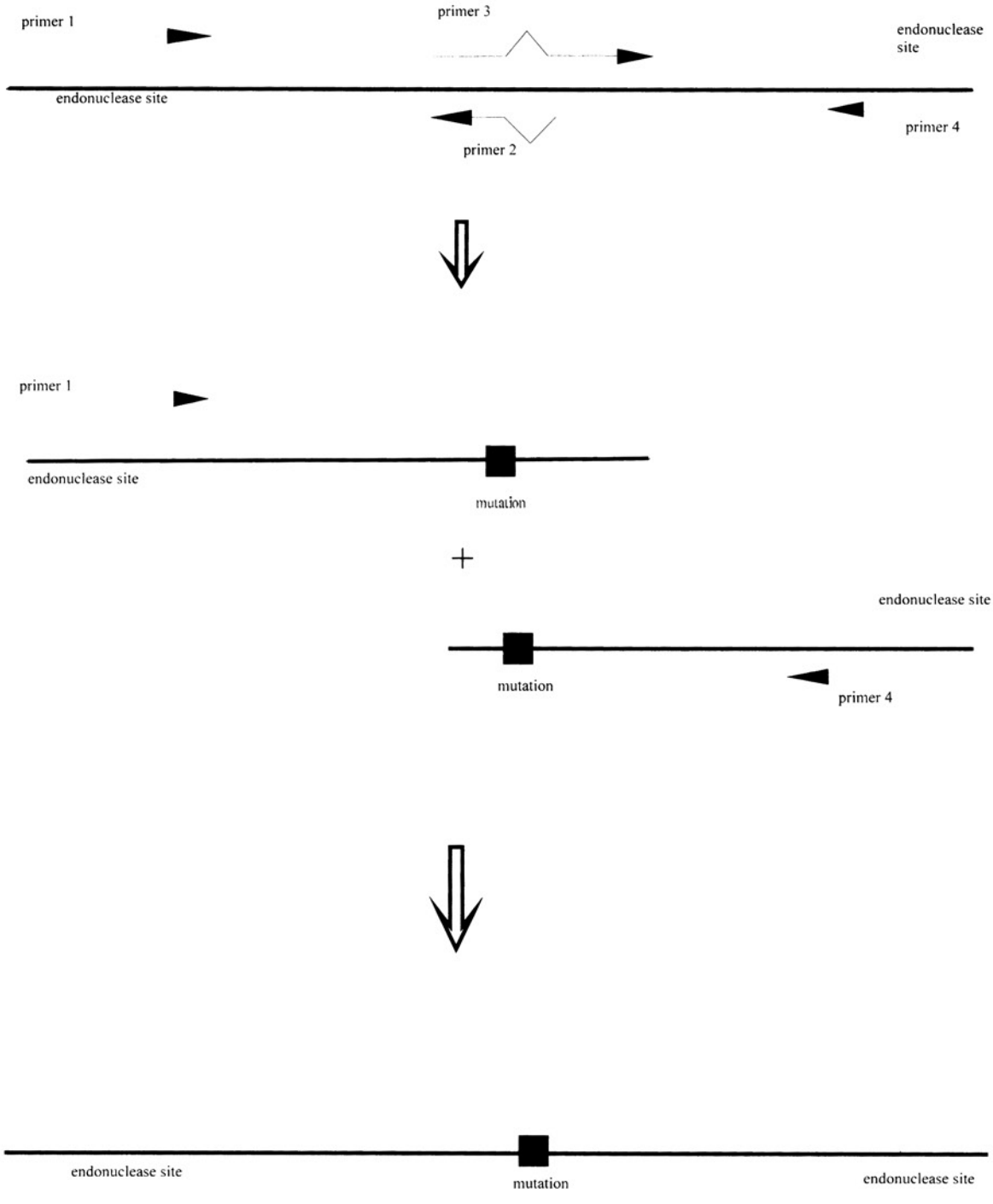
Primer	Sequence
1	CCAGGTTGCTGAAGACAAC
2	TAGACTCCCGTATCCTTCACG
3	GGTACCAGGGAAACAGTGGG
4	CACGGGATTGGAGTTGTTGCT
5	GTAATACGACTCACTATAGGGC
6	CCACTGTTTCCCTGGTACCAG
7	GTACATGAAATCGCACAGTCG
8	ATCGATATGAGTTTCTTGGGGCTTGCA
9	GAATAGTTACTCTTCGGGGT
10	CAAGAAACTCATATCGATGGATGTTAC
11	CGTTTTATTCCTATCGATATTTGGTAC
12	ATCGATAGGAATAAAACGGGGCAAGCA
13	ACTGGTACCAGGGAAACACTACGGCTATACACAATGA
14	ACTGGTACCAGGGAAACACTACGGCTGGACAC
15	ATATCGATATGAATAAAACG
16	CCAAATATTAATAGGAATTTCACG
17	GAAATTCCTATTAATATTTGGTAC
18	ATACACAATGAAATTGCACGA
19	TGCAATTTCATTGTGTATAGCCG
20	GTACCAAATAGTAATAGGAGT

21	CCTATTACTATTTGGTACAAATCG
22	GGACACAAAGAAATTGCACGATTTGTA
23	CAAATCGTGCAATTTCTTTGTGTCCAG
24	ATGGATCCCACGGGGCAAGC
25	CCCCGTGGGATCCATATTTC
26	CGAATTCAGGCGACTGTG
27	CGCCTGAATTCGACGATAG
28	GTTTGATCAAAACTATCGTC
29	TTGATCAAACATCTAGTG
30	GATGAAAACTATAATCCTACTC
31	GAGTAGGATTATAGTTTTCATC
32	ACAATATACAGCAATGGATCCCTGTTCTTC
33	GAACAGGGATCCATTGCTGTATATTGTCTC
34	GAGCCTACACACTAGATATG
35	GTAGGCTCCCATATCCTTC
36	CTACACACTAGATTTGACAG
37	CTGTCAAATCTAGTGTGTAG
38	GCATACAGCGGCCGTTCCCTGCTC
39	GAGCAGGGAACGGCCGCTGTATGC
40	CTGCCCCAGACGCGTGGAGTCTTTTACTGGTAC
41	GGTACCAGGGAAACACTACG
42	GGTACTTCCTATACCCTCAGATG
43	ACACTACGGCTATAGACAAA

44	ATCTGCCGCAGACGCTCC
45	TCGTTGTTGAGGTTAGGGCAC
46	AATGGATCCCTGCTCTTCCAAGCGGCCACCATG
47	AGACTACAACAGGGCCTG

10 mM KCl, 20 mM Tris HCl pH 8.8, 10 mM $(NH4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100)(New England Biolabs), and water to a final volume of 99.5 µl. The mixture was overlaid with mineral oil and heated to 94°C for 4 min. after which 2 units of Vent polymerase were added. Reactions were cycled 25-30 times through 94°C for 30 sec., the chosen annealing temperature for 30 sec., and 72°C for 40 sec. Annealing temperatures were calculated as 5°C below the melting temperature of the oligonucleotide. Melting temperatures were calculated by the equation T_m= 4(G+C) + 2(A+T). This PCR reaction generated the first PCR product (Kwok et al., 1994). A second PCR product was generated using a second set of sense and antisense primers but the same template (see Figure 2). PCR products were electrophoretically separated on agarose gels and the desired DNA bands cut from the gel and eluted with Elu-Quik (Schleicher and Schuell, Keene, NH). This system relies on melting the agarose pieces in buffer at 50°C and binding the DNA to glass beads in sodium perchlorate. For the third PCR reaction, 10 ng of each of PCR products 1 and 2 were added to 2 µl of 10 mM each dXTPs, 10 µl of 10x Vent buffer, and water in a final volume of 99.5 µl. The reactions were heated to 94°C for 4

Figure 2. Three-step mutagenesis PCR. The basic strategy for generating restriction endonuclease sites and point mutations distant from existing restriction endonuclease sites is shown. The first step involves the template and primer 1 and primer 2. The second step involves the same template with primer 3 and primer 4. The third step results in the extension of the products from the first two steps followed by amplification. The bends in the arrows designated primer 2 and primer 3 represent mismatched bases between the oligonucleotide and the template.


primer 1
primer 3
endonuclease site
endonuclease site
primer 4
primer 2
primer 1
endonuclease site
mutation
endonuclease site
mutation
primer 4
endonuclease site
mutation
endonuclease site

min. after which 1 unit of Vent polymerase was added. Extension of the template DNA was accomplished with the same conditions described for the first PCR reaction but with annealing temperatures being 35°C for CGM_3-Kpn and 40°C for all others. After three cycles, 0.5 μg each of the sense and antisense primers (see Table 2) were added and the PCR reactions were continued for 25-30 cycles with new annealing temperatures calculated based on the added oligonucleotide primers.

One-step mutagenesis PCR: These reactions were done using similar parameters to the first PCR of the three-step mutagenesis PCR. Specifics are listed in Table 2. This method was used to create point mutations adjacent to restriction endonuclease sites which were included in one of the primers as demonstrated in Figure 3.

Two-step mutagenesis PCR: This technique (Barettino et al., 1994) is a modification of the three-step mutagenesis PCR in which only one megaprimer is synthesized. This technique was used to create the mutants named MHVR-Mlu and MHVR-Kpn. For the first PCR, the MHVR containing plasmid was linearized with *Not*I and used as template for the generation of the two mutants. Each of the first step reactions was

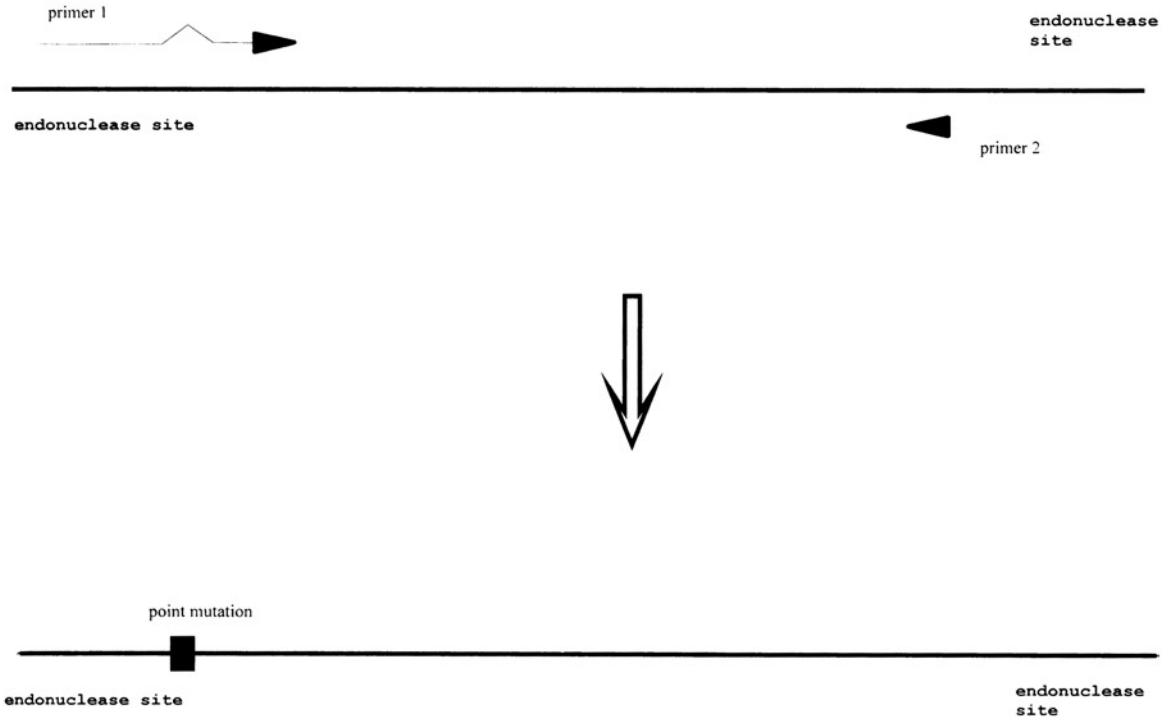
Table 2. List of the templates and oligonucleotides used to generate the mutations. The sequences of the listed oligonucleotides are found in Table 1.

