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Rapid Communication

Heparan sulfate is a binding molecule but not a receptor for CEACAM1-independent infection of murine coronavirus

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Abstract

A highly neurovirulent mouse hepatitis virus (MHV) JHMV strain (wt) with receptor (MHVR)-independent infection activity and its low-virulent mutant srr7 without such activity were found to attach to MHVR-negative, non-permissive BHK cells. To identify the molecule that interacts with JHMV, we focused on heparan sulfate (HS) since it works as a receptor of a mutant MHV-rec1 that infects in an MHVR-independent fashion. The present study indicates that HS interacts with both wt JHMV and srr7 but it does not function as an entry receptor as it apparently does for MHV-rec1. Furthermore, HS failed to serve as an entry receptor in the MHVR-independent infection of wt JHMV, indicating that HS is not a host factor that wt JHMV utilizes in an MHVR-independent infection.

Keywords: MHV; Coronavirus; Heparan sulfate; MHVR-independent infection; Virus receptor

Introduction

The highly neurovirulent JHMV strain of MHV is able to spread from cells infected via the receptor for MHV (MHVR), a carcinoembryonic cell adhesion molecule 1 (Dveksler et al., 1991), to cells without MHVR (MHVR-independent infection) (Gallagher et al., 1992; Taguchi and Matsuyama, 2002), while a mutant srr7 (soluble-receptor-resistant mutant 7) isolated from the JHMV cl-2 strain (wt JHMV) because of its resistance to inactivation by soluble form of MHVR (soMHVR) lacks this ability (Taguchi and Matsuyama, 2002). The mutation responsible for the srr7 phenotype was mapped to S2 (Saeki et al., 1997). MHVR-independent infection is attributed to a unique feature of the S protein of wt JHMV, namely the labile association of S1 with S2. Dissociation of S1 from S2 triggers a conformational changes in S2 and facilitate virus-cell membrane fusion (Gallagher, 1997; Krueger et al., 2001; Matsuyama and Taguchi, 2002). A key condition for this infection may be that the dissociation of S1 takes place in close proximity to MHVR-negative cells, so that the fusion peptide is exposed and penetrates into the adjacent cell membrane. We found that both wt JHMV and srr7 attached to MHVR-negative cells (Watanabe et al., 2006) and infection could be activated by the addition of soMHVR, indicating that wt JHMV and srr7 may bind to molecules other than MHVR. Mutants of the A59 strain of MHV that arose during persistent infection of cell expressing MHVR (Sawicki et al., 1995) were reportedly able to infect MHVR-negative cells (Baric et al., 1999, 1997; Schickli et al., 1997). One of the mutants, MHV-BHK, utilized heparan sulfate (HS) as a receptor (de Haan et al., 2005). This virus has three copies of the putative HS-binding motif in its S protein: one in the S1 as a 7-amino acid insertion that is not present in the original MHV-A59, one in the cleavage site and one in the S2 subunit (de Haan et al., 2005), as illustrated in Fig. 1. These binding motifs are thought to make it possible to use HS as an attachment/entry receptor. There is one copy of the HS-binding motif adjacent to the cleavage site in the S protein of both wt JHMV and srr7 (Fig. 1). This suggests that HS might also interact

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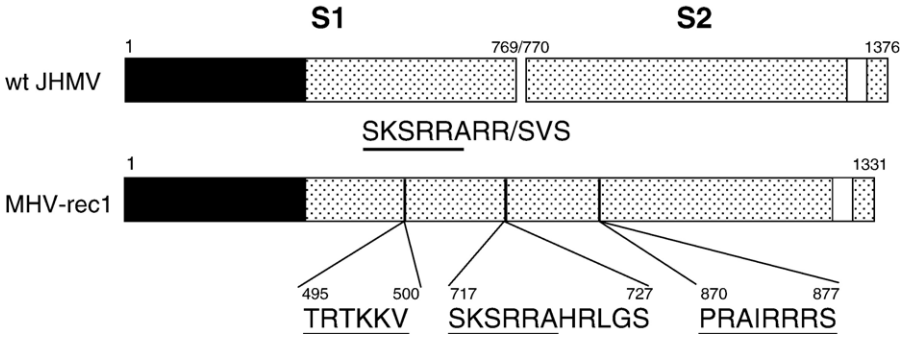

