Receptor Specificity and Receptor-Induced Conformational Changes in Mouse Hepatitis Virus Spike Glycoprotein

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1. INTRODUCTION

Coronavirus spike (S) glycoproteins bind to specific glycoproteins on host cell membranes that serve as virus receptors. Receptors for S glycoproteins of several coronaviruses have been identified (Dveksler, *et al.*, 1991; Dveksler, *et al.*, 1993; Chen, *et al.*, 1995; Yeager, *et al.*, 1992; Delmas, *et al.*, 1992; Tresnan, *et al.*, 1996; Benbacer, *et al.*, 1997). The specificity of virus/receptor interactions is an important determinant of the species-specificity of coronavirus infection, and may play a role in the tissue tropism and virulence of coronavirus diseases (Kolb, *et al.*, 1997; Ballesteros, *et al.*, 1997; Sanchez, *et al.*, 1999). The mechanism of entry has been studied in great detail for several enveloped viruses including influenza A virus, avian leukosis viruses and HIV-1 (Kemble, *et al.*, 1994; Stegmann, *et al.*, 1990; Chen, *et al.*, 1999; Gilbert, *et al.*, 1995; Hernandez and White, 1998; Zhang, *et al.*, 1999; Turner and Summers, 1999). Binding of the spike glycoprotein on the viral envelope to the receptor on the cell membrane may induce specific, pre-programmed conformational changes in the spike protein and/or the receptor that bring together the lipid bilayers of the viral envelope and the cell membrane. A fusion pore is created that expands to permit the entry of the viral nucleocapsid into the cytoplasm, leading to virus

The Nidoviruses (Coronaviruses and Arteriviruses).
Edited by Ehud Lavi *et al.*, Kluwer Academic/Plenum Publishers, 2001.

infection. This chapter will summarize the specificity of the interactions of murine coronavirus MHV and mutants of MHV with cellular receptors, and discuss evidence that receptor binding induces a temperature-dependent conformational change in the MHV S glycoprotein that may play a role in virus entry and MHV-induced cell fusion.

2. MHV RECEPTORS

As receptors for entry into susceptible murine cells, MHV strains use isoforms of several cellular membrane proteins in the immunoglobulin superfamily, formerly called MHVR or Bgp, that are now called CEACAMs (Beauchemin, *et al.*, 1999). The CEACAM1a gene of mice has 4 isoforms that consist of either two or four Ig-like exodomains linked by a transmembrane domain to either a short or a long cytoplasmic tail (Nedellec, *et al.*, 1995). The long tail isoform can be phosphorylated on a tyrosine residue, and both long and short tail isoforms can be phosphorylated on serine residues. It is not known whether phosphorylation of CEACAM1 affects its MHV receptor activity. MHV-resistant adult SJL/J mice express isoforms of a different allele of the receptor, called CEACAM1b (Yokomori and Lai, 1992; Dveksler, *et al.*, 1993). The CEACAM1 glycoproteins are expressed on apical membranes of epithelial cells of the respiratory and enteric tracts, proximal renal tubules, vascular endothelium, and on B cells, macrophages and activated T cells (Godfraind, *et al.*, 1995; Coutelier, *et al.*, 1994). CEACAMs are homophilic cell adhesion proteins that can play a role in signalling (Turbide, *et al.*, 1991; Huber, *et al.*, 1999). A CEA-related murine pregnancy specific glycoprotein called bCEA also has receptor activity for MHV-JHM (Chen, *et al.*, 1995). Several human CEACAM glycoproteins serve as receptors for virulent strains of *Neisseria meningitidis, Neisseria gonorrhoeae,* and *Hemophilus influenzae* (Virji, *et al.*, 1999; Virji, *et al.*, 2000).

3. MECHANISM OF RECEPTOR-DEPENDENT, TEMPERATURE-DEPENDENT VIRUS NEUTRALIZATION

Purified, soluble, anchorless murine CEACAM glycoproteins with a 6 histidine tag can neutralize the infectivity of MHV at 37^{o}C in a concentration-dependent manner (Zelus, *et al.*, 1998; Kubo, *et al.*, 1994). We

found that the receptor isoforms had different virus neutralizing activities and that MHV strains differed in the specificity of receptor interactions. We have used two complementary approaches to study the mechanism of temperature-dependent, receptor-dependent virus neutralization: a liposome flotation assay to detect changes in the hydrophobicity of virions, and a protease susceptibility assay to identify the domain of the viral spike protein that undergoes conformational change.