PCR CONDITIONS

Mutation Name	Step	Template	Sense	Antisense
CGM_3-Kpn	1	$CGM_{3Hind\text{-}Bam}$+MHVR	3	4
	2		5	6
	3		5	7
$CGM_3\bar{c}$ MHVR Cla	1	$CGM_3\bar{c}$ MHVR	8	9
	2		5	10
	3		5	7
MHVR$\bar{c}$ CGM_3 Cla	1	MHVR$\bar{c}$ CGM_3	5	11
	2		12	9
	3		5	7
S_{38} G_{39} G_{41}→T T I		CGM_3-Kpn	13	9
S_{38} G_{39}→T T		CGM_3-Kpn	14	9
IDRK→IDMK		MHVR$\bar{c}$ CGM_3 Cla	15	9
S_{52} M_{54}→I R	1	MHVR	16	9
	2		5	17
	3		5	9
D_{42} $K_{43}S_{69}$→H N G	1	MHVR	18	9
	2		5	17
	3		5	9
T_{50} S_{51} I_{52}→P N S	1	CGM_3+MHVR	20	9
	2		5	21
	3		5	9
N_{43} G_{46}→K A	1	CGM_3+MHVR	22	9
	2		5	23
	3		5	9
T_{98}→I	1	MHVR	26	9
	2		5	27
	3		5	9
T_{91} E_{93}→FQ	1	MHVR	28	9
	2		5	29
	3		5	9
R_{96} R_{97}→V P	1	MHVR	30	9
	2		5	31
	3		5	9
$I_{66}L_{74}$→T F	1	MHVR	32	9
	2		5	33
	3		5	9
M_{77} I_{78} E_{93} V_{106}→A A G E		MHVR	46	9
V_{85}→A	1	MHVR	34	9
	2		5	35
	3		5	9
M_{90}→L	1	MHVR	36	9
	2		5	37

	3		5	9
ΔR fix	1	MHVR	38	9
	2		5	39
	3		5	9

Figure 3. One-step mutagenesis PCR. The basic strategy for generating point mutations near existing restriction endonuclease sites is shown. The bends in the arrow designated primer 1 represents mismatched bases between the oligonucleotide and the template.


primer 1
endonuclease site
endonuclease site
primer 2
point mutation
endonuclease site
endonuclease site

set up with 1 ng of linearized MHVR template, 0.5 µg of sense and antisense primers #40 and #4 for MHVR-Mlu and #41 and #4 for MHVR-Kpn (see Table 1), 2 µl of 10 mM each dXTPs, 10 µl of 10x Vent buffer, and water to a final volume of 99.5 µl. The reaction was overlaid with mineral oil and held at 94°C for 4 min. after which 1 unit of Vent polymerase was added. Reactions were cycled at 94°C for 30 sec., 55°C for 30 sec., and 72°C for 40 sec. for 27-30 cycles. PCR products were electrophoretically separated on agarose gels and the selected DNA bands cut from the gel and eluted with Elu-Quik. For the second step PCR, 1 ng of *Not*I linearized MHVR containing plasmid, 10 ng of the respective megaprimer synthesized in the first PCR reaction, 0.5 µg of sense and antisense primers #5 and #4(see Table 1), 2 µl of 10 mM each dXTPs, 10 µl of 10x Vent buffer, and water in a final volume of 99.5 µl were overlaid with mineral oil and held at 94°C for 4 min. after which 1 unit of Vent polymerase was added. The reactions were then cycled at 94°C for 30 sec., 59°C for 30 sec., and 72°C for 40 sec. 27-30 times.

Sequencing of plasmid DNA: Sequencing reactions were performed using the Taq DyeDeoxy Terminator Cycle Sequencing

Kit (Applied Biosystems). Prior to sequencing, plasmid DNA was precipitated at -70°C for 1 hour with the addition of 0.25 volumes 4 M NaCl, 2.5 volumes ethanol, and 1 µl of glycogen. The precipitated DNA was pelleted by centrifuging at 13,000 g for 15 min. at 4°C. The pellet was washed with 70% ethanol, recentrifuged at 13,000 g for 5 min. at 4°C, and the resultant pellet redissolved in 20 µl of water. The sequencing reaction was set-up with 500 ng of the purified template DNA, 16-24 ng of the appropriate primer (listed in Table 2), 9.5 µl of 10x reaction premix (40 µl 5x TACS buffer, 10 µl dXTPs, 10 µl each of DyeDeoxy A, T, G, and C Terminators, 75 units of AmpliTaq DNA polymerase), and water in a 20 µl reaction. The reactions were overlaid with mineral oil and cycled 25 times at 96°C for 30 sec., 50°C for 15 sec., and 60°C for 4 min. Excess fluorescently-labeled terminator nucleotides were removed by passing the PCR reaction through Select D columns (3Prime - 5 Prime, Boulder, CO). The sequencing reactions were then run on an ABI 392 DNA Sequencer (Applied Biosystems Inc.) at USUHS. All cDNA clones generated were sequenced. Oligonucleotides employed to sequence the cDNA clones are listed in Table 3.

Table 3. Oligonucleotides used to sequence the generated mutations. Oligonucleotide sequences are listed in Table 1.

Oligonucleotide primers used for sequencing

NAME	Oligonucleotides
$CGM_{3\ Hind\text{-}Bam}$ + MHVR	#5 & 42
$CGM_{3\ Bam\text{-}Nsi}$ + MHVR	#43
Rat N-domain + MHVR	#5 & 45
$Rat_{\ Hind\text{-}Bam}$ + MHVR	#5 & 42
$Rat_{\ Bam\text{-}end\ N\text{-}domain}$ + MHVR	#43
$S_{52}\,M_{54}\rightarrow$I R	#1
MHVR-Mlu	#42
$D_{42}\,K_{43}S_{69}\rightarrow$H N G	#1
$T_{50}\,S_{51}\,I_{52}\rightarrow$P N S	#44 & 45
$N_{43}\,G_{46}\rightarrow$K A	#44 & 45
$S_{38}\,G_{39}\,G_{41}\rightarrow$T T I	#42 & 44
$S_{38}\,G_{39}\rightarrow$T T	#44 & 45
$T_{98}\rightarrow$I	#43
$T_{91}\,E_{93}\rightarrow$FQ	#43
$R_{96}\,R_{97}\rightarrow$V P	#43
$I_{66}L_{74}\rightarrow$T F	#43
$M_{77}\,I_{78}\,E_{93}\,V_{106}\rightarrow$A A G E	#43
$V_{85}\rightarrow$A	#43
$M_{90}\rightarrow$L	#43
MHVR-Kpn	#42
CGM_3-Kpn	#42
$CGM_3\,\bar{c}$ MHVR	#1
MHVR $\bar{c}\,CGM_3$	#42
$CGM_3\,\bar{c}$ MHVR Cla	#42
MHVR $\bar{c}\,CGM_3$ Cla	#1 & 42
IDRK→IDMK	#42 & 44
3R3R	#43
CGM_3 Cla	#42 & 44
ΔR fix	#45

Restriction endonuclease digestions: Restriction enzymes *Bam*HI, *Bsp*EI, ClaI, *Hind*III, *Kpn*I, and *Nsi*I were purchased from New England Biolabs. Restriction enzyme *Not*I was purchased from Boehringer Mannheim (Indianapolis, IN). All restriction endonuclease digestions were carried out in the appropriate buffers. Where buffers for double enzyme digestions were not compatible, the first digestion was mixed with an equal volume of 1:1 phenol:chloroform (Ambion, Austin, TX) and phase separated by centrifugation at 10,000 g for 5 min. 2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate were added to the DNA phase. This was stored at -70°C for 1 hour. The precipitated DNA was pelleted by centrifugation at 13,000 g for 15 min at 4°C, followed by a 70% ethanol wash and recentrifugation at 13,000 g for 5 min. at 4°C. The DNA pellet was redissolved in 20 µl of water and the second digestion performed.

Agarose gel electrophoresis: Agarose gels were made at the appropriate percentage using SeaKem GTG agarose (FMC Bioproducts, Rockland, ME) in either TAE (0.04 M Tris-acetate, 0.001 M EDTA pH 8.0) or TBE (0.09 M Tris borate, 0.002 M EDTA pH 8.0) buffer. 1/6 volume of 6x dye (100 mM EDTA pH 8.0, 1% SDS, 30% glycerol, 0.1% bromophenol

blue)(Advanced Biotechnologies Inc., Columbia, MD) was added to the DNA to be separated on the gel. After electrophoresis, agarose gels were stained with ethidium bromide (Oncor, Gaithersburg, MD) and the DNA was visualized with ultraviolet light.

Southern blots: Agarose gels were denatured for 15 min. with 1.5 M NaCl and 0.5 M NaOH and neutralized for 15 min. with 1 M Tris Cl pH 7.4 and 1.5 M NaCl. The DNA was transferred to a Nytran membrane (Schleicher and Schuell), with 20x SSPE overnight by capillary action by the method described by Southern (1975). The DNA was cross-linked to the Nytran membrane for 2 min. under ultraviolet light (254 nm) (Fotodyne, New Berlin, WI) and the membrane was baked for 1 hour at 80°C in a vacuum oven.

DNA ligations: Ligations were performed at a ratio of insert to vector of 3:1 with total DNA per reaction no greater than 300 ng. Insert and vector DNA was added to Ready-to-go T4 DNA ligase tubes (6 units T4 ligase, 66 mM Tris Cl pH 7.6, 6.6 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM spermidine, 10 mM DTT) (Pharmacia Biotech, Uppsala, Sweden), incubated for 45-60 min. at 16°C, and the reaction stopped by a 10 min. incubation at 70°C.

Transformation of competent bacteria: 1 µl of the ligation reaction was added to 50 µl of DH5α competent cells (Life Technologies). Cells were incubated on ice for 30 min., heat-shocked at 37°C for 20 sec., replaced on ice for 2 min., then incubated at 37°C for 1 hour in 950 µl S.O.C. medium (Bethesda Research Laboratories, Gaithersburg, MD). Cells were plated onto agar plates [15 g Bacto-agar in 1 liter Luria-Bertani (LB) broth containing 50 µg/ml ampicillin (Sigma, St. Louis, MO)] (LB broth: 10 g bacto-tryptose, 5 g bacto-yeast extract, 10 g NaCl, pH 7.0 in 1 liter) and incubated at 37°C for 8-12 hours.

Preparation of plasmid DNA: Bacterial colonies were grown in replica on Nytran filters (Schleicher and Schuell) overnight at 37°C. The filters were then denatured in several volumes of 1.5 M NaCl and 0.5 M NaOH for 2 min., neutralized in several volumes of 1.5 M NaCl and 1 M Tris Cl pH 7.4 for 3 min., and probed with ^{32}P-labeled oligonucleotides to verify the presence of the desired insert in the plasmids (Grunstein and Hogness, 1975). Verification was also achieved by restriction endonuclease digestions of plasmid preparations from minipreps. The Wizard 373 Purification System (Promega, Madison, WI)

protocol was followed to isolate plasmid DNA. This is based on an alkaline lysis and binding of plasmid DNA to an ion-exchange resin. The colony containing the desired plasmid was grown overnight at 37°C in 500 ml of super broth (0.089 M $KHPO_4$ pH 7.5, 24 g bacto-yeast extract, 12 g bacto-tryptose, 4 ml glycerol in 1 liter) with ampicillin (Sigma) at a concentration of 50 µg/ml for cesium chloride density gradient separation.

Oligonucleotide labeling: To label oligonucleotides to be used as probes, 1 µg of oligonucleotide (20-30 bases long), 2 µl of 10x kinase buffer (0.5 M Tris Cl pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM EDTA)(New England Biolabs), 200 µCi of $\gamma[^{32}P]$ATP (New England Nuclear, Boston, MA), 10 units of T4 polynucleotide kinase (New England Biolabs), and water to a final volume of 20 µl were incubated for 30 min. at 37°C. The reaction was stopped by adding 2 µl of 0.5 M EDTA pH 8.0. Cross-linked blots and bacterial colonies grown on Nytran filters were prehybridized in Rapid-hyb buffer (Amersham Life Sciences, Arlington Heights, IL) for 30 min. at 45°C. 3-5 µl of labeled oligonucleotide was added to 6 ml of the Rapid-hyb buffer and incubated with the blots or colony filters for 1 hour at 45°C. Blots and filters were

washed extensively at room temperature in 2x SSPE, 0.1% SDS followed by washes at 5°C below the calculated annealing temperature of the oligonucleotide probe in 5x SSPE, 0.1% SDS. After hybridization with the ^{32}P-labeled probes, the blots were exposed to XAR-5 film (Kodak, Rochester, NY) overnight at -70°C.

Cesium chloride density gradient plasmid purification: The 500 ml cultures grown as described under preparation of plasmid DNA were centrifuged at 6,000 g. The resultant pellet was resuspended in 14 ml of GET buffer (50 mM glucose, 25 mM Tris Cl pH 7.5, and 10 mM EDTA) and 28 ml base buffer (0.2% NaOH and 1% SDS). After a 10 min. incubation on ice, 21 ml of acid buffer (3M potassium acetate and 2 M acetic acid pH 5.0) was added followed by another 5 min. incubation on ice. This was centrifuged at 6,000 g for 20 min at 4°C. 2 volumes of 95% ethanol was added to the supernatant and this was incubated on ice for 20 min. Following centrifugation at 5,000 g for 45 min at 4°C, the resultant pellet was resuspended in 10 ml TE buffer (10 mM Tris Cl pH 8.0, 1 mM EDTA pH 8.0) and 6 ml of 7.5 M ammonium acetate and was incubated on ice for 30 min. This was centrifuged for 30 min at 4,000 g at 4°C. 2.5 volumes

of ethanol was added to the supernatant and this was incubated on ice for 20 min. followed by centrifugation at 4,000 g for 15 min. at 4°C. The resultant pellet was resuspended in 5.2 ml TE buffer, 7.8 ml (2.03 g/ml) CsCl TFA (Pharmacia Biotech), and 10 µl 10,000x ethidium bromide. A density gradient was achieved by centrifuging this mixture at 65,000 rpm for 48 hours at 22°C. The DNA band was harvested and an equal volume of pentanol (Sigma) was added to extract the ethidium bromide. The bottom phase containing the DNA was removed and ethanol precipitated.

