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Identification of a Contiguous 6-Residue Determinant in the MHV Receptor That Controls the Level of Virion Binding to Cells

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Received September 17, 1996; returned to author for revision November 4, 1996; accepted January 9, 1997

Murine carcinoembryonic antigens serve as receptors for the binding and entry of the enveloped coronavirus mouse hepatitis virus (MHV) into cells. Numerous receptor isoforms are now known, and each has extensive differences in its amino terminal immunoglobulin-like domain (NTD) to which MHV binds via its protruding spike proteins. Some of these receptor alterations may affect the ability to bind viral spikes. To identify individual residues controlling virus binding differences, we have used plasmid and vaccinia virus vectors to express two forms of MHV receptor differing only in their NTD. The two receptors, designated biliary glycoproteins (Bgp) 1^a and 1^b_{NTD}, varied by 29 residues in the 107 amino acid NTD. When expressed from cDNAs in receptor-negative HeLa cells, these two Bgp molecules were displayed on cell surfaces to equivalent levels, as both were equally modified by a membrane-impermeant biotinylation reagent. Infectious center assays revealed that the 1^a isoform was 10 to 100 times more effective than 1^b_{NTD} in its ability to confer sensitivity to MHV (strain A59) infection. Bgp1^a was also more effective than Bgp1^b_{NTD} in comparative virus adsorption assays, binding 6 times more MHV (strain A59) and 2.5 times more MHV (strain JHMX). Bgp1^a was similarly more effective in promoting the capacity of viral spikes to mediate intercellular membrane fusion as judged by quantitation of syncytia following cocultivation of spike and receptor-bearing cells. To identify residues influencing these differences, we inserted varying numbers of 1^b residues into the Bgp1^a background via restriction fragment exchange and site-directed mutagenesis. Analysis of the resulting chimeric receptors showed that residues 38 to 43 of the NTD were key determinants of the binding and fusion differences between the two receptors. These residues map to an exposed loop (C-C' loop) in a structural model of the closely related human carcinoembryonic antigen. © 1997 Academic Press

INTRODUCTION

Coronaviruses are attractive objects for studies of animal virus entry into cells because there exists a remarkable level of variation in the ligands that engage virions with cells. Several strains of MHV are now recognized (Siddell, 1995) and several receptors are also known (Dveksler *et al.*, 1991; Yokomori and Lai, 1992b; Nedellec *et al.*, 1994; Chen *et al.*, 1995). This abundant natural variation provides the opportunity to compare different receptors for their ability to interact with different virus strains. Such comparative analyses will allow one to identify the determinants responsible for differences in virus:cell interactions.

In this study, we have focussed attention on two allelic isoforms of the MHV receptor, biliary glycoprotein (Bgp) isoforms 1^a (Dveksler *et al.*, 1991; Yokomori and Lai, 1992a) and 1^b (Yokomori and Lai, 1992b; Dveksler *et al.*, 1993a; McCuaig *et al.*, 1993), and we have examined their capacity to interact with purified enveloped coronavirions. The 1^a isoform is expressed in MHV susceptible mice, while mouse strains homozygous for the 1^b isoform are generally resistant to many currently recog-

nized MHV strains (Barthold *et al.*, 1986; Barthold, 1987). These receptors are type I single-pass transmembrane glycoproteins containing an ectodomain structure made up of four immunoglobulin-like domains (Dveksler *et al.*, 1991). The variability between 1^a and 1^b lies primarily in the most membrane-distal domain (Yokomori *et al.*, 1992b, Dveksler *et al.*, 1993a). This amino terminal domain (NTD) is essential for virus binding *in vitro* (Dveksler *et al.*, 1993b) and is similarly required to render cells susceptible to infection by MHV (Dveksler *et al.*, 1995).

Initial studies on the coronavirus-binding functions of Bgp1^a and 1^b indicated that they differ substantially in virion adsorptive capacity—when the two receptors were immobilized on nitrocellulose filters, only the 1^a isoform supported detectable virus-binding (Dveksler *et al.*, 1993a). This finding suggested that one or more of the 29 amino acid differences between the 1^a and 1^b NTDs (Yokomori and Lai, 1992b) are responsible for controlling virion adsorptive capacity, either because they provide direct receptor:virion contact points or because they control the overall conformation of the receptors. If the NTD differences were indeed critical, then identification of the relevant residues could be achieved by constructing 1^a/1^b hybrid cDNAs and then measuring virus binding to the protein products. We have used this homologue scanning mutagenesis approach to pro-

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duce chimeras that have been useful in identifying residues controlling the virus-adsorptive capacity of the MHV receptor.

MATERIALS AND METHODS

Cells and viruses

Baby hamster kidney (BHK), rabbit kidney (RK13), and human carcinoma (HeLa) cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Δ FBS). Murine 17 cl 1 cells (Sturman and Takemoto, 1972) were grown in DMEM containing 5% tryptose phosphate broth (TPB; Difco Laboratories, Detroit, MI) and 5% Δ FBS. Murine astrocytoma (DBT) cells (Hirano *et al.*, 1978) were grown in minimal essential medium (MEM) containing 10% TPB and 5% Δ FBS; these cells were used to measure MHV infectivities by plaque assay. Murine coronavirus strains A59 and JHMX (Makino *et al.*, 1984) were grown in 17 cl 1 cells. Radiolabeled coronaviruses were collected from culture supernatants after a 12–16 hpi pulse with DMEM-1% dialyzed Δ FBS containing 100 μ Ci/ml Tran^[35S] label (ICN Radiochemicals, Irvine, CA). Clarified supernatants were overlaid above 30% (w/w) sucrose cushions in PBS containing 0.01% BSA and subjected to ultracentrifugation (Beckman–Spinco SW28 rotor for 3 hr at 28,000 rpm). Virion-containing pellets were resuspended in ice-cold PBS containing 0.01% BSA and stored in silanized glass vials at -80° .

Synthesis and cloning of MHV receptor cDNA constructs

Methods for construction of vaccinia virus insertion/expression plasmids (pTM3; Moss *et al.*, 1990) encoding MHV receptor isoform Bgp1^a or Bgp1^a lacking codons 10–122 (Bgp1 Δ) have been described previously (Gallagher, 1996). These plasmid vectors were further modified by reinsertion of Bgp NTD sequences into Bgp1 Δ . To this end, the Bgp1^a and 1^b NTD sequences were amplified from outbred CD1 mouse liver RNA by RT-PCR (Kawasaki, 1990) using primers based on the reported Bgp1^a sequence (McCuaig *et al.*, 1993) and products encoding for the 1^a and 1^b NTD were identified by restriction mapping and by sequencing (Sambrook *et al.*, 1989). 1^b NTD sequences were cloned in-frame into the unique *Xma*I site of Bgp1 Δ to generate the chimeric Bgp1^b_{NTD}. Two undesired codon alterations that resulted at the junctions of the insertion (Q10G and V123G) were introduced into a control Bgp1^a construct by performing a parallel insertion of the Bgp1^a NTD into pTM3-Bgp1 Δ . The control recombinant was designated as Bgp1^a_{Q10G,V123G}.