Fig. 1. HS-binding sites on the S protein of wt JHMV and MHV-rec1. S proteins of wt JHMV and MHV-rec1 consisting of 1376 and 1331 amino acids, respectively, are depicted as boxes. The receptor binding site and transmembrane region are shown as closed and open boxes, respectively. Vertical lines in the boxes represent approximate locations of putative HS-binding motifs. Putative HS-binding consensus sequences (XBXBBX or XBXXBBBX; X=any amino acid, B=basic amino acid) are underlined. The S1/S2 cleavage site of wt JHMV is shown by a slash (/).

with JHMV. Thus, we have determined if JHMV, both wt JHMV and srr7, can also utilize HS to attach to MHVR-negative cells. We also addressed whether or not HS is responsible for the MHVR-independent infection by the wt JHMV.

Results

JHMV attachment to MHVR-negative BHK cells

We have previously reported that highly neurovirulent wt JHMV could infect cells lacking MHVR if it was forced to attach to cells by spinoculation, i.e. infection by centrifugation at 3000 rpm for 2 h at 4 °C. We also found that both wt JHMV and srr7 attached to MHVR-negative BHK cells during a standard infection protocol, i.e. without spinoculation (Watanabe et al., 2006). To further confirm these findings, we inoculated 10^5 PFU, corresponding to ca. 10^7 copies of genome of those viruses onto MHVR-negative BHK cells and BHK-R1 cells, which express MHVR, without spinoculation. We then evaluated the copy number of the attached viruses by real-time PCR analysis. As shown in Fig. 2A, about $10^{5.5}$ and 10^5 copies of wt JHMV and srr7, respectively, attached to the BHK cells, which was about 50% of the binding to BHK-R1 cells. This finding clearly indicated that wt JHMV and srr7 attached, even onto MHVR-negative cells. To evaluate the infectivity of the attached virus, 50 nM of soMHVR was added to the culture of BHK cells inoculated with wt JHMV and srr7 and those cells were further incubated for 14 h at 37 °C. As shown in Fig. 2B, srr7 efficiently infected BHK cells in the presence of 50 nM of soMHVR but not at all without soMHVR. Infection of MHVR-negative cells with wt JHMV was greatly enhanced by soMHVR, although a very low level of infection was found without soMHVR (Fig. 2B), which was presumably due to an extremely inefficient MHVR-independent infection after ordinary protocol of infection. These results are in good agreement with our previous findings that soMHVR facilitated the infection of both wt JHMV and srr7 after adsorption onto MHVR-negative cells (Watanabe et al., 2006), suggesting that some molecule(s) on the cell surface other than MHVR allow the attachment of both wt JHMV and srr7.

Binding of JHMV to HS on the cell surface

HS is the major glycosaminoglycan (GAG) found on most cells and was recently reported as an entry receptor for MHV-BHK, a strain that has an extended host range and infects MHVR-negative cells (de Haan et al., 2005). Because JHMV also has one potential HS-binding site, we evaluated the contribution of HS to wt JHMV and srr7 attachment to the cell surface by treating the cells with heparinase. As shown in Fig. 3A, heparinases reduced cell surface HS effectively as shown by FACS analysis, but had little effect on the level of MHVR of BHK-R1 cells. To determine the effect of removing HS on wt JHMV and srr7 attachment of BHK and BHK-R1 cells, the cells, either treated with heparinases or left untreated, were then inoculated with 10^7 copies of viruses and incubated for 1 h at 4 °C. After removal of unattached virus by washing with PBS, cell-associated total RNA was extracted, and the number of viral genomes was measured by real-time PCR. The data in Fig. 3B show that heparinases reduced viral attachment by approximately one-half on both BHK and BHK-R1 cells, suggesting

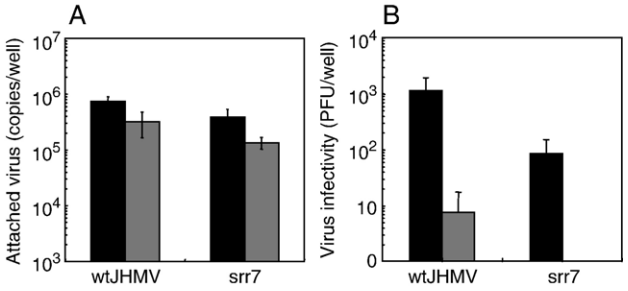

Fig. 2. Binding of JHMV to BHK and BHK-R1 cells. (A) 10^5 PFU (ca. 10^7 genome copies) of wt JHMV and srr7 were inoculated onto BHK-R1 (black column) and BHK (gray column) cells and incubated at 4 °C for 1 h. After 3 washes, total RNA was recovered from cells and the copy number of attached virus was estimated by real-time PCR. (B) BHK cells infected with serially diluted 10^5 PFU of wt JHMV or srr7 as described in (A) were further incubated for 14 h in the presence (black column) or absence (gray column) of 50 nM soMHVR. The number of plaque was counted after staining with crystal violet. Error bars represent standard deviations of the results of three independent experiments.

