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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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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) 1a and 1b, 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 1a isoform was 10 to 100 times more effective than 1b in its ability to confer sensitivity to MHV (strain A59) infection. Bgp1a was also more effective than Bgp1b in comparative virus adsorption assays, binding 6 times more MHV (strain A59) and 2.5 times more MHV (strain JHM X). Bgp1a 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 1b residues into the Bgp1a 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.

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, 1992a; McCuaig et al., 1993), and we have examined their capacity 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 1a (Dveksler et al., 1991; Yokomori and Lai, 1992a) and 1b (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 1a isoform is expressed in MHV susceptible mice, while mouse strains homozygous for the 1b isoform are generally resistant to many currently recognized 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 1a and 1b lies primarily in the membrane-distal domain (Yokomori et al., 1992a, 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 Bgp1a and 1b indicated that they differ substantially in virion adsorptive capacity—when the two receptors were immobilized on nitrocellulose filters, only the 1a isoform supported detectable virus-binding (Dveksler et al., 1993a). This finding suggested that one or more of the 29 amino acid differences between the 1a and 1b 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 1a/1b 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 (ΔFBS). 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% ΔFBS. Murine astrocytoma (DBT) cells (Hirano et al., 1978) were grown in minimal essential medium (MEM) containing 10% TPB and 5% ΔFBS; 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%-dialized ΔFBS containing 100 μCi/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°C.

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 Bgp1a or Bgp1b lacking codons 10–122 (Bgp1Δ) have been described previously (Gallagher, 1996). These plasmid vectors were further modified by reinsertion of Bgp NTD sequences into Bgp1Δ. To this end, the Bgp1aΔ and 1b NTD sequences were amplified from outbred CD1 mouse liver RNA by RT-PCR (Kawasaki, 1990) using primers based on the reported Bgp1a sequence (McCuaig et al., 1993) and products encoding for the 1a and 1b NTD were identified by restriction mapping and by sequencing (Sambrook et al., 1989). 1a NTD sequences were cloned in-frame to the unique XmaI site of Bgp1Δ to generate the chimeric Bgp1aΔNTD. Two undesired codon alterations that resulted at the junctions of the insertion (Q10G and V123G) were introduced into a control Bgp1a construct by performing a parallel insertion of the Bgp1a NTD into pTM3-Bgp1Δ. The control recombinant was designated as Bgp1aQ10G.V123G.

To construct additional Bgp chimeras for use in identifying virus binding determinant(s), restriction fragments between pTM3-Bgp1a and pTM3-Bgp1aNTD were exchanged. 1b 84–122 and 1b 70–122 were made by exchange of Accl–PstI and BamH1–PstI fragments, respectively (see Fig. 7). Additional exchanges were performed after creation of a NdeI site at codon 53 using the megaprimer mutagenesis procedure (Aiyar and Leis, 1993). NdeI site creation altered codon 53 (N53H) in Bgp1a and codons 53 and 54 in Bgp1aNTD (N53H, K54M). 1b Δ52–122 and 1b 10–54 were made by exchange of NdeI–NdeI and NcoI–NdeI fragments, respectively (see Fig. 7). Re-combinant 1b 38–43 was made by oligonucleotide-directed PCR mutagenesis using a primer spanning codons 38–43 and the NdeI site (Scharf, 1990). Nucleotide sequencing methods (Sanger et al., 1977) were employed to confirm that Bgp1a and 1b 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°C. 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). Viruses from well isolated plaques was grown in RK13 cells to generate stocks of recombinant virus (vBgp1a, vBgp1bΔ, vBgp1aNTD, vBgp1aNTDQ10G.V123G, and additional 1a/1b chimeras).