To construct additional Bgp chimeras for use in identifying virus binding determinant(s), restriction fragments between pTM3-Bgp1^a and pTM3-Bgp1^b_{NTD} were ex-

changed. 1^b 84–122 and 1^b 70–122 were made by exchange of *Acc*I–*Pst*I and *Bam*HI–*Pst*I fragments, respectively (see Fig. 7). Additional exchanges were performed after creation of a *Nde*I site at codon 53 using the megaprimer mutagenesis procedure (Aiyar and Leis, 1993). *Nde*I site creation altered codon 53 (N53H) in Bgp1^a and codons 53 and 54 in Bgp1^b_{NTD} (N53H, K54M). 1^b 54–122 and 1^b 10–54 were made by exchange of *Nde*I–*Nde*I and *Nco*I–*Nde*I fragments, respectively (see Fig. 7). Recombinant 1^b 38–43 was made by oligonucleotide-directed PCR mutagenesis using a primer spanning codons 38–43 and the *Nde*I site (Scharf, 1990). Nucleotide sequencing methods (Sanger *et al.*, 1977) were employed to confirm that Bgp1^a and 1^b clones matched those reported for these genes (McCuaig *et al.*, 1993) and to verify the isolation of all of the mutant receptors described herein.

Generation of vaccinia virus recombinants.

Recombinant viruses were prepared by infection of RK13 cells with vaccinia virus strain WR (m.o.i. = 0.05), followed 1 hr later by lipofectin-mediated transfection of the various pTM3-Bgp constructs (Felgner *et al.*, 1987). At 3 days postinfection, cultures were frozen at -80° . After three freeze–thaw cycles, debris was removed by centrifugation and virus in the clarified material was plated on RK13 cells. Plaques arising from specific amplification of recombinant virus were revealed by overlay of cells with mycophenolic acid-containing medium, as described by Falkner and Moss (1988). Virus from well isolated plaques was grown in RK13 cells to generate stocks of recombinant virus (vBgp1^a, vBgp1 Δ , vBgp1^b_{NTD}, vBgp1^a_{Q10G,V123G}, and additional 1^a/1^b chimeras).

Immunoblot analysis of Bgp proteins

Cell monolayers were infected with the respective vBgp recombinants (m.o.i. = 10) and coinfecting with vTF7.3 (m.o.i. = 1). At 20 hr postinfection, lysis buffer (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1 mM PMSF, and 0.3 U/ml aprotinin) was added, and 10- μ l aliquots (equivalent to 2×10^4 cells) were subjected to Western immunoblotting. Receptor was identified using antireceptor antibody 874 (a gift of Dr. M. J. Buchmeier, Scripps Research Institute, La Jolla, CA) which was directed against conserved carboxy-terminal residues 443–458.

Quantitation of relative Bgp cell surface levels

At 20 hr postvaccinia infection, HeLa cells expressing the Bgp molecules were washed three times with ice-cold PBS and incubated for 1 hr at 4° with PBS containing 1 mg/ml NHS-LC biotin (sulfosuccinimidyl-6-(biotinamido) hexanoate; Pierce Co., Rockford, IL). Unreacted reagent was then quenched by the addition of 20 mM

Tris-HCl (pH 7.5) for 15 min at 4°. Monolayers were washed thoroughly with PBS and lysed (2×10^6 cells/ml in lysis buffer). Clarified cytoplasmic extracts (250 μ l) were then mixed with antipeptide antibody 874 (12.5 μ l) and left overnight at 4°. Twenty microliters of Gammabind G-Sepharose (Pharmacia Biotech) was then added for 1 hr at 4°. Sepharose beads were collected by centrifugation, washed twice in RIPA buffer (10 mM Tris-HCl (pH 7.2) 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100), then once in 10 mM Tris-HCl (pH 7.2) containing 0.1% v/v Nonidet P-40. Immunoprecipitated receptors were then subjected to Western blotting, and the biotinylated fraction of each sample was identified by incubation of blots for 1 hr with 0.1% v/v streptavidin-horseradish peroxidase (HRP) conjugate (Pierce Co.) in PBS/3% BSA. Immobilized HRP was detected by incubation in PBS containing 0.5 mg/ml 4-chloro-1-naphthol, 17% v/v methanol, and 0.015% hydrogen peroxide. Blots were photographed (Polaroid 665 film) and signal intensities were quantified with an LKB ultrascan XL densitometer.

Indirect immunofluorescence

Vaccinia-infected BHK cells were challenged with MHV-A59 (m.o.i. = 10) at 10 hr postvaccinia infection. Fourteen hours later, cells were washed with PBS, fixed in acetone, and incubated for 1 hr at 22° with a 1:250 dilution of antispikes Mab 4B11.6 (Collins *et al.*, 1982) in PBS/2% BSA. Bound Mab was detected with FITC-conjugated goat Ig directed against mouse Ig (Cappell, Durham, NC) and cells were photographed using a Leitz fluorescence microscope.

Infectious center assays

cDNA constructs encoding Bgp1^a and Bgp1^b_{NTD} were inserted into plasmid expression vector pUHD-10-3 (Gossen and Bujard, 1992) and the plasmids (0.5 μ g) were transfected by lipofection into aliquots of 3×10^5 HeLa-tTA cells (Gossen and Bujard, 1992). To determine transfection efficiencies, parallel cultures were cotransfected with each pUHD-Bgp construct in conjunction with 0.05 μ g of the β -galactosidase-encoding pCMV- β (Clontech Labs, Inc.). *In situ* x-gal staining (MacGregor *et al.*, 1989) at 36 hr posttransfection revealed 11.8 and 12.5% β -gal-positive cells for the 1^a and 1^b_{NTD} transfectants, respectively. To assess susceptibilities to MHV infection, cells were washed at 36 hr posttransfection with cold serum-free DMEM (SFM) and inoculated with MHV-A59 at 4° for 1 hr. Unbound virus was removed by washing with PBS containing 0.5% BSA and 0.05% Tween 20 and cells were then incubated in DMEM 10% Δ FBS for 2 hr at 37°. Cells were then trypsinized, washed twice with DMEM 10% Δ FBS, and serial dilutions were plated on DBT indicator monolayers. Plaques were visualized after a 2-day incubation period.

Virus binding assays

HeLa cells overexpressing Bgp receptors from vaccinia vectors were washed twice with ice-cold SFM at 20 hpi, then radiolabeled MHV particles were diluted in SFM and added at varying multiplicities. After incubations at 4°, unadsorbed virions were removed and cells were rinsed five times with ice-cold PBS containing 0.5% BSA and 0.05% Tween 20. RIPA buffer was added, and radioactivities associated with the cell lysates and supernatant media were quantified by scintillation counting.