3.1 Liposome Flotation Assay for Conformational Change in S

We investigated whether binding at 37°C of soluble receptor glycoproteins made MHV-A59 virions more hydrophobic so that they would bind to liposomes and float up in a sucrose density gradient. The location of virions in the gradients was determined by immunolabeling of gradient fractions or by assaying for ^{3}H-uridine label from RNA in virions. Table 1 shows that binding of the soluble CEACAM1a receptor with 4 Ig-like domains to MHV-A59 virions at 37°C and pH 6.5 markedly increased the hydrophobicity of virions. No increase in hydrophobicity was observed following incubation without receptor, or at 4°C, pH 6.5, with receptor.

We previously used reactivity with anti-S monoclonal antibodies to show that incubation of MHV-A59 virions at 37°C and pH 8.0, but not pH 6.5, induced a conformational change in the availability of epitopes on the viral S protein (Weismiller, *et al.*, 1990; Sturman, *et al.*, 1990). Therefore, we performed the liposome flotation assay on MHV-A59 virions that had been incubated at 37°C for 30 minutes at pH 8.0 without added soluble receptor. As summarized in Table 1, we found that the MHV-A59 virions increased in hydrophobicity when treated at pH 8.0, 37°C for 30 minutes.

Table 1. Receptor binding or pH 8.0 at 37°C increases hydrophobicity of MHV-A59 Virions*

Incubation Mix	pH	Temperature (°C)	Location of virions in gradient	Hydrophobicity
MHV-A59 + LS	6.5	4	Bottom	Unchanged
		37	Bottom	Unchanged
MHV-A59 + CEACAM1a + LS	6.5	4	Bottom	Unchanged
		37	**Top**	**Increased**
MHV-A59 + LS	8.0	4	Bottom	Unchanged
		37	**Top**	**Increased**

* LS indicates liposomes

3.2 Protease Susceptibility Assay for Conformational Change in S Glycoprotein

We previously showed that incubation of MHV-A59 virions with high concentrations of trypsin cleaved the viral S glycoprotein only at one of many potential trypsin cleavage sites, and that this cleavage was associated with increased cell fusion activity of S (Sturman and Holmes, 1977; Frana, *et al.*, 1985). After incubating MHV-A59 virions for 30 minutes at 37°C, pH 6.5, with soluble MHV receptor CEACAM1a, the mixture was chilled and incubated at 4°C for 30 minutes with 10 ug/ml trypsin. Analysis of the viral S protein and its fragments on SDS-PAGE gels by immunoblotting with monoclonal anti-S antibodies showed that the S2 domain of the spike glycoprotein was proteolytically degraded, while the S1 domain that binds to the receptor was more resistant to protease degradation. Similarly, immunoblotting with monoclonal antibodies showed that S2, but not S1, was degraded after incubation of virions for 30 minutes at 37°C, pH 8.0, without soluble receptor, followed by incubation with trypsin for 30 minutes at 4°C (Table 2). These observations from the protease susceptibility assay indicate that a specific conformational change in the S2 domain of the viral spike glycoprotein can be triggered either by incubation with soluble receptor at pH 6.5, 37°C, or by incubation without receptor at pH 8.0, 37°C.

Table 2. Receptor binding or pH 8.0 at 37°c makes S2 protein of MHV-A59 virions susceptible to degradation by trypsin at 4°c.

Incubation Mix	pH	Temperature (°C)	S1 Protein Degraded?	S2 Protein Degraded?
MHV-A59	6.5	4	No	No
		37	No	No
MHV-A59 + CEACAM1a	6.5	4	No	No
		37	No	**Yes**
MHV-A59	8.0	4	No	No
		37	No	**Yes**

These experiments show that the S glycoprotein of MHV-A59 has a dynamic structure that undergoes pre-programmed conformational change(s) following binding to receptor at 37°C or exposure to alkaline pH at 37°C. These changes are likely to be important for the functions of the S protein including fusion of the viral envelope with cell membranes and/or virus-induced cell fusion.