Immunoblot analysis of Bgp proteins

Cell monolayers were infected with the respective vBgp recombinants (m.o.i. = 10) and coinfectected 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-μl aliquots (equivalent to 2 × 10⁴ 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°C with PBS containing 1 mg/ml NHS-LC biotin (sulfo succinimidyl-6-(biotinamido) hexoanate; Pierce Co., Rockford, IL). Unreacted reagent was then quenched by the addition of 20 mM
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°C with a 1:250 dilution of antispike 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 Bgp1α and Bgp1β were inserted into plasmid expression vector pUHD-10-3 (Gossen and Bujard, 1992) and the plasmids (0.5 μg) were transfected by lipofection into aliquots of 3 × 10⁵ 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 μg 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 1α and 1β TTD 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°C 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% ΔFBS for 2 hr at 37°C. Cells were then trypsinized, washed twice with DMEM 10% ΔFBS, 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°C, 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-Bgp1α 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°C, 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% ΔFBS, and 0.6-ml (3 × 10⁵ cell) aliquots were overlaid on to confluent 5-cm² monolayers of HeLa cells that were coinfectected 20 hr earlier with vTF7.3 and vBgp recombinants. After 3 hr at 37°C, 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-Bgp1α 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. Bgp1Δ lacks the NTD; replacement with NTD sequences from Bgp1Δ NT (Bgp1Δ NT) and Bgp1Δ NT (Bgp1Δ NT) 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; Nussbaum 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 Bgp1Δ, 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 Bgp1Δ (Bgp1Δ) 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 Bgp1Δ (McCuaig et al., 1993) were then placed back into the truncated cDNA to generate a hybrid capable of encoding the chimeric molecule Bgp1Δ NT. 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 Bgp1Δ containing the mutations (designated Bgp1Δ NT) was prepared by ligating the NTD sequences of Bgp1Δ 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 Bgp1Δ NT, Bgp1Δ NT, and Bgp1Δ (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 Bgp1Δ had a similar, slightly more
MHV infection was assessed by inoculation of the MHV with known MHV proteins (data not shown). In initial proportion of biotinylated receptor among the immunoreceptors immunoprecipitated the receptor proteins. The relative infection with the reagent and then we lysed cells and visualized the proteins on intact cells at 20 hr postvaccinia.

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 Bgp1α or Bgp1β were introduced into plasmid expression 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 10^3 PFU/ma 3 x 10^3 cells) were generated even by A59-challenged HeLa cells (hatched bars). This was clearly due to a failure to quantitatively remove nonspecifically 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 Bgp1α and 1β differed significantly in their capacity to stimulate formation of infectious centers. Infectious centers among Bgp1α transfectants (solid bars) were 3 to 4 log higher than controls, while Bgp1β transfectants (open bars) were only 1 to 2 log 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 69,000, 3,000, and 200 PFU/ml for the 1α, 1β, and control transfectants, respectively. Thus, when expressed in the HeLa-tTA cells, Bgp1α was utilized as an MHV-A59 receptor much more effectively than Bgp1β.

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[S] 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°C 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 Bgp1α, 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 Bgp1α or 1β than 1α, 9.5 and 3% respectively, after 1 hr. Subtraction of nonspecific adsorption values revealed that the Bgp1β was 6-fold less effective than Bgp1α 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<sub>NTD</sub>, (2) Bgp1<sub>Q10G,V123G</sub>, (3) Bgp1<sub>Q12</sub>, (4) Bgp1Δ.

This difference in efficiency of binding was maintained throughout the 8-hr, 4˚C incubation period. Moreover, this pattern whereby Bgp1<sup>a</sup> bound more virus than Bgp1<sup>b</sup> 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˚C, as binding differences between the two receptor isoforms were readily discernible 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<sup>a</sup> and Bgp1<sup>b</sup> 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<sup>a</sup> and 1<sup>b</sup> residues. This was accomplished through a series of restriction fragment exchanges to produce recombinant receptors containing varying numbers of 1<sup>b</sup> residues within a Bgp1<sup>a</sup> 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<sup>b</sup> residues from positions 54 to 122 were as effective as 1<sup>a</sup> in binding, while the reciprocal chimeras containing 1<sup>b</sup> 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<sup>a</sup> residues 38 to 43 were changed to 1<sup>b</sup> residues by oligonucleotide-directed mutagenesis. The resulting chimera (1<sup>b</sup> 38–43) exhibited a weak binding efficiency that was equivalent to that of
Bgp1b<sub>NTD</sub> (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<sup>a</sup> and Bgp1<sub>b NTD</sub> receptors, we readily identified a correlation between virus binding capacity and syncytium formation. Upon infection by MHV-A59, stable HeLa-Bgp1<sup>a</sup> transfectants fused rapidly into polykaryons, while the corresponding HeLa-Bgp1<sub>b NTD</sub> 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 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,
this assay involved infection of stable HeLa-Bgp1<sup>a</sup> 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 coinfectected 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.

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<sup>a</sup> (Fig. 9a, panel A) and the Bgp1<sup>a</sup>Q10G,V123G mutant. Fewer blue syncytia were seen in the Bgp1<sup>b</sup>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<sup>a</sup> was 5–6 times the level found in Bgp1<sup>b</sup>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. 8. Amino acid alignment of the N-terminal domain of murine biliary glycoproteins and human carcinoembryonic antigen. The large arrow at position 107 marks the junction between the NTD and domain 2, while the numbers indicate endpoints of the various 1α/1β exchanges. The square encloses the hypervariable region containing residues determining the efficiency of virus binding. Potential N-linked glycosylation sites are underlined. Regions predicted to form the β strands (A–G) comprising the framework of the immunoglobulin-like NTD of human CEA are indicated as dashed lines below the amino acid sequence (Bates et al., 1992).

an analogous set of experiments correlating receptor sequences with biological functions such as virion binding capacity can reveal the molecular determinants of the virus:receptor interaction.