Intercellular fusion assay

The cell fusion-dependent reporter gene (β -galactosidase) activation assay of Nussbaum *et al.* (1994) was adapted for studies of MHV fusion as described previously (Gallagher, 1996), with minor modifications. In brief, stably transfected HeLa-Bgp1^a cells were infected with MHV-A59 (m.o.i. = 10) and with vaccinia virus strain WR (m.o.i. = 10). After 1 hr at 37°, these cells were further transfected by lipofection with the reporter gene construct pG1NT7 β -gal (kindly provided by Dr. Richard A. Morgan, National Center for Human Genome Research, Bethesda, MD). Five hours later (before the onset of MHV A59-induced syncytium formation yet after pG1NT7 β -gal DNA amplification via vaccinia-encoded polymerases) the cells were trypsinized, suspended in DMEM 10% Δ FBS, and 0.6-ml (3×10^5 cell) aliquots were overlaid on to confluent 5-cm² monolayers of HeLa cells that were coinfecting 20 hr earlier with vTF7.3 and vBgp recombinants. After 3 hr at 37°, the mixed monolayers were fixed and stained with x-gal for *in situ* localization of β -gal activity (MacGregor *et al.*, 1989). Alternatively, the monolayers were lysed by addition of 0.5% NP-40 in PBS and the quantity of β -galactosidase in each lysate was calculated using a colorimetric enzyme assay involving hydrolysis of chlorophenyl red β -galactopyranoside (Nussbaum *et al.*, 1994). OD 590 values were normalized by comparing the hydrolysis rates for each sample with that obtained for a standard preparation of *E. coli* β -galactosidase (Calbiochem, La Jolla, CA) and were expressed as nanograms/well.

RESULTS

Synthesis of chimeric MHV receptors from vaccinia virus vectors

A method for receptor overexpression on cell surfaces was necessary to reproducibly measure the binding of MHV particles to a series of different receptors. HeLa cell lines in which the MHV receptor gene was stably and constitutively expressed (HeLa-Bgp1^a cells) have been developed (Gallagher, 1996); however, we found that clones varied dramatically in receptor levels (data not shown). Therefore we anticipated difficulty in identifying a series of stable HeLa cell transfectants in which differ-

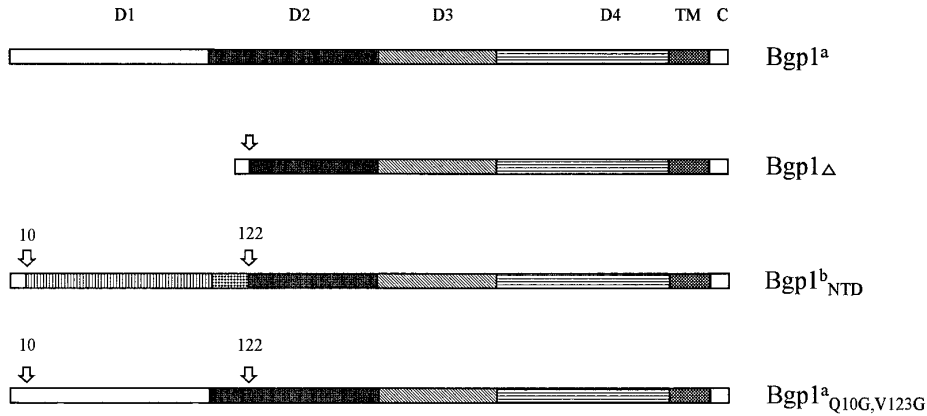


FIG. 1. Schematic representation of MHV receptor mutants. D1 to D4 indicate the four immunoglobulin-like ectodomains, TM is transmembrane region, C is cytoplasmic tail. Bgp1 Δ lacks the NTD; replacement with NTD sequences from Bgp1^b (Bgp1_{RNTD}) and Bgp1^a (Bgp1_{Q10G,V123G}) affected the junction codons 10 and 123.

ent receptors are each present at the same levels. Thus to increase the likelihood of equivalent receptor production, different receptor cDNAs were expressed from vaccinia virus vectors. Such vectors have historically produced high levels of surface glycoprotein (Broder *et al.*, 1994; Nusbaum *et al.*, 1995) and their preparation is known to be relatively straightforward (Falkner and Moss, 1988).

A set of cDNAs capable of encoding four distinct forms of MHV receptor were each recombined into the vaccinia virus genome using insertion vector pTM3 (Elroy-Stein and Moss, 1990; Moss *et al.*, 1990) and recombinant viruses (designated vTM3-Bgp) were selected as described under Materials and Methods. The use of the pTM3 vector places the cDNAs under the transcriptional control of a bacteriophage T7 promoter; thus expression of MHV receptors required coinfection of HeLa cells with vTM3-Bgp and vTF7.3, which encodes bacteriophage T7 RNA polymerase (Fuerst *et al.*, 1986).

The four translation products predicted from this expression scheme are depicted in Fig. 1. The mature Bgp1^a, after removal of its signal peptide, has an ectodomain composed of four immunoglobulin-like domains, designated D1 to D4 (Dveksler *et al.*, 1991). A deletion mutant of Bgp1^a (Bgp1 Δ) was prepared by excision of nucleotides encoding the bulk of the virus-binding D1 domain as well as 15 amino acids of the D2 domain. Corresponding sequences from the allelic Bgp1^b (McCuaig *et al.*, 1993) were then placed back into the truncated cDNA to generate a hybrid capable of encoding the chimeric molecule Bgp1^b_{NTD}. These cloning manipulations resulted in two unwanted codon changes (Q10G and V123G) at the exchange junctions (see arrows in Fig. 1). To test the contribution that these two changes might have on interaction with virions, cDNA for a Bgp1^a containing the mutations (designated Bgp1^a_{Q10G,V123G}) was prepared by ligating the NTD sequences of Bgp1^a into the deletion mutant.

The carboxy-terminal 16 amino acids are identical for all four receptors; and an antipeptide antibody (874) raised against these cytoplasmic residues was available to us for use in monitoring receptor levels. Initial tests using this anti-C-terminal antibody were performed by immunoblotting BHK and HeLa cell lysates collected 20 hr after coinfection with vTF7.3 and vTM3-Bgp. The results (Fig. 2) revealed identical patterns of immunoreactive protein in lysates expected to contain Bgp1^b_{NTD}, 1^a_{Q10G,V123G}, and 1^a (lanes 1–3, respectively). A sharp band at c. 55 kDa was detected, which likely represent underglycosylated form(s) of the 424 amino acid proteins. The series of bands ranging from 80 to 120 kDa indicated that equivalent levels of the various glycoforms of receptor were present in each infected culture. Lysates expected to contain Bgp1 Δ had a similar, slightly more