Transfection of plasmid DNA into BHK-21 cells: 3 µg of plasmid DNA was incubated with 18 µl of lipofectamine (2 mg/ml) (Life Technologies, Gaithersburg, MD) in 3 ml of OPTIMEM medium (MEM Eagles 1x with HEPES buffer, 2400 mg/l Na bicarbonate, L-glutamine) (Life Technologies) for 45 min. The DNA-lipofectamine complex was added to $6x10^5$ BHK-21 cells (American Type Culture Collection, Rockville, MD) grown on glass coverslips in a 60mm dish and incubated for 6 hours at 37°C after which 3 mls of BHK medium (Dubelco's modified Eagles medium with 10% fetal bovine serum, 10% tryptose phosphate buffer, 1% L-glutamine, 1% pen/strep fungizone mix) supplemented to 20% fetal bovine serum was

added. After an overnight incubation at 37°C, the media were removed and the cells were incubated for 24 hours at 37°C in regular BHK medium.

Detection of recombinant proteins on the surface of transfected cells by immunofluorescence: BHK-21 cells transfected with the cloned cDNAs were grown on glass coverslips. Transfected cells which were to be immunofluorescently stained for surface expression of recombinant proteins, were washed in PBS buffer with calcium and magnesium (Bio-Whitaker, Walkersville, MD). Cells were then fixed at 4°C in 2% paraformaldehyde for 1 hour. Cells were rehydrated in PBS with calcium and magnesium containing 2% normal goat serum (NGS)(Life Technologies) for 10 min. at room temperature. Coverslips were then incubated at 37°C for 1 hour with a 1:50 dilution of rabbit polyclonal anti-MHV receptor antibody (655) preabsorbed with paraformaldehyde fixed BHK cells. Coverslips were washed with PBS with calcium and magnesium containing 2% NGS and then incubated at 4°C for 45 min. with a 1:50 dilution of rhodamine labeled goat anti-rabbit antibody (Organon Teknika Corp., Durham, NC). Coverslips were washed with PBS with calcium and magnesium containing 2% NGS and mounted onto

glass slides with Aqua-mount (Lerner Laboratories, Pittsburgh, PA) prior to examination in a Zeiss fluorescent microscope (Carl Zeiss Corp., Thornwood, NY).

Detection of MHV antigens by immunofluorescence: BHK-21 cells transfected with the cloned cDNAs were grown on glass coverslips. MHV-A59 was propagated in the 17Cl1 line of spontaneously transformed BALB/c 3T3 cells and plaque assayed in L2 cells. The transfected BHK-21 cells were infected with MHV-A59 at a multiplicity of infection (MOI) of 5 in 0.8 ml of BHK medium. Transfected cells to be assessed for protection by mouse monoclonal anti-MHV receptor antibody (MAb CC1) were pre-treated one hour before MHV-A59 infection with a 1:1 dilution of MAb CC1 hybridoma supernatant in BHK medium. The virus containing medium was aspirated off of the coverslips after one hour and 3 mls of BHK medium was added for 8-12 hours. Coverslips were than washed with PBS with calcium and magnesium. Cells were fixed in acetone at -20°C for 10 min. and were rehydrated in PBS with calcium and magnesium containing 2% NGS for 10 min. at room temperature. Coverslips were then incubated at 37°C for 1 hour with a 1:50 dilution of polyclonal anti-MHV-A59 convalescent mouse serum in PBS with calcium and magnesium

containing 2% NGS. Coverslips were washed three times with PBS with calcium and magnesium containing 2% NGS and then incubated at 4°C for 45 min. with a 1:100 dilution of rhodamine labeled goat anti-mouse antibody (Organon Teknika Corp.). Coverslips were washed with PBS with calcium and magnesium containing 2% NGS and mounted onto glass slides with Aqua-mount prior to examination in a Zeiss fluorescent microscope.

Production of recombinant proteins: BHK-21 cells were infected with vaccinia virus (vTF7-3) at a MOI of 10 (Fuerst et al., 1986). Virus containing medium was apirated off after 1 hour and fresh BHK medium was added. Two hours later, this medium was aspirated off and cells were transfected with plasmid DNA using lipofectamine reagent as described above. 24 hours post-infection, cells were lysed in 300 µl of RIPA buffer (0.1 M NaCl, 0.001 M EDTA pH 7.4, 0.01 M Tris pH 7.4, 0.1% Nonidet P40, 0.1% deoxycholate). Proteins were separated on 8% SDS-polyacrylamide gels (Novex, San Diego, CA) by the method of Laemmli (1970). Proteins were then transferred to nitrocellulose in transfer buffer (12 mM Tris base, 96 mM glycine, 20% methanol pH 8.3) and the membrane was placed in B-3 buffer (0.15 M NaCl,

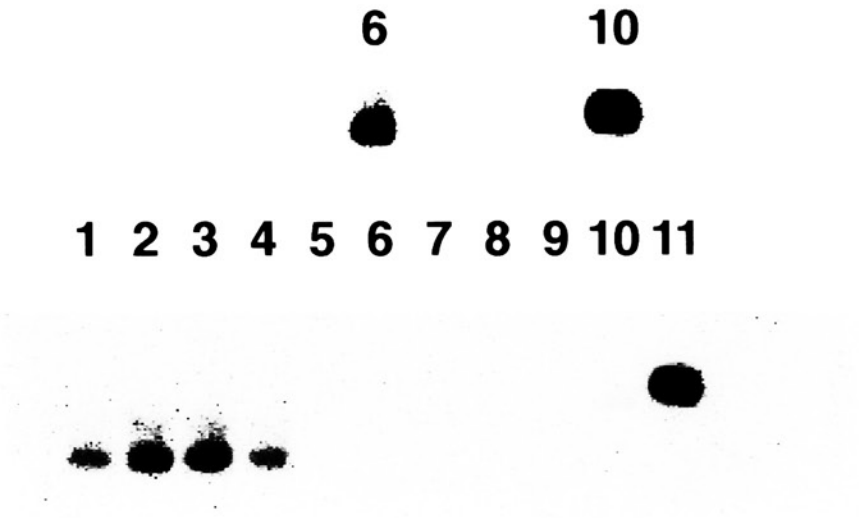
0.001 M EDTA, 0.05 M Tris base, 0.05 M Tris Cl, 0.05% polyoxyethylene sorbitan monolaurate, 0.1% bovine serum albumin, pH 7.4) supplemented with 3% bovine serum albumin (Sigma) for blocking overnight at 4°C.

Immunoblot analysis: B-3 blocked membranes to be analyzed for MAb CC1 binding were first incubated for 1 hour with a 1:50 dilution of mouse MAb CC1 in B-3 buffer at room temperature. Blots were washed three times with B-3 buffer and then incubated at room temperature for one hour with a 1:500 dilution of rabbit affinity purified anti-mouse IgG (Cappel, Cochranvelle, PA) in B-3 buffer. Blots were washed in B-3 buffer and were then incubated for 1 hour with 80 μCi/ml (3.2 μCi total) ^{125}I-labeled Staphylococcal protein A (New England Nuclear). Blots were washed again with B-3 buffer and exposed to XAR-5 film. B-3 blocked membranes to be analyzed for recombinant proteins using polyclonal antibody were first incubated at room temperature with a 1:250 dilution of rabbit polyclonal anti-MHV receptor antibody (655) in B-3 buffer. After washing, the blots were incubated with ^{125}I-labeled Staphylococcal protein A and autoradiographed as described above.

RESULTS

Amplification and hybridization of the *Bgp1*^{a} and *Bgp1*^{b} genes: Genomic DNA from SJL/J, SWR, A/J, C57BL/6, and C3H strains of inbred mice was amplified by PCR with sense oligonucleotide primer #1 and antisense oligonucleotide primer #2 (Table 1). These primers were chosen as they are both complimentary to regions of the N-domain of all four splice variants ((4d), (2d), $(4d)_L$, and $(2d)_L$) of MHVR (*Bgp1*^{a} gene product) and mmCGM2 (*Bgp1*^{b} gene product) (Dveksler et al., 1991; McCuaig et al., 1993). As a control for possible contamination, a reaction with water as template was included. Plasmids containing the cDNAs of all murine BGPs (MHVR, mmCGM2, bgp2, and mmCGM3) were digested with *Hind*III and *Bam*HI. This resulted in a DNA fragment consisting of the first 386 bp of the 411 bp N-domain (as measured in MHVR) which includes the area amplified by PCR from the genomic DNA. The PCR products and *Hind*III-*Bam*HI digested plasmids were separated by electrophoresis on a 2% agarose gel in duplicate. Staining with ethidium bromide showed the expected 198 bp PCR product. The DNA was transferred to Nytran membranes and the duplicate Southern blots were hybridized with ^{32}P-labeled primer #43 and with ^{32}P-labeled

Figure 4. Southern blot analysis of the PCR products generated after amplification of genomic DNA. Genomic DNA from C3H (lane 1), C57BL/6 (lane 2), SWR (lane 3), A/J (lane 4), and SJL/J (lane 6) strains of mice was amplified by PCR. Duplicate blots were probed with either ^{32}P-labeled oligonucleotide #47 (top) or ^{32}P-labeled oligonucleotide #43 (bottom). 2 μg of plasmids containing Bgp2 (lane 8), mmCGM3 (lane 9), mmCGM2 (lane 10), and MHVR (lane 11) cDNAs were digested with *Hind*III and *Bam*HI and electrophoresed as controls. Water (lane 5) was used as template for PCR reactions as a control for possible contamination. PCR products are 198 base pairs long and *Hind*III-*Bam*HI digested products are 400 base pairs long.


6
10
1 2 3 4 5 6 7 8 9 10 11

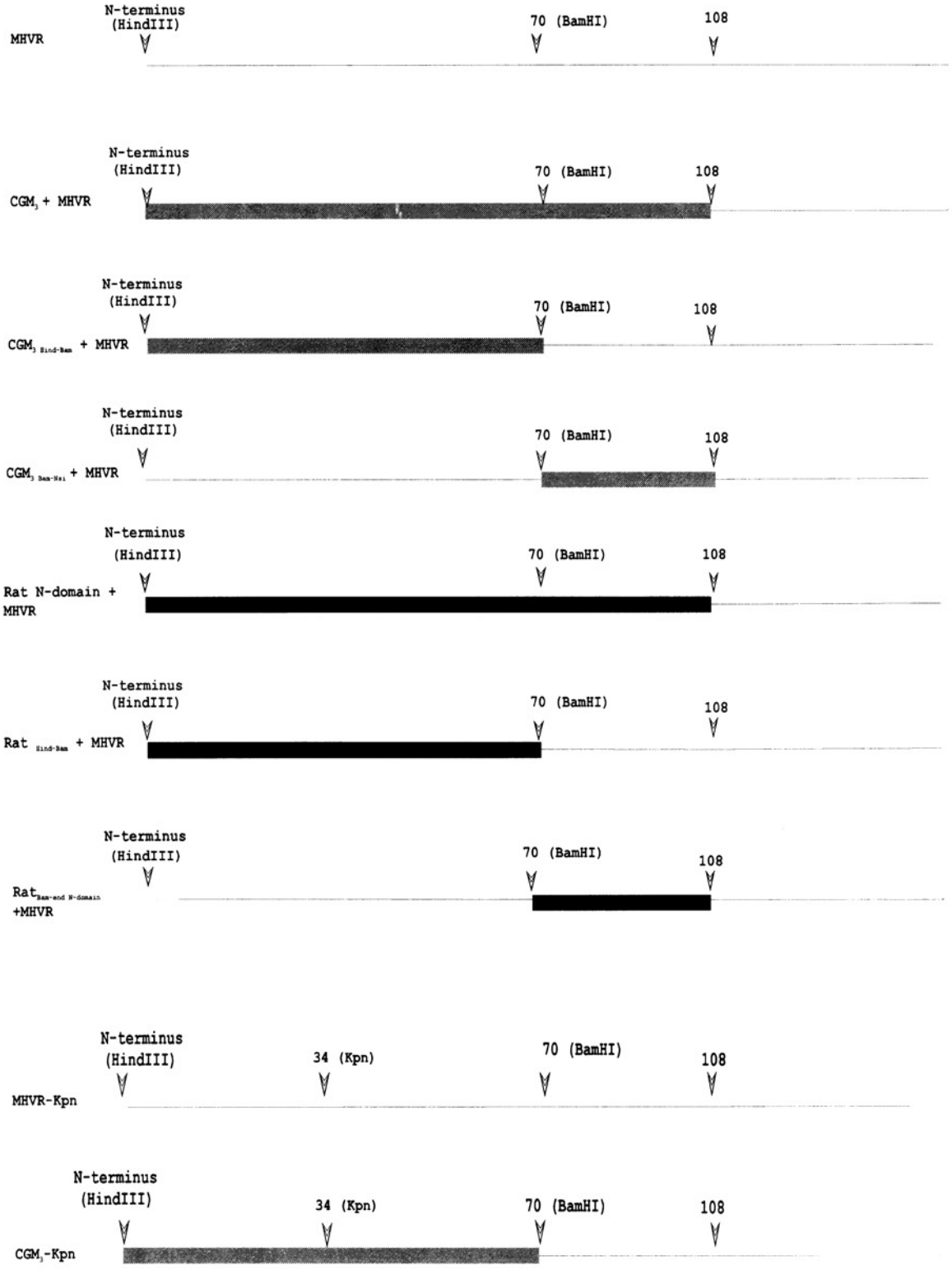
#47 respectively. These oligonucleotides were selected because #43 is MHVR specific and #47 is mmCGM2 specific. Fig 4. shows the results of an overnight exposure of the duplicate blots. PCR products obtained after amplification of SJL/J genomic DNA did not hybridize to the oligonucleotide specific for MHVR while all the PCR products generated by amplification of genomic DNA isolated from all other inbred strains showed strong hybridization. PCR products obtained after amplification of SJL/J genomic DNA did hybridize to the oligonucleotide specific for mmCGM2 whereas none of the other PCR products obtained after amplification of genomic DNA of the other mouse strains did. It is therefore concluded that *Bgp1*^{a} and *Bgp1*^{b} are alleles of the same gene.

