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NEUTRALIZATION OF MHV-A59 BY SOLUBLE RECOMBINANT RECEPTOR GLYCOPROTEINS

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1. ABSTRACT

The interaction of viruses with specific receptors is an important determinant of viral tissue tropism and species specificity. Our goals are to understand how mouse hepatitis virus (MHV) recognizes its cellular receptor, MHVR, and how post-binding interactions with this receptor influence viral fusion and entry. Murine cells express a variety of cell surface molecule in the biliary glycoprotein (Bgp) family that are closely related to the MHVR. When these proteins are expressed at high levels in cell culture, they function as MHV receptors. We used a baculovirus expression system to produce soluble recombinant murine Bgp receptors in which the transmembrane and cytoplasmic domains have been replaced with a six-histidine tag. The soluble glycoproteins were purified to apparent homogeneity and shown to react with antisera to the native receptor. We compared the virus neutralizing activities of various soluble receptor glycoproteins. Soluble MHVR [sMHVR(1–4)] had 10–20 fold more virus neutralizing activity the soluble protein derived from the $Bgp1^b$ glycoprotein [$sBgp1^b$(1–4)], from MHV-resistant SJL mice. The sMHVR(1–4) glycoprotein was 60–100 fold more active than a truncated receptor molecule containing only the first two immunoglobulin-like domains, sMHVR(1,2). The observation that sMHVR lacking domains 3 and 4 neutralizes MHV-A59 very poorly suggests that these domains may influence virus binding or subsequent steps associated with neutralization.

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2. INTRODUCTION

Mouse hepatitis viruses (MHV) provide an excellent model system in which to study the role of receptor variation in virus biology and pathogenicity. This group of coronaviruses causes inapparent infection or a variety of murine diseases in mice including diarrhea, hepatitis, splenolysis, immunological dysfunction, thymic atrophy, and acute and chronic neurological disorders (Barthold, 1986; Wege *et al.*, 1982). MHV is efficiently spread within colonies of laboratory mice, and most MHV strains readily infect murine cell lines (including 17 Cl 1 cells, L2 cells and DBT cells) causing cell fusion, lysis and death (Frana *et al.*, 1985). Infection by MHV is initiated by binding of the viral attachment protein, S, to a specific virus receptor glycoprotein, MHVR (Dveksler *et al.*, 1991; Williams *et al.*, 1991; Collins *et al.*, 1982). Analysis of deletion mutant has shown that the viral S protein binds to the N-terminal domain (domain 1) of MHVR (Dveksler *et al.*, 1993b). The anti-MHVR monoclonal antibody MAb-CC1 also binds to domain 1, and blocks virus binding and infection (Dveksler *et al.*, 1993b). Binding is followed by S-mediated fusion of the viral envelope and host cell membranes, allowing the viral nucleocapsid to enter the cytoplasm (Sturman *et al.*, 1990).

MHVR, also referred to as $Bgp1^a$, is a biliary glycoprotein of the carcinoembryonic antigen (CEA) family of the immunoglobulin (Ig) superfamily (Brümmendorf *et al.*, 1994; Nedellec *et al.*, 1994; Rudert *et al.*, 1992). MHVR consists of four Ig-like extracellular domains, a transmembrane domain and either a long or short cytoplasmic tail (McCuaig *et al.*, 1993; Dveksler *et al.*, 1991). It is a cell adhesion protein that is expressed in fibroblasts and epithelial cells of many murine tissues (Godfraind *et al.*, 1995; Coutelier *et al.*, 1994; Benchimol *et al.*, 1989). The identification of MHVR as the receptor for MHV has led to the identification of MHV receptor activities for several related murine glycoproteins in the Bgp family (Figure 1) (Dveksler *et al.*, 1993a; Yokomori and Lai, 1992). These include a two domain splice variant of MHVR; an allelic protein called $Bgp1^b$ or mmCgm2, with four and two domain splice variants; and Bgp2; and brain CEA (Chen *et al.*, 1995; Nedellec *et al.*, 1994; McCuaig *et al.*, 1993).