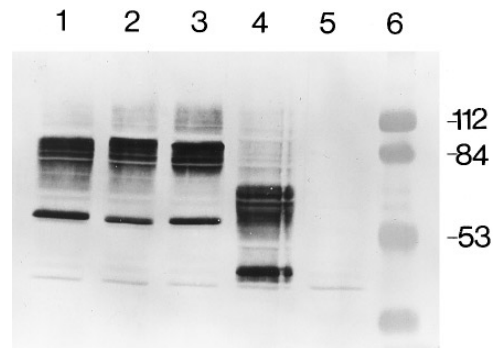


FIG. 2. Immunoblot analysis of MHV receptors expressed from vaccinia vectors. BHK cells were coinfecting with vTF7.3 (m.o.i. = 1) and with respective vTM3-Bgp vaccinia recombinants (m.o.i. = 10). At 20 hr postinfection, cells were lysed and cytoplasmic extracts (equivalent to 10^5 cells per lane) were probed by Western immunoblotting with an antipeptide antibody directed against conserved carboxy-terminal residues of Bgp. Lanes: (1) Bgp1^b_{NTD}, (2) Bgp1^a_{Q10G,V123G}, (3) Bgp1^a, (4) Bgp1 Δ , (5) vTF7.3 alone, (6) molecular weight markers, kilodaltons. Identical profiles of immunoreactive protein were observed in similarly infected HeLa cell lysates.

TABLE 1

Relative Cell Surface Levels of Recombinant Receptors

Receptor recombinants ^a	Relative surface density ^b
vBgp1 ^a	1.0
vBgp1 Δ	1.05
vBgp1 _{NTD} ^b	1.1
vBgp1 _{D10G,V123G} ^a	0.95
vTF7.3	0

^a Indicated vaccinia recombinants were used to express Bgp molecules in HeLa cells.

^b Portion of receptors present on cell surface were identified at 20 hr postinfection by a biotinylation process as described under Materials and Methods. Signals on nitrocellulose filters which represent biotinylated receptors (50- to 110-kDa size range) were quantitated by densitometry. Values are normalized to those found in lysates from vBgp1^a-infected cells.

intense immunoreactive protein profile, with the apparent molecular weights of the bands lower by approximately 20 kDa (lane 4).

To determine whether the four different MHV receptors were displayed on the cell surface at similar levels, we first made the assumption that each receptor contains similar numbers of exposed amines available for conjugation with the membrane-impermeant biotinylation reagent sulfo-NHS-LC biotin. With this in mind, we derivatized the proteins on intact cells at 20 hr postvaccinia infection with the reagent and then we lysed cells and immunoprecipitated the receptor proteins. The relative proportion of biotinylated receptor among the immunoprecipitates was then assessed by Western blotting using streptavidin-horseradish peroxidase as a detection reagent. By this method, the relative abundance of biotinylated receptors were similar in all cultures; densitometric scans of the blots revealed less than 10% variation in signal intensities (Table 1), suggesting similar surface receptor levels.

Function of chimeric MHV receptors during infection

The ability of the four receptors to support a productive MHV infection was assessed by inoculation of the MHV strain A59 onto the vaccinia infected cell monolayers. Fourteen hours later, MHV-A59 spike was visualized by indirect immunofluorescence. All cells within cultures shown to express the complete receptors contained spike antigen (Fig. 3, panels 1, 2, and 3) while the truncated receptor failed to support MHV-A59 infection (panel 4), in concert with the results of Dveksler *et al.* (1993b).

Additional examination of the various receptors for their ability to confer susceptibility to infection involved infectious center assays. To this end, cDNAs encoding Bgp1^a or Bgp1_{NTD}^b were introduced into plasmid expres-

sion vector pUHD-10-3 and transfected into HeLa-tTA cells (Gossen and Bujard, 1992) by lipofection. After 36 hr, the cells were challenged with MHV-A59 at a range of input multiplicities, and the relative number of cells that became infected was assessed by infectious center assays. The results (Fig. 4) revealed that some infectious centers ($10^{0.5}$ to 10^2 per 3×10^5 cells) were generated even by A59-challenged HeLa cells (hatched bars). This was clearly due to a failure to quantitatively remove non-specifically adsorbed virions as numerous immunofluorescence tests confirmed that HeLa-tTA cells could not be infected by MHV A59 (data not shown). More importantly, the results of Fig. 4 showed that Bgp1^a and 1_{NTD}^b differed significantly in their capacity to stimulate formation of infectious centers. Infectious centers among Bgp1^a transfectants (solid bars) were 3 to 4 log₁₀ higher than controls, while Bgp1^b transfectants (open bars) were only 1 to 2 log₁₀ higher. Moreover, these differences in receptor effectiveness could not be eliminated by increasing input multiplicities during virus challenge. Finally, we observed a straightforward correlation between infectious centers and virus yields; 24 hr after challenge of the Bgp transfectants with MHV A59 at 10 PFU per cell, yields were 69000, 3000, and 200 PFU/ml for the 1^a, 1_{NTD}^b, and control transfectants, respectively. Thus, when expressed in the HeLa-tTA cells, Bgp1^a was utilized as an MHV-A59 receptor much more effectively than Bgp1_{NTD}^b.

Virus binding capacities of the chimeric MHV receptors

With the HeLa cell monolayers each expressing a different MHV receptor on the cell surface, straightforward virus adsorption assays became feasible. Thus we collected particles from supernatants of MHV-infected 17c11 cells that had been incubated for 4 hr in the presence of trans-[³⁵S] label, and we concentrated the radiolabeled virions by ultracentrifugation. Virion preparations were judged to be free of unincorporated radioactivity because all [³⁵S] was acid precipitable; additionally the virions were deemed radiochemically pure as all electrophoretically separated [³⁵S] proteins had mobilities consistent with known MHV proteins (data not shown). In initial experiments, aliquots of [³⁵S]MHV-A59 were allowed to bind to HeLa cells bearing different MHV receptors at 4° for varying time periods. Cells were then rinsed and the percentage of added radioactivity remaining bound to the monolayers was determined. The results (Fig. 5) revealed a gradual increase in the levels of nonspecific adsorption of virions to cells displaying Bgp1 Δ , from 0.8% after 15 min, to 1.6% by 1 hr, and 3.5% by 8 hr. MHV-A59 bound far more avidly to cells with Bgp1^a or 1_{NTD}^b; 9.5 and 3%, respectively, after 1 hr. Subtraction of nonspecific adsorption values revealed that the Bgp1_{NTD}^b was 6-fold less effective than Bgp1^a in virion adsorptive capacity.