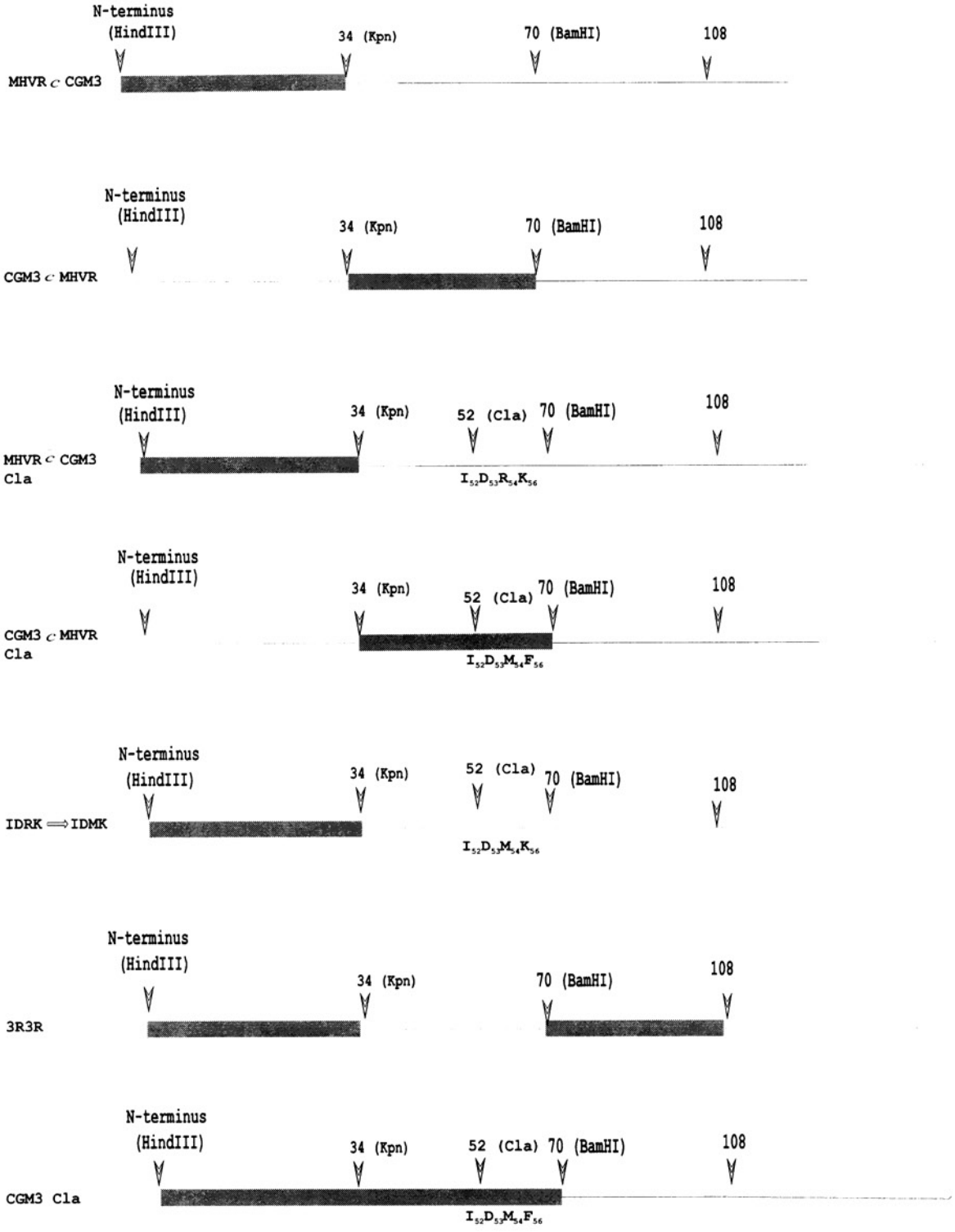
Identification of the virus and monoclonal antibody binding sites on the mouse hepatitis virus receptor MHVR.

Recombinant chimeric proteins with mmCGM3 and rat bgps. To identify the amino acids in the MHVR protein which bind to the MHV-A59 spike protein S, chimeric proteins were constructed using naturally occurring common restriction

enzyme sites between a member of the murine CEA family which does not function as a receptor for MHV-A59 (mmCGM3) and the functional MHVR receptor. All the chimeras were produced in an effort to alter the N-domain of the protein because previous work showed that the virus binds to the N-domain of MHVR (Dveksler et al., 1993b). Figure 5 shows a diagram of the different hybrids constructed using the MHVR and $mmCGM_3$ cDNAs. All hybrids contain the entire second, third, fourth, transmembrane, and cytoplasmic domains of MHVR. The N-domain consists of 108 amino acids and was artificially divided at amino acid 70 due to the presence of a common *Bam*HI site. Similarly, chimeric proteins were created using MHVR and the rat short bgp cDNA. Rat bgps do not function as receptors for MHV-A59. The resultant hybrids are also diagrammed in Fig. 5. Receptor-negative BHK cells were transfected with these hybrid cDNAs under the CMV promoter in the pcDNA3 eukaryotic expression vector. To determine the functional receptor activities of these recombinant proteins, BHK cells transfected with these plasmids were challenged with MHV-A59, fixed with acetone, and immunofluorescently stained with mouse polyclonal anti-MHV

Figure 5. Diagrammatic representation of the chimeras produced between MHVR and mmCGM3 and MHVR and rat bgp. The thin line represents MHVR, the shaded, thicker line mmCGM3 and the black, thicker line rat bgp. All chimeras were constructed with MHVR from the end of the N-terminal domain (amino acid 108) to the carboxy-terminus which is not shown in the diagrams.


MHVR
N-terminus (HindIII)
70 (BamHI)
108
CGM3 + MHVR
CGM3 Hind-Bam + MHVR
CGM3 Bam-Nsi + MHVR
Rat N-domain + MHVR
Rat Hind-Bam + MHVR
Rat Bam-end N-domain +MHVR
34 (Kpn)
MHVR-Kpn
CGM3-Kpn


N-terminus
(HindIII)
34 (Kpn)
70 (BamHI)
108
MHVR c CGM3
N-terminus
(HindIII)
34 (Kpn)
70 (BamHI)
108
CGM3 c MHVR
N-terminus
(HindIII)
34 (Kpn)
52 (Cla)
70 (BamHI)
108
MHVR c CGM3
Cla
I52D53R54K56
N-terminus
(HindIII)
34 (Kpn)
52 (Cla)
70 (BamHI)
108
CGM3 c MHVR
Cla
I52D53M54F56
N-terminus
(HindIII)
34 (Kpn)
52 (Cla)
70 (BamHI)
108
IDRK ⟹ IDMK
I52D53M54K56
N-terminus
(HindIII)
34 (Kpn)
70 (BamHI)
108
3R3R
N-terminus
(HindIII)
34 (Kpn)
52 (Cla)
70 (BamHI)
108
CGM3 Cla
I52D53M54F56

and rhodamine-labeled goat anti-mouse antibody (Fig. 6). Monoclonal anti-MHVR antibody (MAb CC1) binds to the N-domain of MHVR at a different site than MHV-A59 does. MAb CC1 binding protects MHVR expressing cells from infection with MHV-A59. To determine whether these recombinant proteins bound MAb CC1, cells transfected with cDNAs shown to encode for functional virus receptors were pre-treated with MAb CC1 prior to MHV-A59 challenge. If MAb CC1 was able to bind to the mutant receptor, the cells transfected with the cDNAs would no longer be susceptible to infection (Fig. 6). To further explore MAb CC1 binding, recombinant proteins were produced using the vaccinia virus T7 system. Through a combination of infection with this virus and transfection of BHK cells with the hybrid cDNAs under the T7 promoter, large quantities of protein were obtained. Following SDS-PAGE separation these proteins were transferred to nitrocellulose. Western blots with polyclonal anti-MHVR antibody and MAb CC1 were done (Fig. 7). All glycoproteins were recognized by polyclonal antibody 655. Results of the immunoblots suggest that the first 70 amino acids are the most critical for virus binding but something in the area of amino acids 70-108 contributes

Figure 6. Immunofluorescence labelling of hamster cells transfected with the designated chimeric cDNA clones. Immunofluorescence of cells represents the ability of the chimeras to act as MHV-A59 receptors, the failure of MAb CC1 to protect pretreated cells from MHV-A59 infection, or the surface expression of the chimeric proteins as measured by polyclonal antibody 655.