SJL mice are homozygous for $Bgp1^b$, do not express MHVR, and are highly resistant to infection by MHV-A59. A cell line derived from SJL mice (PSJLSV) is also resistant to infection by MHV-A59 (Yokomori and Lai, 1992). Intestinal brush border membranes (BBM) isolated from SJL mice contain high levels of Bg1b glycoprotein but do not bind MHV-A59 as well as BBM isolated from MHV-susceptible BALB/c mice, which express MHVR (Williams *et al.*, 1991; Boyle *et al.*, 1987). This suggests that $Bgp1^b$ is a less efficient receptor for MHV-A59 than MHVR (Dveksler *et al.*, 1993a; Yokomori and Lai, 1992; Boyle *et al.*, 1987). When recombinant $Bgp1^b$ is expressed in PSJLSV or MHV-resistant hamster cells (BHK), these cells become susceptible to infection by MHV-A59, demonstrating that $Bgp1^b$ can serve as a receptor when expressed at high levels (Dveksler *et al.*, 1993a; Yokomori and Lai, 1992). Sequence comparison of the N-terminal, virus-binding domains of MHVR and $Bgp1^b$ revealed that 29 of the 108 amino acids differ, and mutational analysis of this region has shown that it is responsible for the observed differences in receptor activity (Rao and Gallagher, 1997; Wessner *et al.*, 1997; Dveksler *et al.*, 1993a; Yokomori and Lai, 1992). Other Bgp glycoproteins (Figure 1) also function as receptors for MHV-A59 when expressed at high levels in BHK cells, although less efficiently than MHVR or $Bgp1^b$ (Chen *et al.*, 1995; Nedellec *et al.*, 1994; Dveksler *et al.*, 1993a). These alternative receptors may be more efficient receptors for different MHV isolates. The combination of several alternative virus receptors, with differing tissue distributions and virus binding capabilities, with many well characterized and pathogenically

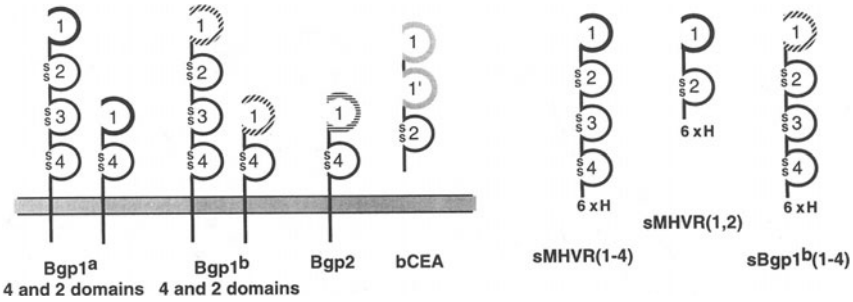

Figure 1. The various murine Bgp molecules are shown schematically, with the Ig-like domains numbered, beginning with the N-terminal domain. These glycoproteins differ markedly in the amino acid sequences of domain 1, the virus-binding domain, which is represented here by different shading patterns. Naturally occurring, membrane associated Bgps are shown on the left and the truncated, soluble Bgp molecules expressed using a baculovirus expression system are shown to the right. The names of the soluble proteins indicate which of the immunoglobulin-like domains are present, and the six-histidine tail present on each molecule is represented as 6xH.

distinct viruses makes the MHV system a fascinating arena for the investigation of virus-receptor interactions. The study of these virus-receptor interactions is important not only for understanding virus binding and the post-binding events of fusion, but also for elucidating the effects of receptor variation upon the pathogenesis of MHV infection.