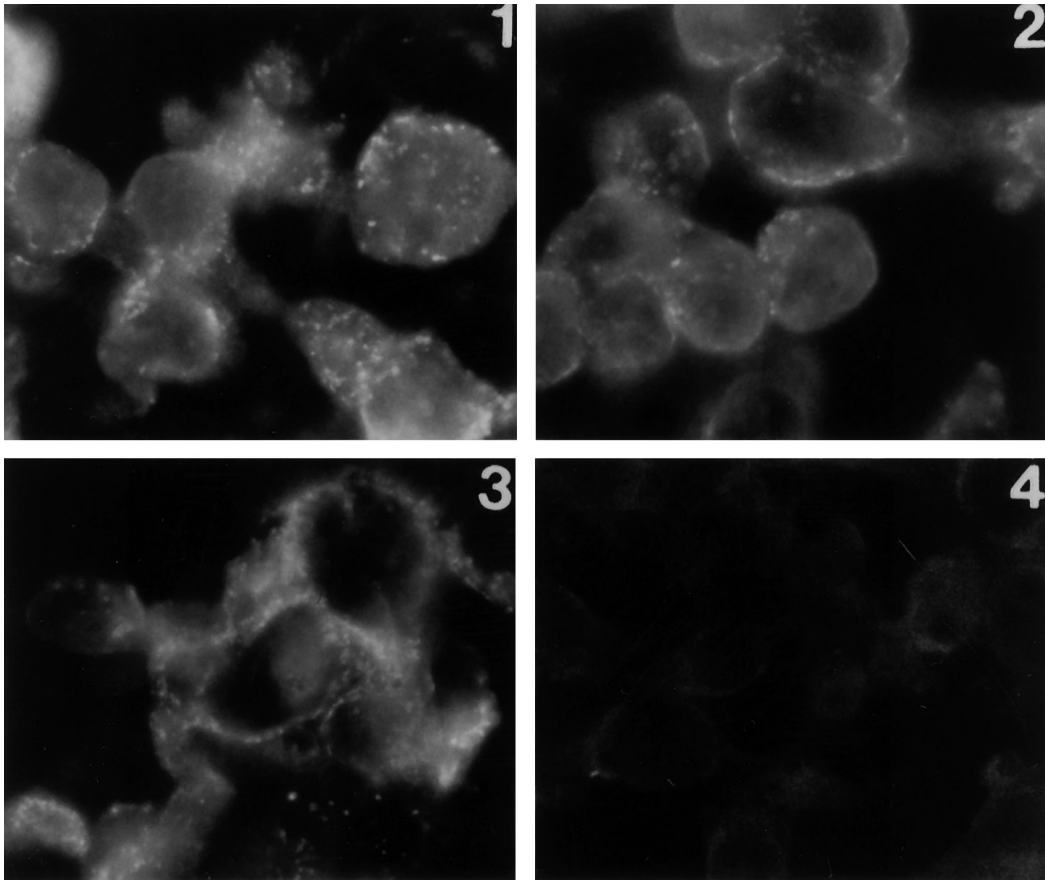


FIG. 3. Demonstration of MHV (strain A59) antigens in cells expressing full-length Bgp proteins. BHK cell monolayers grown on glass coverslips were infected with vaccinia vectors to allow for cell surface expression of Bgps. 10 hr later, cells were inoculated with MHV A59. After a 14-hr incubation period, cells were fixed in absolute acetone and A59 spike proteins were detected by immunofluorescence using anti-spike Mab 4B11.6 (Collins *et al.*, 1982) and FITC-conjugated goat anti-mouse immunoglobulin. (1) Bgp1^{NTD}. (2) Bgp1^{G10G,V123G}. (3) Bgp1^a. (4) Bgp1^Δ.

This difference in efficiency of binding was maintained throughout the 8-hr, 4° incubation period. Moreover, this pattern whereby Bgp1^a bound more virus than 1^b_{NTD} remained constant over a 10-fold range of virus input multiplicities (data not shown).

In all subsequent assays, we chose to interact [³⁵S] virions with receptor-bearing cells for 1 hr at 4°, as binding differences between the two receptor isoforms were readily discernable under these conditions. Our next assay involved testing the related strain JHMX for binding. For JHMX, a similar pattern of virus adsorption emerged (Fig. 6). The amount of virion adsorption, however, was lower for JHMX and the difference between Bgp1^a and Bgp1^b_{NTD} in JHMX adsorptive capacity was only 2.5-fold, comparatively lower than the 6-fold difference observed when strain A59 was used.

Mapping the MHV receptor determinants that control virus adsorption

The two receptors contain a limited number of amino acid differences and show measurable differences in virus binding capacity. This provided an opportunity to

identify the residues involved in virus binding by systematically exchanging 1^a and 1^b residues. This was accomplished through a series of restriction fragment exchanges to produce recombinant receptors containing varying numbers of 1^b residues within a Bgp1^a background (Fig. 7). These receptors were expressed in HeLa cells from vaccinia vectors and then tested for their ability to bind [³⁵S] labeled MHV particles. Chimeric receptors containing 1^b residues from positions 54 to 122 were as effective as 1^a in binding, while the reciprocal chimeras containing 1^b residues 10 to 54 were ineffective (Table 2).

To further narrow down residues controlling receptor binding efficiency, we focused on codons 38 to 43, as this region is the most variable among the presently sequenced Bgps (see Fig. 8; boxed region). Additionally this stretch of six residues is predicted to form a protruding loop connecting two β strands that form the framework of the immunoglobulin-like CEA domain (Bates *et al.*, 1992). Thus the Bgp1^a residues 38 to 43 were changed to 1^b residues by oligonucleotide-directed mutagenesis. The resulting chimera (1^b 38–43) exhibited a weak binding efficiency that was equivalent to that of

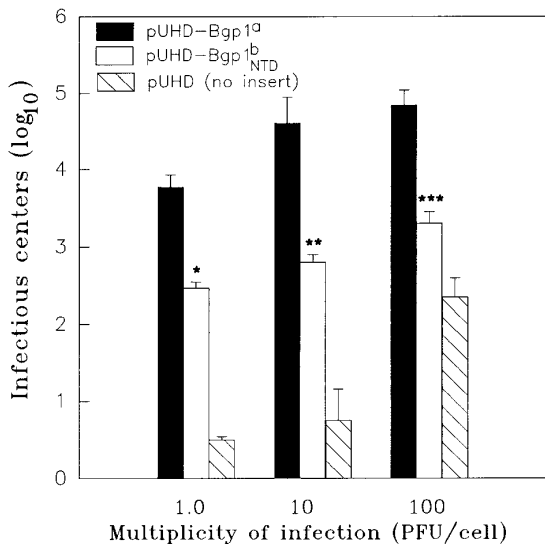


FIG. 4. Quantitation of infectious centers following inoculation of HeLa-Bgp transfectants with MHV (strain A59). Aliquots of 3×10^5 HeLa cells were transfected with the indicated plasmids by lipofectin and then challenged 36 hr later with varying doses of MHV-A59 for 1 hr at 4° . Inoculum virus was removed, and after a 2-hr penetration period at 37° , cells were trypsinized and serial dilutions were plated on DBT cell monolayers for development of infectious centers. *P* values relative to the Bgp1^a values were obtained by Student's *t* test. *, *P* < 0.01; **, *P* < 0.001; ***, *P* < 0.005.

Bgp1^b_{NTD} (Table 2). Thus a key determinant of binding differences between the isoforms was present within these six residues.