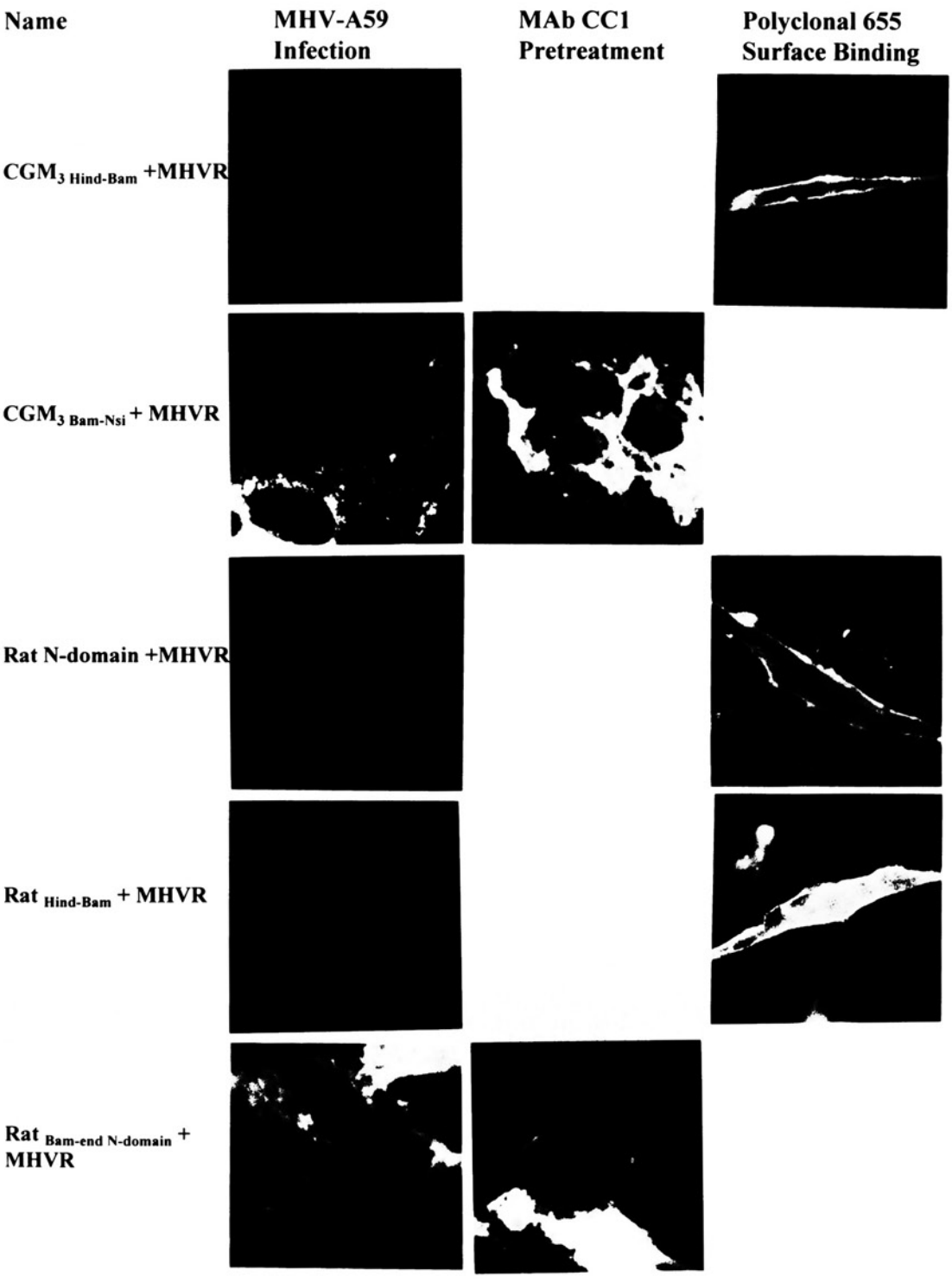
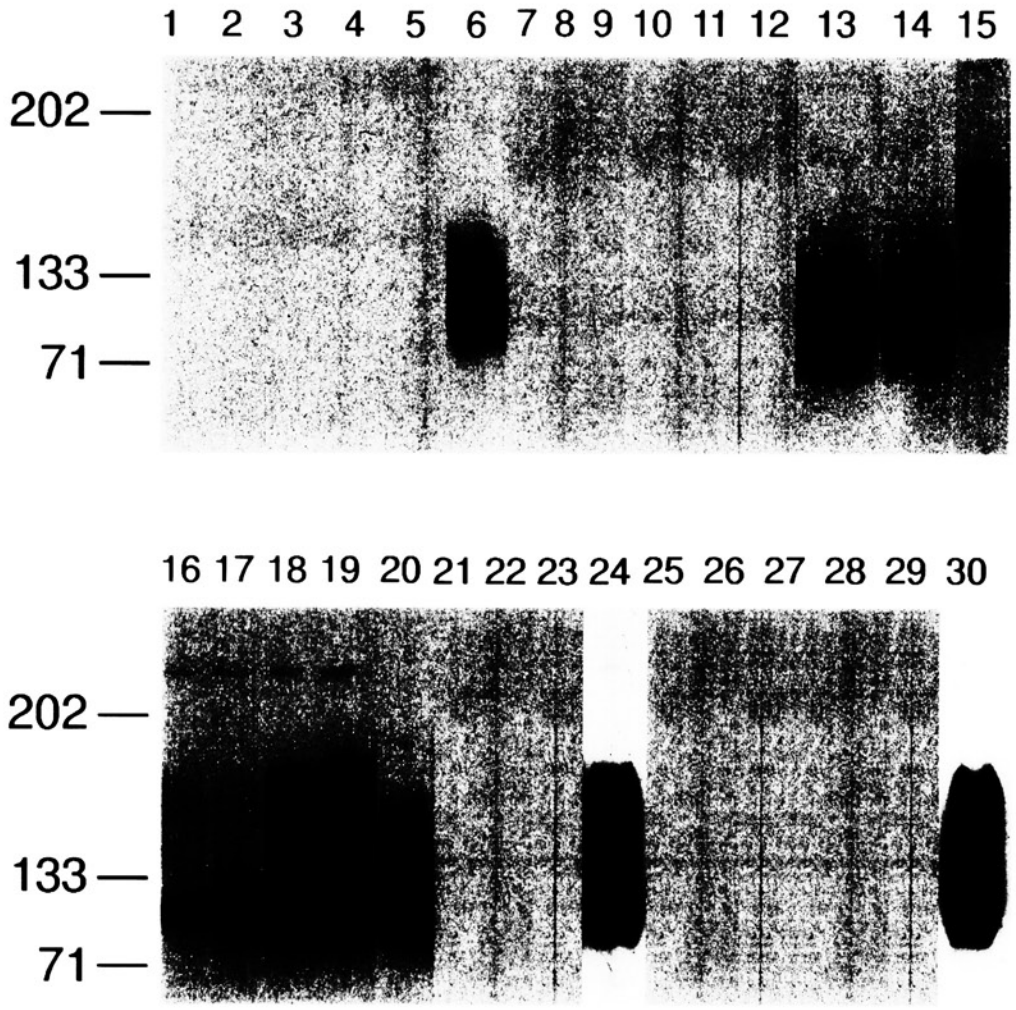

Name
MHV-A59 Infection
MAb CC1 Pretreatment
Polyclonal 655 Surface Binding
CGM3 Hind-Bam +MHVR
CGM3 Bam-Nsi + MHVR
Rat N-domain +MHVR
Rat Hind-Bam + MHVR
Rat Bam-end N-domain + MHVR

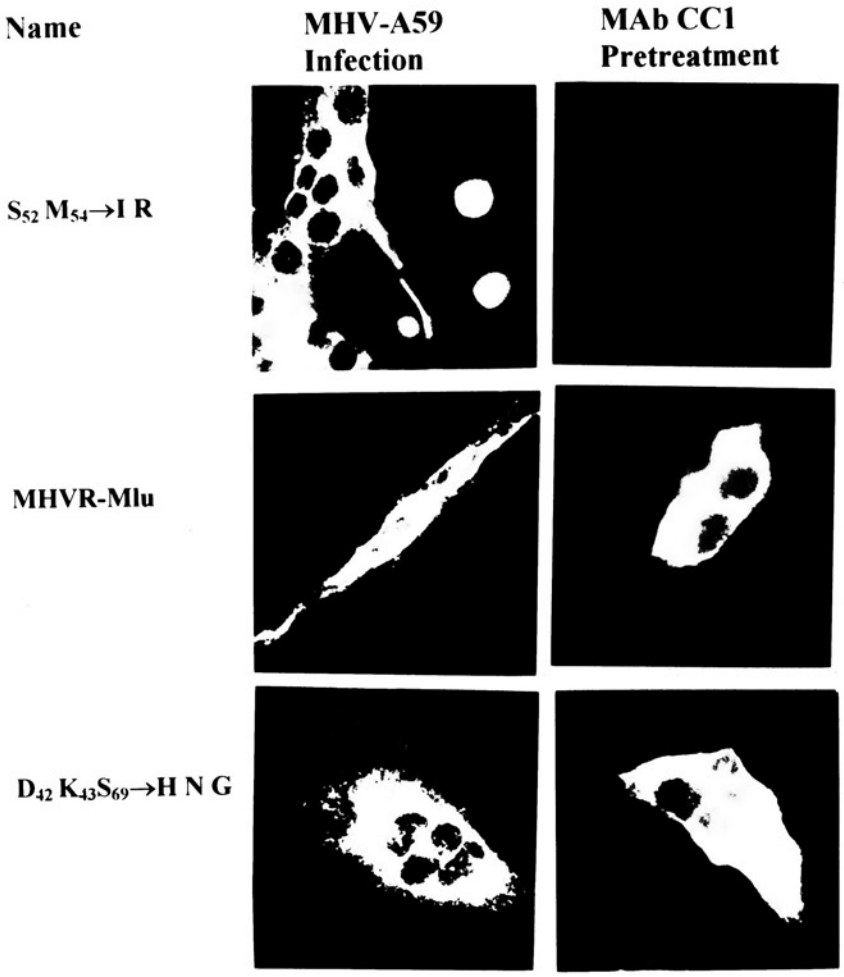
Figure 7. Recognition of mutant glycoproteins by MAb CC1. $CGM_{3Hind\text{-}Bam}$+MHVR lane 1), $CGM_{3Bam\text{-}Nsi}$+MHVR (lane 2), Rat N-domain+MHVR (lane 3), Rat $_{Hind\text{-}Bam}$+MHVR (lane 4), Rat $_{Bam\text{-}end\ N\text{-}domain}$+MHVR (lane 5), $S_{52}M_{54}\rightarrow$I R (lane 6), MHVR-Mlu (lane 7), $D_{42}K_{43}S_{69}\rightarrow$H N G (lane 8), $T_{50}S_{51}I_{52}\rightarrow$P N S (lane 9), $N_{43}G_{46}\rightarrow$K A (lane 10), $S_{38}G_{39}G_{41}\rightarrow$T T I (lane 11), $S_{38}G_{39}\rightarrow$T T (lane 12), $T_{98}\rightarrow$I (lane 13), $T_{91}E_{93}\rightarrow$F Q (lane 14), $R_{96}R_{97}\rightarrow$V P (lane 15), $I_{66}L_{74}\rightarrow$T F (lane 16), $M_{77}I_{78}E_{93}V_{106}\rightarrow$A A G E (lane 17), $V_{85}\rightarrow$A (lane 18), $M_{90}\rightarrow$L (lane 19), MHVR-Kpn (lane 20), CGM_3-Kpn (lane 21), $CGM_3\bar{c}$MHVR (lane 22), MHVR$\bar{c}CGM_3$ (lane 23), $CGM_3\bar{c}$MHVR Cla (lane 24), MHVR$\bar{c}CGM_3$ Cla (lane 25), IDRK→IDMK (lane 26), 3R3R (lane 27), CGM_3 Cla (lane 28), ΔR fix (lane 29) and MHVR (lane 30) mutant proteins were immunoblotted with MAb CC1. Molecular mass standards in kilodaltons are indicated.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
202
133
71
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
202
133
71

to MAb CC1 binding. To demonstrate surface expression of the proteins that do not encode for functional MHV receptors, transfected cells with the correspondent cDNAs were fixed with 2% paraformaldehyde and immunofluorescently stained using rabbit poylclonal anti-MHVR antibody (655) and rhodamine-labeled goat anti-rabbit antibody (Fig. 6). Polyclonal anti-MHVR (655) has been shown to recognize the fourth domain of MHVR which is present in all of the recombinant proteins generated (Dveksler, unpublished results).

Point mutations in amino acids 1-70. Based on tertiary structure analysis of other immunoglobulin superfamily proteins and the virus binding sites of some of these (ICAM-1, PVR, CD4)(Arthos et al., 1989; Freistadt et al., 1991; McClelland et al., 1991; Register et al., 1991; Staunton et al., 1990) point mutations were introduced to change amino acids in presumed exposed areas of the MHVR receptor. The amino acids in MHVR were changed to be like those amino acids in mmCGM3 in an effort to destroy receptor activity. In none of the generated mutant glycoproteins was functional MHV receptor activity eliminated (Fig. 8). MHVR-Mlu (L26Q, A27T, L28R, A30V, A32Y) and D_{42} K_{43} $S_{69}\rightarrow$H N G did lose the

Figure 8. Immunofluorescence labelling of hamster cells transfected with MHVR cDNA with the designated point mutations between amino acids 1 and 70. Immunofluorescence of cells represents the ability of the mutant glycoproteins to act as MHV-A59 receptors, the failure of MAb CC1 to protect pretreated cells from MHV-A59 infection, or the surface expression of the mutated proteins as measured by polyclonal antibody 655.


Name
MHV-A59 Infection
MAb CC1 Pretreatment
S_{52} M_{54}→I R
MHVR-Mlu
D_{42} $K_{43}S_{69}$→H N G

ability to bind MAb CC1. Using similar rationale and techniques, point mutations were created in the mmCGM$_3$ cDNA to change mmCGM3 amino acids into those of MHVR in an effort to generate a recombinant protein with functional receptor activity. As seen in Fig. 9 S_{38} G_{39} $G_{41}\rightarrow$T T I did gain receptor activity. In an effort to determine which of the three amino acids was important for MHV-A59 binding, S_{38} $G_{39}\rightarrow$T T was created, but as seen in Fig. 9 it did not act as a functional receptor. This suggested the importance of I41.

Point mutations in amino acids 70-108. To determine if amino acids located between residues 70 and 108 -including the putative prominant FG loop- were critical, point mutations were made to transform a functional receptor into a nonfunctional one by changing MHVR amino acids to be like those of mmCGM3 or rat bgps. None of these point mutations altered receptor activity or MAb CC1 binding(Fig. 10).

***Kpn* sites and chimeras.** To narrow down which of the first 70 amino acids of the N-domain are essential for virus binding to MHVR, a *Kpn*I restriction endonuclease site was introduced using the three-step or two-step PCR mutagenesis techniques in CGM$_{3\ \text{Hind-Bam}}$ + MHVR and MHVR (see Fig. 5). The

Figure 9. Immunofluorescence labelling of hamster cells transfected with mmCGM$_3$ cDNA with the designated point mutations between amino acids 1 and 70. Immunofluorescence of cells represents the ability of the mutant glycoproteins to act as MHV-A59 receptors, the failure of MAb CC1 to protect pretreated cells from MHV-A59 infection, or the surface expression of the mutated proteins as measured by polyclonal antibody 655.