3. RESULTS AND DISCUSSION

To pursue our investigations of MHV-receptor interactions, we developed a baculovirus expression system for production of large amounts of soluble, recombinant MHVR and Bgp1^b glycoproteins (Figure 1, designated with the 's' prefix). Using PCR-mutagenesis techniques, we replaced the transmembrane and cytoplasmic domains of the glycoproteins with six histidine residues. The secreted glycoproteins were purified to apparent homogeneity by nickel affinity and ion exchange chromatography. The apparent molecular weights of the soluble proteins on SDS-PAGE gels were approximately double those predicted on the basis of their amino acid compositions, due to extensive glycosylation. A high degree of glycosylation is also seen in MHVR purified from Swiss Webster mouse liver and in two murine Bgps expressed in a baculovirus system (Ohtsuka *et al.*, 1996; Williams *et al.*, 1991). Immunoblot analysis demonstrated that sMHVR(1–4) and sMHVR(1,2) were recognized by the anti-MHVR MAb-CC1. Binding of MAb-CC1 is very dependent upon the proper tertiary configuration of domain 1 of MHVR as shown by mutagenesis studies (Wessner *et al.*, 1997; Dveksler *et al.*, 1996; Dveksler *et al.*, 1993b). These results indicate that the soluble, recombinant Bgps secreted from insect cells were properly folded and processed.

Soluble receptors for polioviruses, rhinoviruses and coronaviruses neutralize infectious virus in a concentration-dependant manner (Dveksler *et al.*, 1996; Ohtsuka *et al.*, 1996; Greve *et al.*, 1991; Kaplan *et al.*, 1990). Similar virus neutralization studies were conducted to compare the MHV receptor activities of the soluble four domain glycoproteins, sMHVR(1–4) and sBgp1^b(1–4) (Figure 2A). 5000 PFU of MHV-A59 was incubated with varying amounts of purified receptor for one hour at 37°C and the surviving virus was

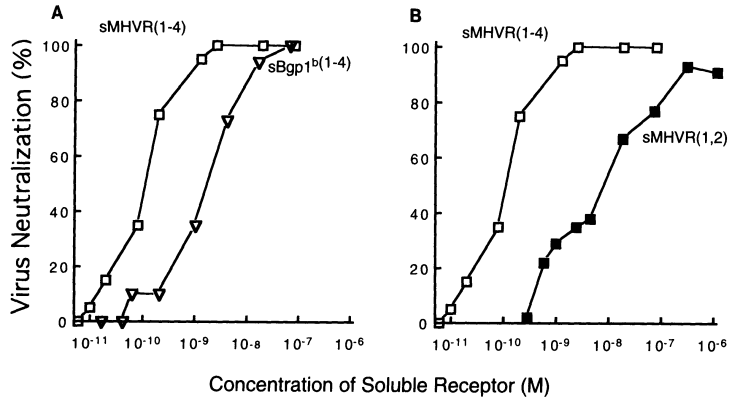

Figure 2. Neutralization of MHV-A59 by soluble receptors. A) The ability of the soluble four domain receptors, sMHVR(1–4) (□) and sBgp1^b(1–4) (▽), to neutralize MHV-A59 was determined by plaque assay on 17 Cl 1 cells. B) The neutralizing activity of the soluble two-domain sMHVR(1,2) (■) is compared to that of the four domain sMHVR(1–4) (□).

quantitated by plaque assay using murine 17 Cl 1 cells. Control reactions contained virus incubated with buffer alone. For the experiments presented in Figure 2, the 50% inhibitory dose (ID_{50}) is used to compare the neutralizing activities of the soluble receptors; the results of many experiments established the ranges given in the discussion. sMHVR(1–4) glycoprotein had the most neutralizing activity (ID_{50}=0.1 nM), while in repeated experiments, sBgp1^b(1–4) had 10–20 fold less virus neutralizing activity (ID_{50}=1.7 nM). The higher neutralization activity of sMHVR(1–4) compared to sBgp1^b(1–4) correlates with the higher receptor activity of anchored MHVR compared to Bgp1^b when expressed in murine tissues and transfected BHK cells. Rao *et al.* (1997) created a chimeric receptor in which domain 1 of MVHR was replaced with domain 1 of Bgp1^b and compared its receptor activity to that of MHVR when these two receptors were expressed in HeLa cells. MHVR had 10–100 fold more virus receptor activity than the Bgp1^b chimera, a result comparable to the neutralizing activity of the soluble glycoproteins. Our results are quite different from those of Ohtsuka *et al.* (1996), who have found a greater difference between the receptor activities of soluble Bgps comprised of the first and fourth domains of MHVR and Bgp1^b, sMHVR(1,4) and sBgp1^b(1,4) in the nomenclature used here. Anchored two domain (1,4) proteins are naturally occurring isoforms generated by alternative RNA splicing (Figure 1) which have receptor activity when expressed at high levels in hamster cells (Dveksler *et al.*, 1993a). Ohtsuka *et al.* (1992) incubated soluble sMHVR(1,4) and sBgp1^b(1,4) glycoproteins in concentrated insect cell culture supernatants with MHV-JHM virus, and plaqued the survivors on DBT cells. In this system, the sMHVR(1,4) neutralized MHV-JHM 500 times more effectively than soluble sBgp1^b(1,4). One possible explanation for much larger difference in virus neutralizing activity may be their use of MHV-JHM virus, while we used MHV-A59. MHV-JHM has been reported to utilize the Bgp1^b receptor less efficiently than MHV-A59 (Pasick *et al.*, 1992; Yokomori and Lai, 1992). MHV-JHM also has different receptor preferences than MHV-A59 in that MHV- JHM cannot infect cells transfected with brain CEA, while MHV-A59 can (Chen *et al.*, 1995). Taken together, these results show that these two strains of MHV have different interactions with Bgp1^b. This supports the hypothesis that