Correlation between virus binding capacity and promotion of membrane fusion

In performing these experiments comparing the efficiency of Bgp1^a and Bgp1^b_{NTD} receptors, we readily identified a correlation between virus binding capacity and syncytium formation. Upon infection by MHV-A59, stable HeLa-Bgp1^a transfectants fused rapidly into polykaryons, while the corresponding HeLa-Bgp1^b_{NTD} cells were much less susceptible. This was readily evident by simple microscopic examination of infected cell monolayers anywhere from 10 to 30 hr postinfection. However, previously published information suggested that measurements of additional receptor mutants for virus binding and fusion promotion capacities might not reveal a direct relationship between these two properties. First, evidence for the uncoupling of virus binding and fusion activation functions has been demonstrated in studies of mutant retrovirus receptors (James *et al.*, 1996; Lifson *et al.*, 1988; Truneh *et al.*, 1991). Second, two reports focusing on the highly fusogenic MHV JHM strain have provided evidence that membrane fusion induced by this strain occurs to a limited extent even in the absence of murine Bgp receptors (Gallagher *et al.*, 1992; Nash and Buchmeier, 1996). Given these complex findings we wanted to measure the relative abilities of our Bgp mutants to

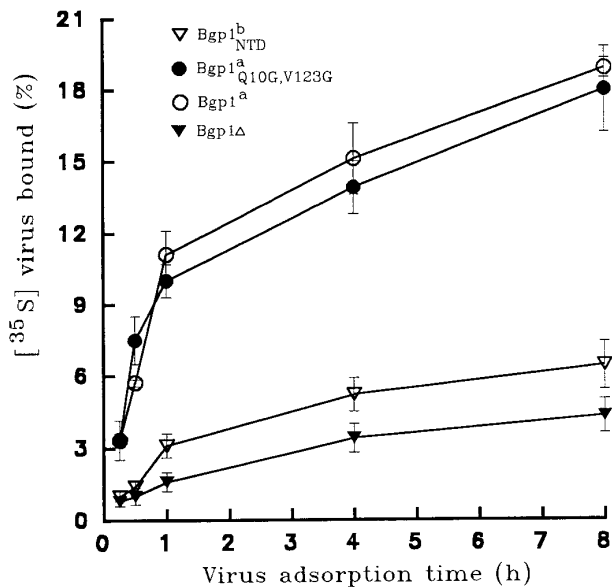


FIG. 5. Kinetics of virus binding to overexpressed Bgps. At 20 hr postvaccinia infection, aliquots of 5×10^5 HeLa cells bearing the indicated Bgp molecules were incubated at 4° for varying time periods with [³⁵S]MHV-A59 (5×10^5 cpm/ml). After each time period, unadsorbed virions were removed and cells were washed extensively with cold PBS containing 0.5% BSA and 0.05% Tween 20. Cells were then lysed and the radioactivity associated with the lysates was calculated as a percentage of total [³⁵S] virus added. The error bars represent standard deviations; average of two experiments.

enhance intercellular fusion with S-expressing cells. To do this, we employed a cell fusion-dependent reporter gene activation assay (Nussbaum *et al.*, 1994). In brief,

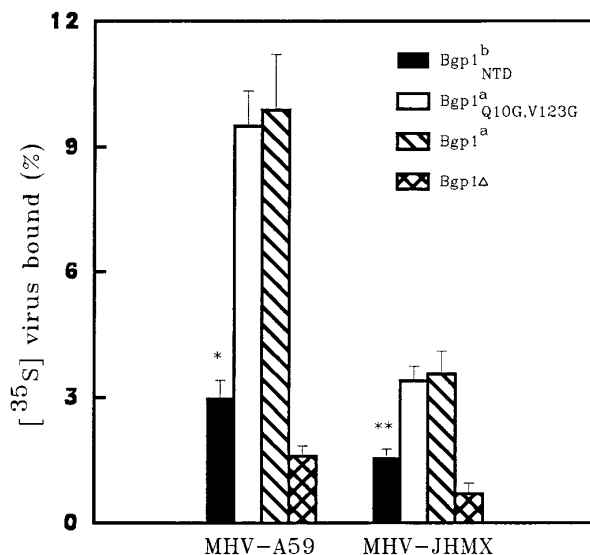


FIG. 6. Quantitation of [³⁵S]MHV binding to overexpressed Bgps. HeLa cells displaying the indicated Bgp molecules were incubated at 4° for 1 hr with [³⁵S]MHV strains A59 or JHMx (both at 5×10^5 cpm/ml). Radioactivities associated with the cells were then determined as described in the legend to Fig. 5. The error bars represent standard deviations; average of five experiments. Comparisons were made by Student's *t* test. *, *P* < 0.005 versus Bgp1^a; **, *P* < 0.01 versus Bgp1^a.

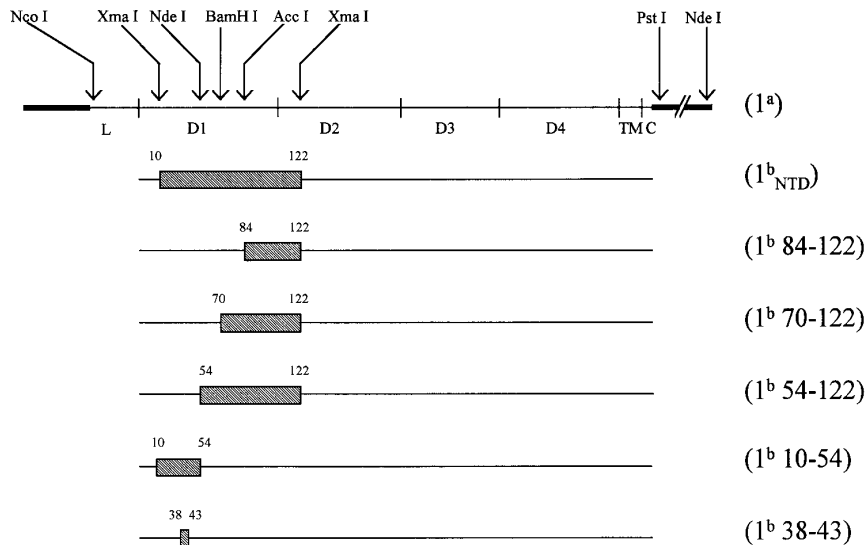


FIG. 7. Schematic representations of Bgp1^a cDNA and chimeric receptors. Top line represents Bgp1^a cDNA; thicker flanking lines represent vector sequences. The indicated restriction enzymes were used to exchange cDNA fragments encoding Bgp1^a residues with Bgp1^b. ^b codons are depicted by hatched boxes.

this assay involved infection of stable HeLa-Bgp1^a transfectants with vaccinia virus (strain WR) and MHV strain A59 and then loading a fraction of the cells with a transcriptionally silent β -galactosidase gene under the control of the phage T7 promoter. These cells, which bear S proteins on their surface, were then overlaid at subconfluent densities onto monolayers of HeLa cells coinfecting with vTF7.3 and the respective vTM1-Bgp recombinants. Transfer of the T7 polymerase to the S-expressing cells causes β -galactosidase expression, which can be measured and taken as an indicator of the amount of fusion between receptor and spike-bearing cells.