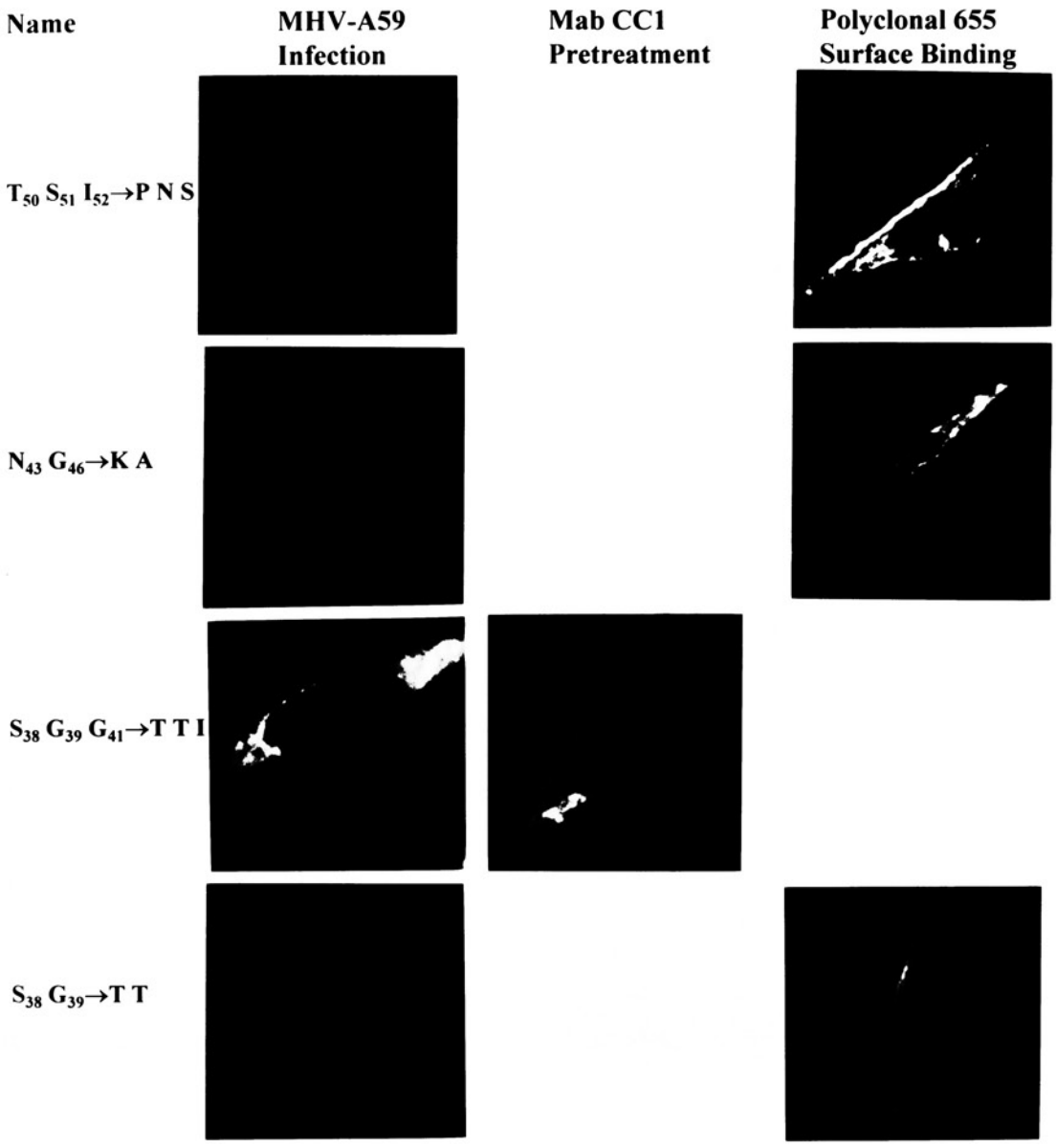
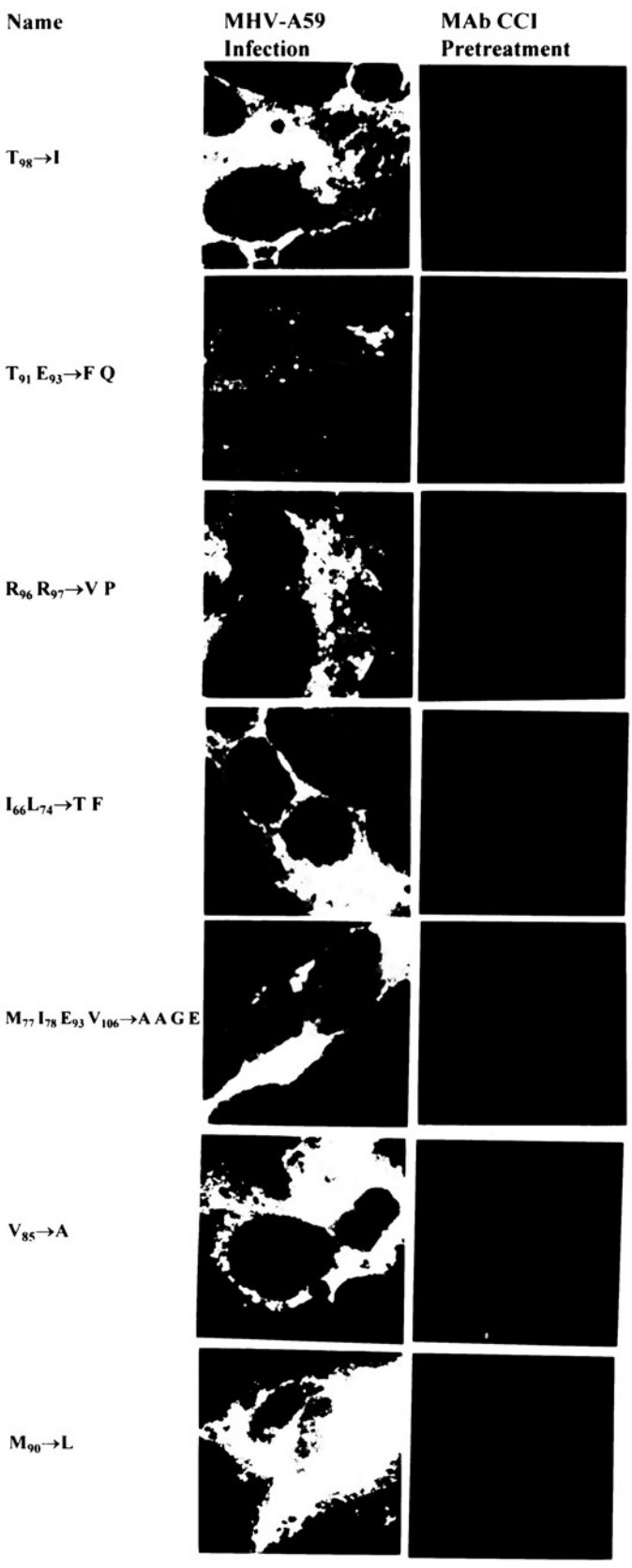

Name
MHV-A59 Infection
Mab CC1 Pretreatment
Polyclonal 655 Surface Binding
T50 S51 I52→P N S
N43 G46→K A
S38 G39 G41→T T I
S38 G39→T T

Figure 10. Immunofluorescence labelling of hamster cells transfected with MHVR cDNA with the designated point mutations between amino acids 70 and 108. Immunofluorescence of cells represents the ability of the mutant glycoproteins to act as MHV-A59 receptors, the failure of MAb CC1 to protect pretreated cells from MHV-A59 infection, or the surface expression of the mutated proteins as measured by polyclonal antibody 655.


Name
MHV-A59 Infection
MAb CCI Pretreatment
$T_{98}\rightarrow I$
$T_{91}\ E_{93}\rightarrow F\ Q$
$R_{96}\ R_{97}\rightarrow V\ P$
$I_{66}L_{74}\rightarrow T\ F$
$M_{77}\ I_{78}\ E_{93}\ V_{106}\rightarrow A\ A\ G\ E$
$V_{85}\rightarrow A$
$M_{90}\rightarrow L$

creation of the *Kpn*I site mutated K34Q in both the CGM_3 chimera and MHVR proteins. This single change did not alter receptor activity or MAb CC1 binding (Fig. 11). These plasmids were digested with *Hind*III and *Kpn*I and the liberated DNA pieces exchanged to create the hybrids MHVR $\bar{c}$ CGM_3 and CGM_3 $\bar{c}$ MHVR (Fig. 5). This also did not alter the receptor activity of the parent plasmid but did affect MAb CC1 binding (Fig. 11). This suggests the importance of amino acids 34 to 70 in receptor activity in confirmation of the I41 mutation described above and confirms the MHVR-Mlu results of the importance of residues amongst the first 34 amino acids for MAb CC1 binding.

***Cla* sites and mutations.** To further narrow down the amino acids involved in MHV-A59 binding between 34 and 70, *Cla*I restriction endonuclease sites were introduced using three-step mutagenesis techniques in plasmids MHVR $\bar{c}$ CGM_3 and CGM_3 $\bar{c}$ MHVR. The creation of the *Cla*I site at amino acid 52 created the point mutations S_{52} N_{53} M_{54} $F_{56}\rightarrow$I D R K in MHVR $\bar{c}$ CGM3 Cla and N_{53} R_{54} $K_{56}\rightarrow$D M F in CGM_3 $\bar{c}$ MHVR Cla (Fig. 5). These mutations altered both the receptor activity of the protein and its ability to bind MAb CC1 as expressed on

Figure 11. Immunofluorescence labelling of hamster cells transfected with the designated *Kpn*I chimera cDNAs. Immunofluorescence of cells represents the ability of the chimeras to act as MHV-A59 receptors, the failure of MAb CC1 to protect pretreated cells from MHV-A59 infection, or the surface expression of the chimeric proteins as measured by polyclonal antibody 655.

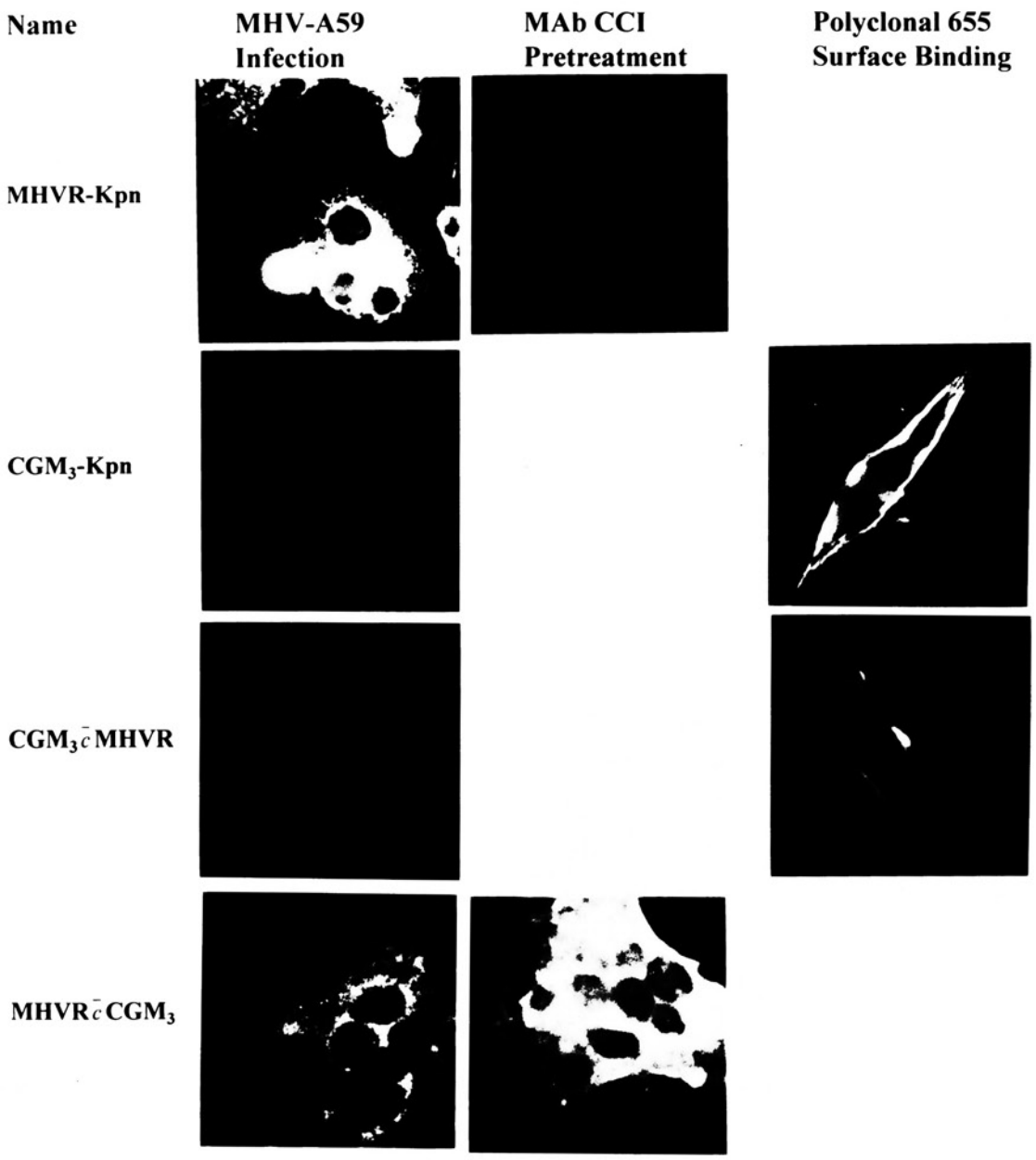
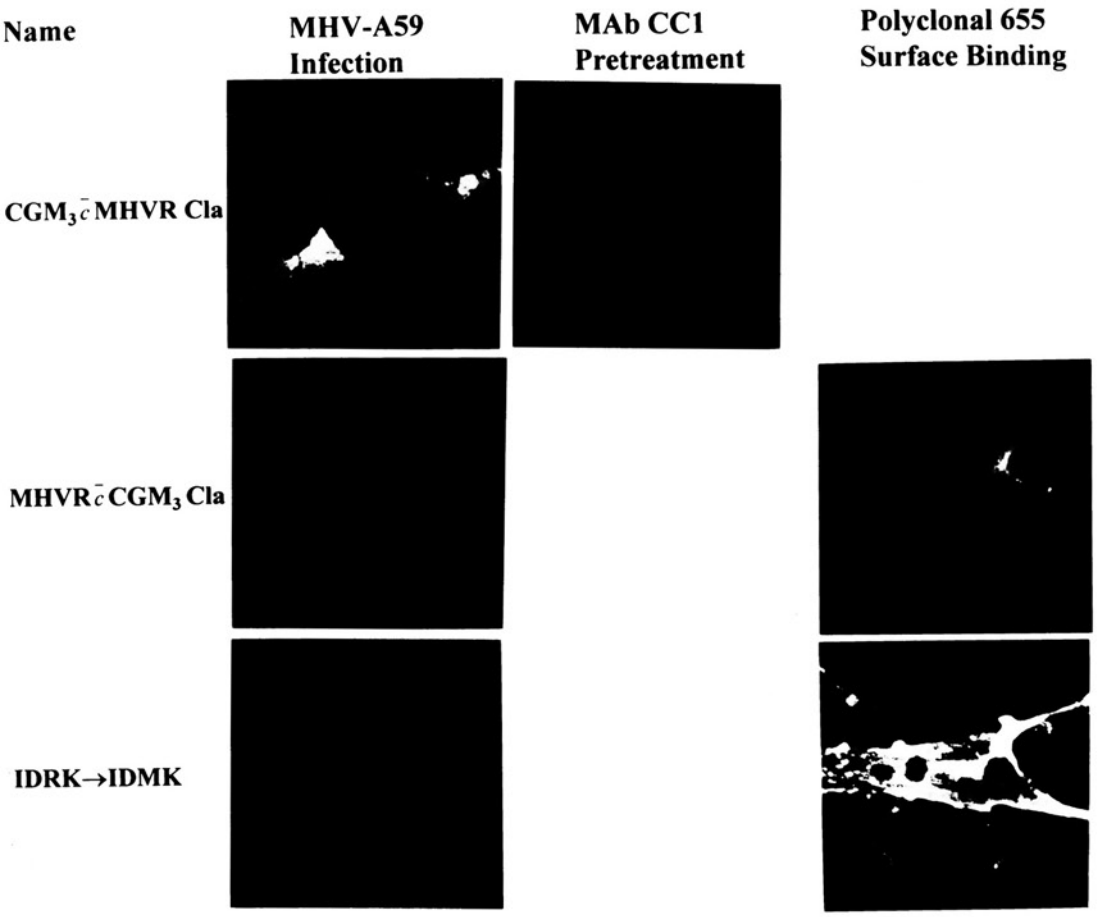