different strains of MHV may preferentially use different subsets of the MHVR "family" of receptors, which may affect the pathogenesis of individual viral strains.

The soluble two domain MHVR [sMHVR(1,2)] was constructed for biophysical and crystallographic studies. When purified, sMHVR(1,2) appeared to be properly processed and glycosylated, as demonstrated by its immunoreactivity and apparent molecular weight. However, it had 60 to 100 fold less virus neutralizing activity than the four domain sMHVR(1–4) (Figure 2B, ID_{50} = 8 nM) even though both molecules contain the same domain 1, the site of virus binding (Dveksler *et al.*, 1993b). The observation that sMHVR(1,2) has very low virus neutralizing activity is in agreement with the fact that membrane-anchored MHVR(1,2) transiently expressed in BHK cells is a very inefficient receptor for MHV-A59 (Wessner *et al.*, 1997). The low neutralizing activity of sMHVR(1,2) relative to sMHVR(1–4) is due to the absence of domains 3 and 4, which may alter the conformation of domain 1 and reduce its ability to bind the virus. Bgp glycoproteins appear to be signaling molecules that transmit information from the N-terminus outside the membrane to the C-terminus inside the cell via conformational changes. Thus, changes in distal regions of the protein might affect the conformation and the virus neutralizing activity of domain 1. Similarly, for the measles receptor, CD46, removal of the third and fourth of the seven short consensus repeats decreased the ability of the two N-terminal repeats to interact with measles virus (Devaux *et al.*, 1997). Virus neutralization assays reflect not only virus binding, but also post-binding events associated with membrane penetration and uncoating (Colston and Racaniello, 1995; Greve *et al.*, 1991; Kaplan *et al.*, 1990). For MHVR(1–4) and MHVR(1,4), we suggest that there may be an important structural and functional significance for the domain X-domain 4 linkage. This linkage may resemble an immunoglobulin hinge, which may play an important role in post-binding conformational changes associated with virus uncoating or penetration. This structural feature would be absent from the sMHVR(1,2) protein, and may explain its low virus binding and neutralizing activities.

4. CONCLUSIONS

We have produced and purified soluble Bgps that can neutralize MHV-A59, although with very different efficiencies: sMHVR(1–4) is 15–20 times more active than $sBgp1^b$(1–4) and 60–100 times more active than the two domain sMHVR(1,2). These differences in virus neutralization activity reflect the relative abilities of the anchored molecules to support MHV infection *in vivo* and when over expressed in BHK cells. These results, show that the soluble Bgps produced in the baculovirus system accurately reflect the behavior of Bgps produced by mammalian cells and are therefore a realistic model system for studying the biophysical parameters of virus-receptor interactions. Additional studies are underway to determine if the differences in virus neutralization are due to differences in virus binding affinities, or if post-binding events associated with membrane fusion and viral uncoating also affect neutralizing capability. The soluble receptors will also be used to investigate the receptor preferences of the various MHV strains to investigate the basis for their different biological properties and tissue tropisms.

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