TABLE 2

Relative Virus Binding and Cell Fusion Promoting Activities of Recombinant Receptors

Receptor recombinants ^a	[³⁵ S] Virus bound (%) ^b	Cell fusion (ng of β -galactosidase/well) ^c
vBgp1 ^a	10.4 (1.2)	12.9
(Δ 10–122)	1.5 (0.5)	3.5
(1 ^b NTD)	3.1 (0.3)	4.9
(1 ^b 84–122)	10.2 (1.1)	11.2
(1 ^b 70–122)	9.8 (0.9)	11.4
(1 ^b 54–122)	9.4 (1.0)	11.5
(1 ^b 10–54)	3.1 (0.2)	5.2
(1 ^b 38–43)	2.9 (0.3)	5.9

^a Indicated Bgp chimeras were expressed on HeLa cell surfaces from vaccinia recombinants.

^b Percent [³⁵S]MHV-A59 absorbed after a 1 hr, 4° incubation on vBgp-infected HeLa cells (20 hpi). Standard deviations are in parentheses.

^c Intercellular fusion between receptor and spike-bearing cells was determined after a 3 hr cocultivation period, as described in the legend to Fig. 9. The data is an average of three independent experiments.

The results obtained from a subset of these tests is shown in Fig. 9. When cells were fixed and stained with x-gal (MacGregor *et al.*, 1989), intense blue clusters were abundantly evident in monolayers expressing Bgp1^a (Fig. 9a, panel A) and the Bgp1^a_{O10G,V123G} mutant. Fewer blue syncytia were seen in the Bgp1^b_{NTD}-expressing monolayers (Fig. 9a, panel C), while blue cells were rare in the cultures lacking the Bgp NTD altogether (Fig. 9a, panel B). Quantitation of the β -galactosidase activity in detergent lysates prepared from each monolayer at various times after cell mixing revealed the potency of each receptor in promoting fusion (Fig. 9b). From 2 to 4 hr after cocultivation, specific β -galactosidase expression in cultures harboring Bgp1^a was 5–6 times the level found in Bgp1^b_{NTD}-containing cultures, in concert with the sixfold binding differences between these receptors. Quantitative fusion assays were continued using cell monolayers bearing the Bgp chimeras listed in Table 2. In every situation tested, the relationship between binding and fusion promotion was direct.

DISCUSSION

Studies of murine coronavirus entry into cells provide unique opportunities to probe structure–function relationships in both virion attachment proteins and their cellular receptors. These viruses exhibit considerable natural strain variation; a variation that is largely localized to the spike (virion attachment) protein. Straightforward correlations between spike sequences and biological functions such as membrane fusion allow for identification of relevant determinants (Bos *et al.*, 1995; Gombold *et al.*, 1993; Hingley *et al.*, 1994). Because murine coronavirus receptors exhibit genetic variation in the domains known to interact with viral spikes (Dveksler *et al.*, 1995),

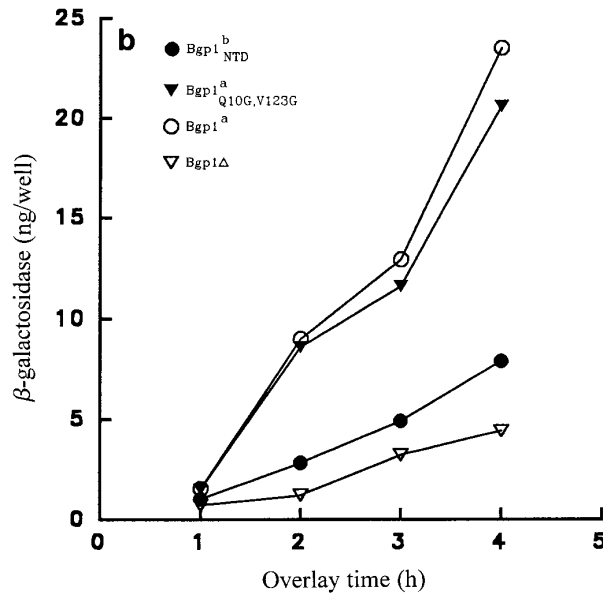
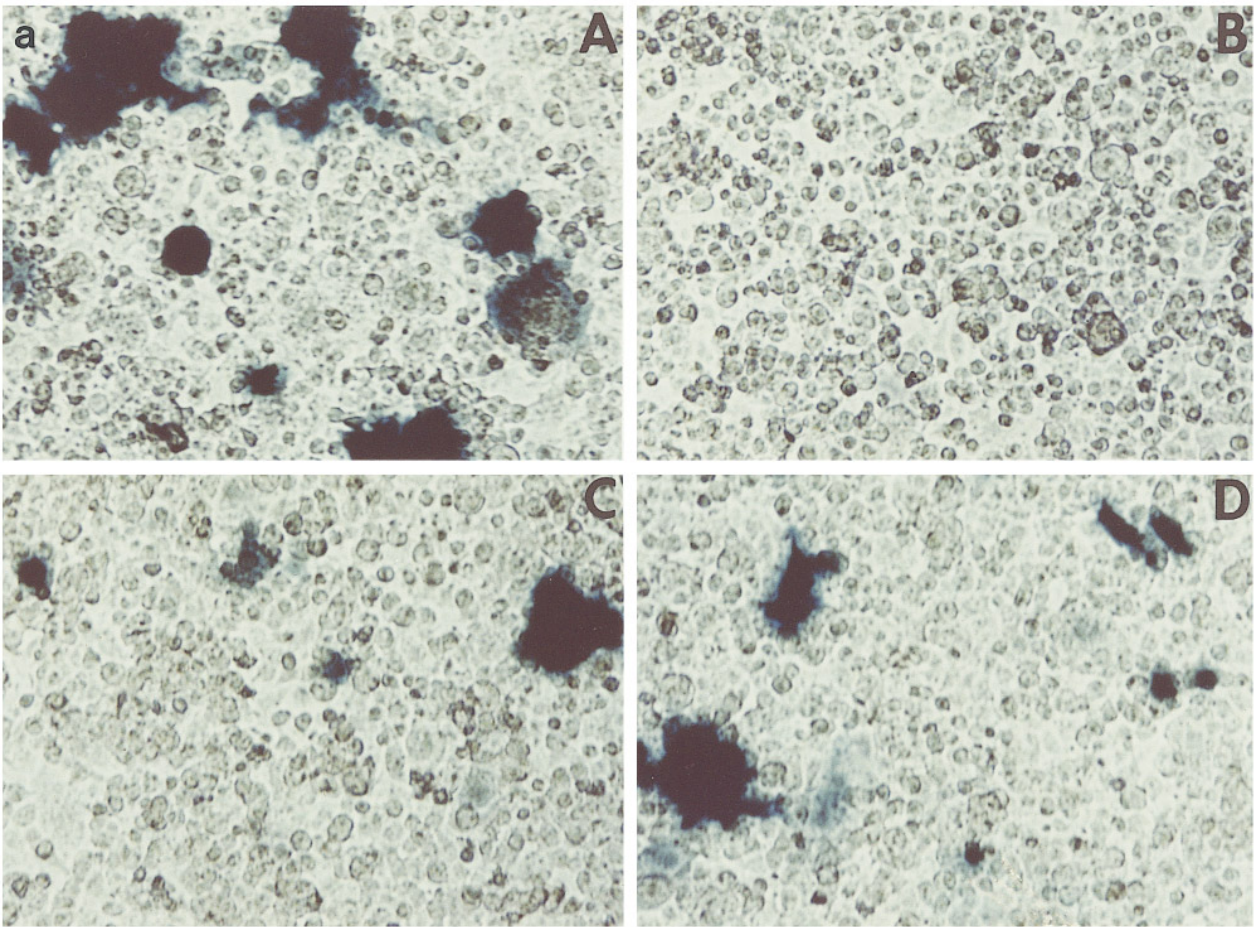