Name
MHV-A59 Infection
MAb CCI Pretreatment
Polyclonal 655 Surface Binding
MHVR-Kpn
CGM3-Kpn
CGM3c̄MHVR
MHVRc̄CGM3

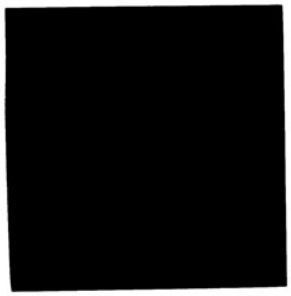
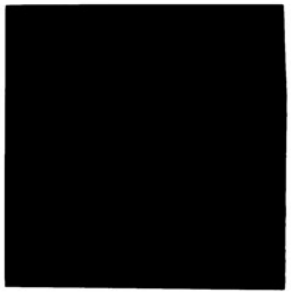
Figure 12. Immunofluorescence labelling of hamster cells transfected with the designated *Cla*I chimera cDNAs. Immunofluorescence of cells represents the ability of the chimeras to act as MHV-A59 receptors, the failure of MAb CC1 to protect pretreated cells from MHV-A59 infection, or the surface expression of the chimeric proteins as measured by polyclonal antibody 655.


Name
MHV-A59 Infection
MAb CC1 Pretreatment
Polyclonal 655 Surface Binding
CGM3c̄MHVR Cla
MHVRc̄CGM3 Cla
IDRK→IDMK

transfected cells (Fig. 12). This area is very prominent on tertiary structures of other immunoglobulin superfamily virus receptors (Bernhardt et al., 1994; Giranda et al., 1990; Wang et al., 1990) and although S52, M54, F56, and I52 had been previously mutated with no effect, a new mutation (I_{52} D_{53} R_{54} $K_{56}\rightarrow$I D M K) was created in MHVR $\bar{c}$ CGM_3 Cla in which R54 was mutated into M54 thus bringing only one amino acid difference between the previous two *Cla* mutation sites. Like its parent plasmid, this too was a nonfunctional receptor (Fig. 12). This might suggest the crucial role of F56 in receptor activity, but this amino acid was deleted in the ΔR fix mutant which had some residual receptor activity (Fig. 13). This lends itself to the suggestion that there are various sites of interaction between the virus and the N-domain of the receptor.

Multiple sites of interaction. To test the possibility of multiple sites of interaction between the receptor and virus, two new chimeric proteins were created. For the first, following a *Bam*HI and *Bsp*EI digestion, the liberated DNA piece from $CGM_{3\ Bam\text{-}Nsi}$ + MHVR was ligated into the MHVR $\bar{c}$ CGM_3 plasmid to create 3R3R (Fig. 5). This turned a functional receptor into a nonfunctional one by changing

Figure 13. Immunofluorescence labelling of hamster cells transfected with the 3R3R, CGM_3 Cla and ΔR fix cDNAs. Immunofluorescence of cells represents the ability of the chimeric and deletion glycoproteins to act as MHV-A59 receptors, the failure of MAb CC1 to protect pretreated cells from MHV-A59 infection, or the surface expression of the chimeric proteins as measured by polyclonal antibody 655.

Name	MHV-A59 Infection	MAb CC1 Pretreatment	Polyclonal 655 Surface Binding
3R3R			
CGM_3 Cla			
ΔR fix			

amino acids 70-108 (Fig. 13). For the second chimera, the *Kpn-Bam* DNA piece of CGM_3 $\bar{c}$ MHVR Cla was inserted into MHVR $\bar{c}$ CGM_3 Cla to create CGM_3 Cla (Fig. 5). The result was a nonfunctional receptor in which the net difference between it and the functional CGM_3 $\bar{c}$ MHVR Cla was amino acids 1-34 (Fig. 13). A summary of results is shown in Table 4.

Table 4. Summary of virus receptor activity, expression on the cell membrane, and MAb CC1 binding of the recombinant mutated glycoproteins and chimeras generated.

NAME	Functional MHV-A59 receptor activity	MAb CC1 protection	Surface expression*	MAb CC1 binding on Immunoblots
$CGM_{3\ Hind\text{-}Bam}$ + MHVR	neg		pos	neg
$CGM_{3\ Bam\text{-}Nsi}$ + MHVR	pos	neg		neg
Rat N-domain + MHVR	neg		pos	neg
$Rat_{\ Hind\text{-}Bam}$ + MHVR	neg		pos	neg
$Rat_{\ Bam\text{-}end\ N\text{-}domain}$ + MHVR	pos	neg		neg
$S_{52}\ M_{54}\rightarrow$I R	pos	pos		pos
MHVR-Mlu	pos	neg		neg
$D_{42}\ K_{43} S_{69}\rightarrow$H N G	pos	neg		neg
$T_{50}\ S_{51}\ I_{52}\rightarrow$P N S	neg		pos	neg
$N_{43}\ G_{46}\rightarrow$K A	neg		pos	neg
$S_{38}\ G_{39}\ G_{41}\rightarrow$T T I	weakly pos	neg		neg
$S_{38}\ G_{39}\rightarrow$T T	neg		pos	neg
$T_{98}\rightarrow$I	pos	pos		
$T_{91}\ E_{93}\rightarrow$F Q	pos	pos		
$R_{96}\ R_{97}\rightarrow$V P	pos	pos		
$I_{66} L_{74}\rightarrow$T F	pos	pos		
$M_{77}\ I_{78}\ E_{93}\ V_{106}\rightarrow$A A G E	pos	pos		
$V_{85}\rightarrow$A	pos	pos		
$M_{90}\rightarrow$L	pos	pos		
MHVR-Kpn	pos	pos		pos
CGM_3-Kpn	neg		pos	neg
$CGM_3\ \bar{c}$ MHVR	neg		pos	neg
MHVR $\bar{c}\ CGM_3$	pos	neg		neg
$CGM_3\ \bar{c}$ MHVR Cla	weakly pos	pos		pos
MHVR $\bar{c}\ CGM_3$ Cla	neg		pos	neg
IDRK→IDMK	neg		pos	neg
3R3R	neg		pos	neg
CGM_3 Cla	neg		pos	neg
ΔR fix	weakly pos	neg		neg

*Expression of the mutated glycoproteins on the surface of transfected cells was only determined for the glycoproteins lacking MHV-A59 receptor activity.

DISCUSSION

***Bgp1*^{a} and *Bgp1*^{b} are allelic variants of the *Bgp1* gene:** MHVR is the name of the cloned cDNA and protein products of the *Bgp1*^{a} gene. mmCGM2 is the name of the cloned cDNA and protein products of the *Bgp1*^{b} gene. MHVR and mmCGM2 differ in the N-domain by 29 amino acids. Dveksler et al. (1993a) showed by RNA-PCR that the BALB/c, C3H, and C57BL/6 inbred strains of mice expressed isoforms of MHVR but did not express isoforms of mmCGM2 and that SJL/J inbred mice expressed isoforms of mmCGM2 but not isoforms of MHVR. McCuaig et al. (1993) demonstrated that CD1 outbred mice express isoforms of both MHVR and mmCGM2. In the effort to rule out the possibility that these findings are the result of alternative splicing in different strains of mice, genomic DNA from C3H, C57BL/6, SWR, A/J, BALB/c (data not shown), and SJL/J strains of mice were amplified by PCR and hybridized to ^{32}P-labeled oligonucleotides specific for either MHVR or mmCGM2. Figure 4 demonstrates that the mmCGM2 specific oligonucleotide hybridized only to PCR products from SJL/J genomic DNA whereas the MHVR specific oligonucleotide hybridized to all of the other PCR amplified

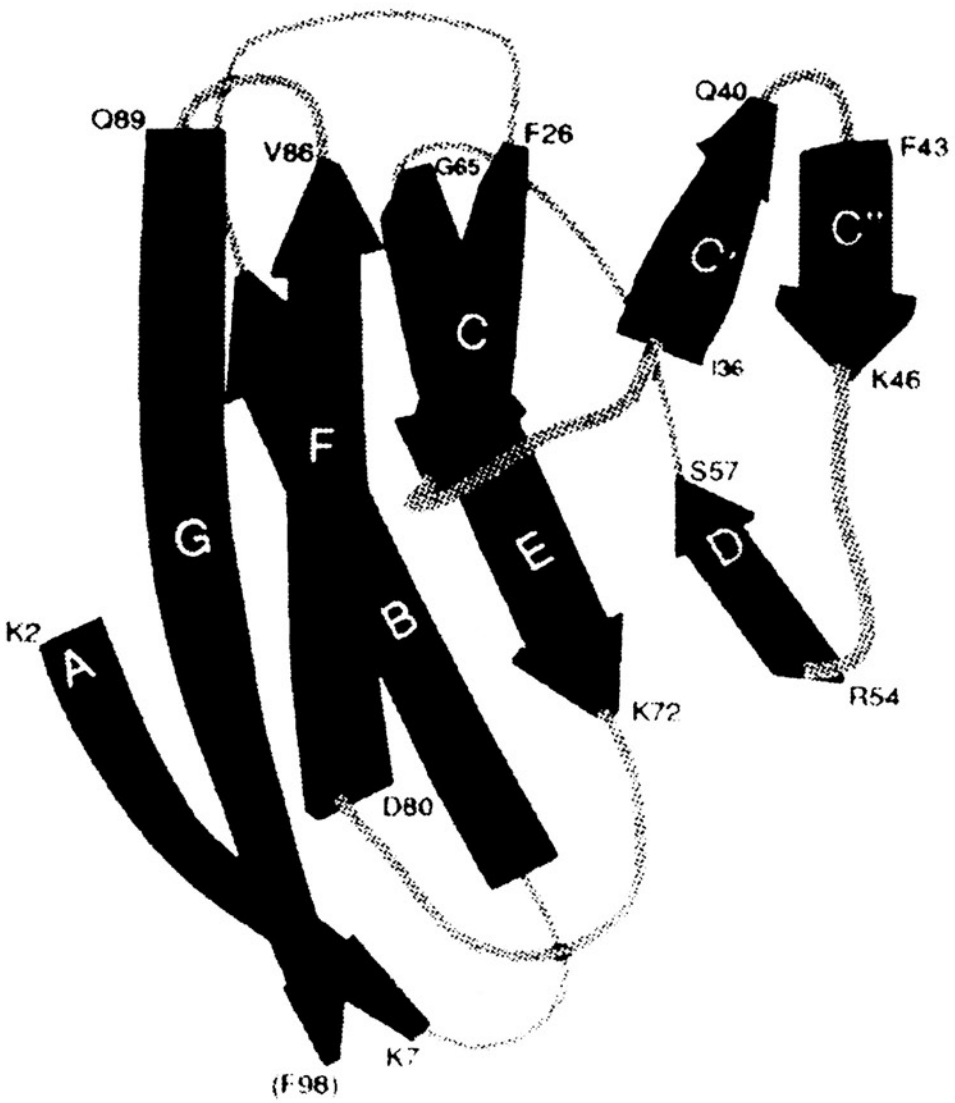
genomic DNAs but not to the PCR products generated after amplification of adult SJL/J genomic DNA. From this data it is concluded that *Bgp1*^{a} and *Bgp1*^{b} are alleles of the same gene.