3.3 Alternative Trypsin Cleavage Site in H716D S Glycoprotein

Viruses isolated from persistently infected glial cells had a H716D mutation adjacent to the cleavage site in the MHV-A59 spike glycoprotein and had a small plaque, delayed fusion phenotype on the 17 Cl 1 line of murine fibroblasts (Gombold, *et al.*, 1993). Targeted RNA recombination was used to introduce this mutation into the genome of wild type MHV-A59 (Kuo, *et al.*, 2000; Weiss and Hingley, in preparation). We studied the effects of soluble receptor at 37°C on the hydrophobicity of mutant virions and on trypsin cleavage of the viral S protein. We found that this virus, which differs from wild type MHV-A59 by only one amino acid, behaved quite differently from the wild type virus in both the liposome flotation assay and the protease assay. The H716D mutant virions floated part way to the top of the gradient at 4°C, pH 6.5, even without any soluble receptor. The protease cleavage assay detected a conformational change in S of H716D that was dependent upon soluble receptor and 37°C, or pH 8.0 and 37°C. As expected, trypsin treatment of H716D virions did not generate S1 and S2 peptides of approximately 90 kDa. In the presence of the receptor or pH 8.0 at 37°C, H716D S protein was cleaved at a new site to form a peptide of approximately 125 kDa. Immunoblotting with mapped monoclonal antibodies to S showed that this peptide contains epitopes in S1 and the N-terminal region of S2. This alternative or secondary cleavage site in S that is exposed by treatment with soluble receptor or pH 8.0 at 37°C may facilitate fusion of the viral envelope with host cell membranes, and/or for virus-induced cell fusion. The small plaque, delayed fusion phenotype of this virus may be due to the effect of the mutation in S on the dynamics of the mutant S glycoprotein.

4. ROLE OF S GLYCOPROTEIN IN ALTERED SPECIES SPECIFICITY OF MHV FROM PERSISTENTLY INFECTED CELLS

Coronaviruses can cause persistent infection *in vitro* and *in vivo*. Persistent infection of murine cell lines with MHV results in a carrier culture that is characterized by downregulation of the expression of the MHV receptor CEACAM1 and selection of small plaque, sometimes temperature

sensitive viruses, many of which have mutations in the S glycoprotein (Holmes and Behnke, 1981; Sawicki, *et al.*, 1995; Schickli, *et al.*, 1997; Lavi, *et al.*, 1987; Gombold, *et al.*, 1993; Gallagher, 1991; Chen and Baric, 1996). A small plaque virus (MHV/BHK) isolated from murine cells persistently infected with MHV-A59 utilizes the CEACAM1a receptor less efficiently than wild type virus (Schickli, *et al.*, 1997). Several other MHV isolates from persistently infected murine cells also have a broadly extended host range and can infect cell lines from hamsters, rats, monkeys, humans, and/or cats in addition to mice (Schickli, *et al.*, 1997; Baric, *et al.*, 1997; Baric, *et al.*, 1999). Multiple mutations were identified in the S proteins of these viruses. MHV/BHK virus differed markedly from the parental MHV-A59 virus in the liposome flotation and protease susceptibility assays. The marked conformational changes induced in MHV-A59 S protein by soluble receptor at 37°C were not observed for MHV/BHK, and the S protein of MHV/BHK showed a conformational change at pH 5.0 and 37 °C, rather than pH 8.0 like MHV-A59.

We co-infected murine cells with wild type MHV-A59 and with the MHV/BHK virus, and selected recombinants that grew in hamster cells at 39.5°C. These recombinant viruses were characterized for plaque morphology, cell fusing activity, reactivity with anti-S monoclonal antibodies, restriction fragment pattern in the S gene, susceptibility to protease cleavage of S, and host range. The S genes were sequenced to identify the site of recombination in S between the two parental genomes. The recombinant that had the least amount of the MHV/BHK S gene but still could be propagated in hamster cells had the N-terminal 1493 nucleotides of MHV/BHK with the remainder of the gene identical to MHV-A59. This region contained a 21 base insertion that encoded amino acids TRTKKVP as well as 24 point mutations upstream of the insert. Thus, the N-terminal approximately 410 amino acids of the S protein are responsible for the extended host range of MHV/BHK. This is similar to the observation that MHV-JHM binds to the CEACAM1 receptor on murine cells by the N-terminal 330 amino acids of the S glycoprotein (Kubo, *et al.*, 1994). Interspecies transmission of murine coronavirus depends upon mutations in the N-terminal domain of the S protein. It will be interesting to determine which of the mutations in the recombinant virus containing this domain of MHV/BHK is/are responsible for the extended host range of this virus. The evolution of coronaviruses to generate viruses that can infect and become adapted to new hosts probably depends on selection of mutations in this region of the S protein. How likely a receptor-jumping event is to occur in nature may depend upon how many mutations are required to recognize a receptor in the new host and what selective pressures in growth of the virus favor the replication of the variant virus over the parental virus.

ACKNOWLEDGEMENTS

The authors are grateful for the excellent technical assistance of Fenna Phibbs. Isabel Laparc Goffart selected the H716D mutant. This work was funded by NIH grants AI 25231NS30606 and NS 21954.

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