FIG. 9. Quantitation of intercellular fusion between spike- and receptor-bearing HeLa cells. Stable HeLa-Bgp1^a transfectants were infected with MHV strain A59 (m.o.i. = 2) and concomitantly transfected by lipofection with pG1NT7- β gal, which produces the β -gal product only in the presence of T7 RNA polymerase. 5 hr later, the spike-bearing cells were trypsinized and overlaid onto HeLa cell monolayers that had been infected 20 hr earlier with vTF7.3 and the indicated vTM3-Bgp recombinants. The resulting intercellular fusion levels were quantitated by measuring β -gal enzyme activities produced by the mixing of T7 RNA polymerase (in vTF7.3/vTM3-Bgp cytosol) with pG1NT7- β gal (in MHV-A59 cytosol). (a) Cocultivated cells were fixed after a 3-hr incubation and incubated with x-gal substrate to reveal the β -gal product *in situ* (A) Bgp1^a, (B) Bgp1 Δ , (C) Bgp1^b_{NTD}, (D) Bgp1^a_{Q10G,V123G}. (b) At the indicated times after overlay, cell monolayers lysed with PBS containing 0.5% NP-40. β -gal activity in the lysates was measured by a colorimetric assay and the OD_{590 nm} values were normalized by comparison with OD_{590 nm} produced by a preparation of purified β -galactosidase.

remembered that a tremendous number of possible receptor chimeras can be generated, each with a unique combination of 1^a and 1^b residues. It therefore remains possible that investigations of additional 1^a/1^b combinations will reveal a role for residues outside the 38–43 stretch in binding, perhaps because some patterns will impact overall receptor conformation.

Exchange of Bgp1^a residues 38–43 with those from 1^b also eliminates a potential N-linked glycan addition site at residue 37. However it has been established from thorough mutagenesis studies in the Holmes laboratory that a carbohydrate at this position does not contribute to virus binding (Dveksler *et al.*, 1995). Identification of these critical amino acid residues led us to attempt blockade of binding with synthetic peptides. To date we have been unable to interfere with the virus:receptor interaction using synthetic Bgp1^a peptide 33–45, implying a requirement for the immunoglobulin-like framework region in presenting a defined conformation of the residues.

According to a predicted three-dimensional model for the corresponding human CEA NTD (Bates *et al.*, 1992), residues 38 to 43 would represent a “loop” that is held in place by two of the beta strands (strands C and C′) that comprise a portion of the framework region (Fig. 8). This C-C′ loop is further predicted to protrude from the internal framework and thus it is reasonable to infer its direct interaction with the viral spike. Loop regions in the immunoglobulin-like receptor for HIV and poliovirus are critical for virus binding. The binding site of HIV-1 on CD4 has been localized to extensions made up of the C′-C″ strands and its interconnecting loop (Moebius *et al.*, 1992; Choe and Sodroski, 1992; Harrison, 1994). Poliovirus binding is influenced by mutations in the C′-C″, C″-D, and D-E loops (Aoki *et al.*, 1994; Morrison *et al.*, 1994). Additional comparisons of receptor structure and function will require definitive resolution of the Bgp1^a receptor through crystallographic methods. This is fast becoming a realistic possibility as a system for production and purification of reasonably large quantities of soluble recombinant Bgp1^a are now in place (Gallagher, 1997).

An understanding of the role that the Bgp receptor plays in mediating postbinding events such as virus-induced membrane fusion has been complicated by variable virus strain-specific responses. For example, spikes encoded by the JHM strain of MHV are exceptionally potent in mediating intercellular fusion and can perform this function to a limited extent even in the absence of receptor binding (Gallagher *et al.*, 1992; Nash and Buchmeier, 1996). In contrast, fusion mediated by the A59 spike protein is limited and is primarily receptor dependent. In this report we have used a quantitative fusion assay to show that A59-infected HeLa cells fuse together with partner cells bearing either Bgp1^a or 1^b and that the relative fusion levels corresponded directly with

the binding capacities of the receptors. In fact we have come to similar conclusions using MHV JHM in place of A59—while receptor independent fusion was observed with JHM, enhancement of this endogenous activity was most pronounced in the presence of receptors with 1^a residues 38–43. At present we have no evidence of separate domains on the Bgp molecule that are individually responsible for binding and induction of membrane fusion. However, such separate domains may eventually be identified, as studies of both the HIV and poliovirus receptors have revealed specific regions that induce changes in virion conformation required for membrane penetration (James *et al.*, 1996; Morrison *et al.*, 1994).

Finally, the results from these quantitative binding assays may shed light on the determinants of murine coronavirus tropism *in vivo*. JHMX can infect murine brain tissue more readily than A59 (Lavi *et al.*, 1986; Robb and Bond, 1979), and the overall abundance of Bgp1^a and 1^b in brain is very low (Yokomori and Lai, 1992a, 1992b). Given that JHMX is actually less avid than A59 in the Bgp binding properties, it appears likely that the different *in vivo* tropisms of these strains are not dictated simply by their capacity to bind these Bgps but rather by utilization of alternative receptors or by additional events occurring subsequent to binding.

ACKNOWLEDGMENTS

We thank Kathryn V. Holmes (University of Colorado Medical Center, Denver, CO) for providing anti-receptor MAb CC1 and Michel J. Buchmeier (Scripps Research Institute, La Jolla, CA) for providing anti-receptor antibody 874 and anti-spike antibody 4B11.6. Our thanks also go to Edward Berger and Bernard Moss (National Institute of Health) for providing vaccinia recombinant vTF7.3. We are grateful to Manfred Gossen and Herman Bujard for the HeLa-tTA cells. This work was supported by NIH Grant R29-NS-31616 and by a grant from the Schweppe Foundation of Chicago.

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