Previous qualitative assays in which virions were allowed to interact with denatured and immobilized MHV receptors showed that allelic receptor isoforms termed Bgp1α and 1β do indeed differ in virion adsorptive capacity (Boyle et al., 1987; Dveksler et al., 1993a), thereby providing the opportunity for determining which nonhomologous region(s) control the differences. Our initial goal was to establish assays in which we could accurately measure the relative virus binding capacities of these two receptors. We anticipated that the success of these assays might require abundant receptor levels because previous attempts to measure virion binding to murine cells endogenously expressing Bgps were not sensitive enough to reveal any specific adsorption (Yokomori et al., 1993; Wilson and Dales, 1988). Our binding assays therefore involved interaction of radiolabeled virions to native receptors that were overexpressed on the surface of HeLa cells from vaccinia vectors. By this method, specific binding measurements were obtained, even though the proportions of cell-associated [35S] virions were relatively low (Fig. 6). Adsorption might be limited in part by slow diffusion to cell monolayers, as prolonged incubation periods gradually increased binding (Fig. 5). Ligand densities also likely play a role—recombinant Bgp1α that is immobilized onto Sepharose at extraordinarily high densities will bind the majority of [35S] virions in our recently developed in vitro receptor binding assays (Gallagher, 1997).

We established conditions in which parallel HeLa cell monolayers displayed equivalent amounts of either Bgp1α or a chimeric Bgp1αNTD on the cell surface. These two receptors varied in 30 positions of the 112 residue N-terminal region. When purified [35S] MHV strain A59 was adsorbed to HeLa cells at 4°C for 1 hr, 9% of added radioactivity bound specifically to Bgp1α, while 1.4% bound to Bgp1β. When identical assays were performed using [35S] MHV strain JHMX, 2.8% bound specifically to Bgp1α, while 0.8% bound Bgp1β (Fig. 6). These differences between the two receptors in virus binding capacity impact the outcome of MHV infection. We found that transfection of either Bgp1α or Bgp1β genes into HeLa cells conferred sensitivity to MHV A59 infection, but Bgp1β was inefficiently utilized as revealed by quantitation of infectious centers (Fig. 4). This latter finding was generally consistent with a variety of previous observations. Chen and Baric (1996) reported that challenge of stable BHK-Bgp1α transfectants with MHV A59 led to titers of 10⁸ PFU/ml after 1 day, while parallel challenge of BHK-mmCGM2 (Bgp1β) transfectants yielded only 10⁴ PFU/ml. In similar tests, Compton (1994) found analogous (but less dramatic) differences between the two stably transfected BHK cell lines in their support of MHV A59 infection. Finally, Yokomori and Lai (1992b) found that Cos7 cells transiently transfected with Bgp1α or 1β supported very low levels of MHV A59; the 1α transfectants were marginally superior as virus hosts.

We have exchanged portions of Bgp1α cDNA with those from Bgp1β and have assessed the resulting hybrid gene products for virus binding capacity. Our most informative recombinant receptor was a Bgp1α in which only residues 38–43 were specific to 1β. This chimera was indistinguishable from the complete Bgp1αNTD in its support of virus binding (Table 2; see data highlighted by bold type), thus strongly suggesting that these six residues are the critical determinants controlling virus adsorption levels. Despite this compelling data, it must be...
FIG. 9. Quantitation of intercellular fusion between spike- and receptor-bearing HeLa cells. Stable HeLa-Bgp1a 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) Bgp1a, (B) Bgp1Δ, (C) Bgp1NTD, (D) Bgp1Q100G,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<sub>590</sub> values were normalized by comparison with OD<sub>590</sub> 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 1a and 1b residues. It therefore remains possible that investigations of additional 1a/1b 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 Bgp1a residues 38–43 with those from 1b 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 Bgp1a 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 Bgp1a 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 Bgp1a 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 Bgp1a or 1b 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 1b 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. JHM X can infect murine brain tissue more readily than A59 (Lavi et al., 1986; Robb and Bond, 1979), and the overall abundance of Bgp1a and 1b in brain is very low (Yokomori and Lai, 1992a, 1992b). Given that JHM X 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.

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