Virus and monoclonal antibody binding sites: Surface glycoproteins in the immunoglobulin (Ig) superfamily are used as cellular receptors by a number of viruses (White and Littman, 1989). As members of this superfamily, intercellular adhesion molecule-1 (ICAM-1), poliovirus receptor (PVR), CD4, and MHVR share sequence and structural homology with Igs. The specificity of an Ig for an antigen is determined primarily by the structure of its complimentarity determining regions. These three regions, CDR1, CDR2 and CDR3, are approximately located between amino acids 31-34, 53-57, and 94-98 (based on IgG) within the variable region (N-terminal domain) of the Ig. The structure of these regions compliments that of the antigen (Capra and Edmundson, 1977).

ICAM-1 is a glycoprotein expressed at the cell surface consisting of five Ig-like domains. The N-terminal domain (N-domain) is 84 amino acids long and reveals homology to the V domain of IgG. The extra four domains show homology to the IgG constant regions. Through the

creation of chimeras with murine ICAM-1 (which does not bind human rhinoviruses) it has been shown that the N-domain is responsible for virus binding (McClelland et al., 1991; Staunton et al., 1990). Based on structural alignment with IgG, the N-domain of ICAM-1 was modeled (Giranda et al., 1990). Multiple point mutations were then generated to reveal that multiple areas at the N-terminal portion of the tertiary structure of the N-domain form a major part of the human rhinovirus binding site. Mutations in ICAM-1 that diminished virus binding, are located in the BC loop (D26QPK/KEDL), D β strand (K40D and L43E), FG loop (P70DG/GTV), and in the G β strand (T75AK/SAS)(McClelland et al., 1991). Register et al. (1991) found amino acids 28-30, 67 (in the F β strand), and 70 to be important for virus binding. IgG fold structure analysis predicts the BC loop and the FG loop to be prominent structures at the N-terminal end of the tertiary structure of the molecule (See Fig. 14 which shows the similar structure of the N-domain of the homologous CD4 molecule). These are likely candidates to contact the rhinovirus canyon.

Figure 14. CD4 N-domain model. Domains 2-4 and the carboxy-terminus would be underneath the diagrammed model. The number of the individual residue varies from one Ig-like protein to another, but the secondary and tertiary structures of the virus receptors discussed here (ICAM-1, PVR, and MHVR) are homologous. Adapted from Wang et al., 1990.


Q89
V86
G65
F26
Q40
F43
C'
C''
C
I36
K46
F
S57
G
E
D
B
K2
A
K72
R54
D80
K7
(F98)

PVR is also a member of the Ig superfamily with three Ig-like domains. Freistadt et al. (1991) determined through deletion analysis that the C-terminal domain (domain 3) was not important for polio virus binding. Bernhardt et al. (1994) showed that mutations in the C'C" loop (F78), C"D loop (Q82,S87), and DE loop (L99-E102) of PVR impaired poliovirus binding. Based on alignment of the N-domain sequences of PVR with IgG and model building, the C'C", C"D, and DE areas provides a large, prominently exposed region likely to interact with poliovirus.

CD4 has four Ig-like domains. All four of these domains are homologous to Ig VJ domains. The N-terminal domain (V1) of CD4 has been illustrated through epitope mapping (Peterson and Seed, 1988) and x-ray crystallography (Wang et al., 1990; Ryu et al., 1990) to fold analogously to the Ig V region (Fig. 14). Landou et al. (1988) have demonstrated through human/mouse chimeras (murine CD4 does not bind HIV gp120) that it is the V1 domain of CD4 which is critical for binding gp120 of the HIV. By creating point mutations, Arthos et al. (1989) showed that residues 41 to 55 were critical for high affinity binding to gp120. These residues lie at the base of the "CDR2 loop" within the C'C"

turn and C″ β strand. Thus, mutations in this region are likely to disrupt the conformation of the CDR2-homology region. Analogous to the BC and FG loops of ICAM-1 which likely interact with the canyon of rhinoviruses and the C′C″, C″D, and DE area of PVR which likely interacts with poliovirus, Arthos et al. (1989) postulate that the CD4 loop containing the residues homologous to the Ig CDR2 region also contacts a small pocket within the HIV gp120. More recently, Moebius et al. (1992) created point mutation (K35A, F43A, K46A, and S49A) in this CDR2-homology region which altered gp120 binding. These mutations are located in the C′ β strand, the C′C″ turn, the C″ β strand, and the C″D loop. This area forms an exposed structure which interacts with gp120 (Fig. 14). Interestingly, replacement of four positively charged residues in the CDR1 and CDR3 regions by neutral amino acids also impaired gp120 binding. These positively charged amino acids were shown on the atomic structure model to fold around F43 in the CDR2 region (C′C″ prominence) thus creating a pocket for gp120 binding. A W62Y substitution also impaired gp120 binding because the bulky tryptophan side chain is buried beneath the C′C″ turn and appears to disrupt the conformation of this turn.

It is also known through the creation of deletion mutants and chimeric proteins that virus binds to the N-terminal domain (N-domain) of MHVR (Dveksler et al., 1993b; Dveksler et al., 1995). Also, just as glycosylation is found to be unimportant for human rhinovirus binding to ICAM-1 (McClelland et al., 1991), poliovirus binding to PVR (Bernhardt et al., 1994), and HIV binding to CD4 (Fenouillet et al., 1989), Dveksler et al. (1995) demonstrated that when all three of the potential N-linked glycosylation sites in the N-domain were mutated (N37,55,70/Q37,55,70), the resultant glycoprotein still acted as a functional MHV receptor.

Analysis of the first group of chimeric glycoproteins reported here suggests that of the 108 amino acids that constitute the N-domain of MHVR, it is the first 70 amino acids which are essential for MHV-A59 binding since substitution of these amino acids for those of other rodent BGPs resulted in glycoproteins that lacked MHV receptor activity. Substitution of MHVR amino acids 71-108 with amino acids from mmCGM3 or rat bgp did not alter the functional ability of these chimeric proteins to act as MHV-A59 receptors on the surface of hamster cells. All these

chimeras however did not bind the monoclonal anti-MHVR antibody MAb CC1.

In an effort to pin-point which of these amino acids may be critical for virus binding, two groups of point mutations were generated within the first 70 amino acids. The first group consisted of glycoproteins in which mutations changed MHVR amino acids into those of mmCGM3. Of this group, none of the introduced mutations destroyed their ability to function as MHV receptors; however, the mutants MHVR-Mlu, which changed L26Q, A27T, L28R, A30V, and A32Y, and D_{42} K_{43} $S_{69}\rightarrow$H N G did not bind MAb CC1. In conjunction with the results obtained with the chimeric glycoproteins in which amino acid substitutions 1-70 or 70-108 also eliminated MAb CC1 binding, this strongly suggests that MAb CC1 binding is dependent on a conformational association in which amino acids on both sides of residue 70 are important and that one of the interacting areas is between amino acids 26-32 and another is probably at D42K43 or S69. The D42K43 residues which lie in the CC' loop are most likely implicated in MAb CC1 binding. S69G mutates a small polar side group into a small nonpolar one on the back-side of the molecule in the DE loop but the *Kpn*I insertion mutates the

adjacent positively charged K70 side group into a neutral Q with no effect on MAb CC1 binding. Three dimensional model analysis is needed to be more definitive in ruling-out S69 however.

The second group of point mutations generated within the first 70 amino acids were made in the $CGM_{3Hind-Bam}$+MHVR chimera which did not function as a receptor. These mutations changed mmCGM3 amino acids into those of MHVR in an effort to restore receptor activity. Within this group, S_{38} G_{39} $G_{41}\rightarrow$T T I was able to act as a functional receptor for MHV-A59 but S_{38} $G_{39}\rightarrow$T T did not. These results identify the first point of importance for MHV-A59 binding - I41.

Through the use of the *Kpn*I site inserted into the MHVR and CGM_3 cDNA clones, chimeras in which the first 34 amino acids were switched did not have altered functions as MHV-A59 receptors. By switching these first 34 amino acids from MHVR residues to CGM_3 residues, MHVR$\bar{c}$$CGM_3$ did loose its ability to bind MAb CC1. These findings support the MHVR-Mlu results (the only previously described mutant to solely involve any of the first 34 amino acids).

Introduction of *Cla*I sites into the cDNAs of the *Kpn* chimeras described above resulted in amino acid changes

S52I, N53D, M54R, and F56K in MHVR$\bar{c}$CGM$_3$ and N53D, R54M, and K56F in CGM$_3\bar{c}$MHVR. S52, M54, and F56 in MHVR and I52 in CGM$_3$ had been previously mutated to I, R, P, and S respectively without altering MHV-A59 or MAb CC1 binding. In the new mutants however, MHV-A59 binding is reversed suggesting a critical site between amino acids 52-56. In conjunction with the above mentioned point mutations, this suggests an interaction between amino acids 52-56 and the area between amino acids 70-108 or 1-34. Both of the new chimeric Cla mutants possess I52 and D53 suggesting the importance of either M54 or F56 in virus binding. When R54 is mutated back to M in MHVR$\bar{c}$CGM$_3$ Cla (IDRK→IDMK), neither receptor activity nor its ability to bind MAb CC1 is altered suggesting the importance of F56; yet, ΔR fix in which amino acids 55-58 are deleted still acts as a functional receptor thus eliminating F56 as a "critical" residue. CGM$_3\bar{c}$MHVR Cla also bound MAb CC1. This suggests an interaction between M54 or F56 and the area between amino acids 1-34 or 70-108 for MAb CC1 binding also.

When the first 34 amino acids of the functional CGM$_3\bar{c}$MHVR Cla molecule are substituted (CGM$_3$ Cla), receptor

activity and MAb CC1 binding are lost again pointing to an important interaction between residues within the first 34 amino acids and the remainder of the N-domain. It would be tempting at this point to speculate that the interaction is between amino acids 52-56 and 26-32. This would conveniently coincide structurally with the critical C'C" ridge of the CD4 glycoprotein and its interacting CDR1 homolog area, but when amino acids 70-108 are substituted in the functional MHVR$\bar{c}$CGM$_3$ receptor, this new chimera (3R3R) becomes nonfunctional.

From homologous structural analogy, I41 and S52NMNF residues contribute to the C' and C" antiparrallel strands and resultant ridge. This area in CD4 is homologous to the CDR2 region of κ light chains. Like CD4 and PVR, this is a likely area for virus binding. ΔR fix which deletes residues N55FTG, located at the bottom of the C" β strand and first portion of the C"D loop, still acts as a virus receptor albeit a weak one. This suggests the more N-terminal portions of the C'C" area as being more important in MHV-A59 binding.

MHVR-Mlu (L26ALGAFA/QTRGVFY) has mutations in the prominent BC loop which altered MAb CC1 but not MHV-A59

binding. This area is close to, if not inclusive of the CDR1 homolog and is important in ICAM-1 binding of human rhinoviruses and structurally interacts with the gp120 binding "pocket" at F43 of CD4 (Moebius et al., 1992).

The amino acid residues within the first 34 amino acids which altered virus binding in CGM_3 Cla and the additional 38 amino acids between residues 71-108 which altered virus binding in 3R3R may be important in conformational interactions which, when altered, eliminates MHV-A59 viral S protein binding. This would correlate to the replacement of positively charged residues in CD4 within these same structural regions which impaired affinity for HIV gp120.

Finally, it can not be ruled out that not only is MHVR conformation prior to initial S protein contact important; but, subsequent to virus contact, a stable MHV-A59 - MHVR interaction may be dependent on transient conformational intermediates induced upon each other.

These results create important implications in treating and preventing MHV infections Mouse strains can transgenically be created which possess the *Bgp1*^{b} allele in place of *Bgp1*^{a} resulting in MHV-resistant mice, soluble peptides with the MHV binding site can be created to act as

competitive inhibitors, and MHVR can be a potential site for targeting chemotherapeutic agents.

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