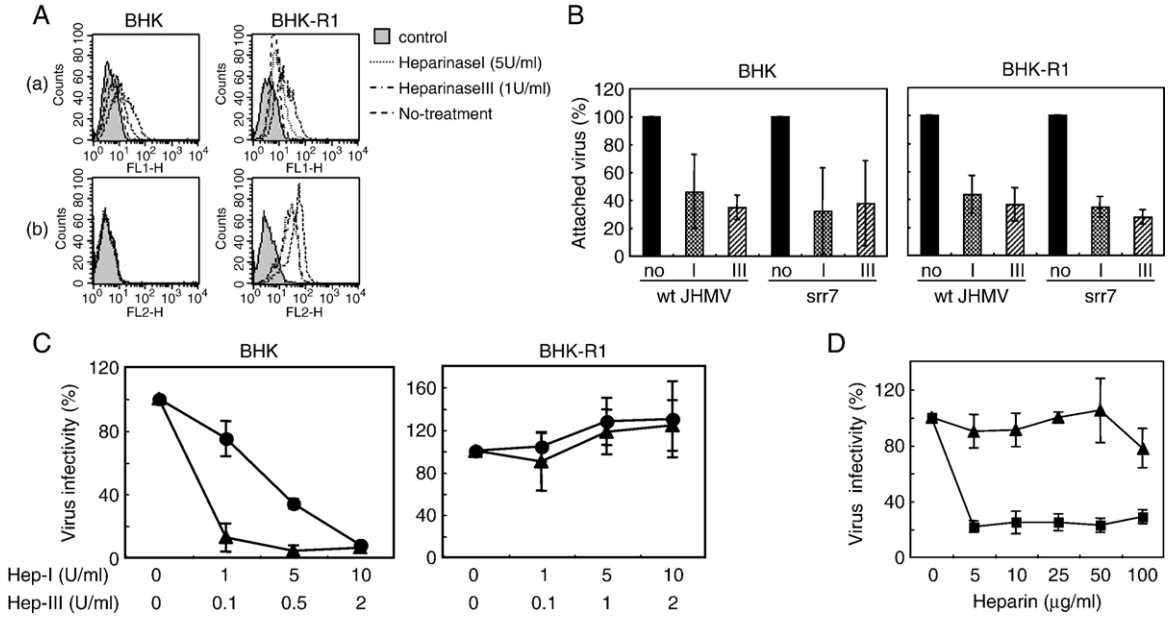

Fig. 3. Effects of heparinase (A–C) and heparin (D) treatment on virus attachment to BHK and BHK-R1 cells. (A) Effect of heparinase treatment on the amount of cell surface HS (a) and MHVR (b). Cells were treated with heparinase I (5 U/ml) or III (1 U/ml) at 37 °C for 1 h, and treated cells were stained with anti-HS or anti-MHVR MAbs followed by fluorescence-conjugated secondary antibodies. Fluorescence intensities were analyzed by FACSCalibur. (B) BHK cells were treated with either heparinase I (10 U/ml) or III (2 U/ml) at 37 °C for 1 h. Those cells were then inoculated with ca. 10^7 copies of JHMVs and incubated at 4 °C for 1 h. The copy number of cell-attached virus was estimated by real-time PCR using total RNA extracted from the infected cells. Values are represented as relative % against the copy number of viruses attached to cells that were not treated with heparinase. Error bars represent standard deviations of the results of three independent experiments. (C) Effect of heparinase treatment on srr7 attachment as examined by soMHVR-mediated infection. BHK and BHK-R1 cells were treated by heparinase I (Hep-1) (▲) or III (Hep-III) (●) at various concentrations (U/ml) at 37 °C for 1 h and were inoculated with 2×10^4 and 200 PFU of srr7, respectively. Cells were further incubated for 15 h in DMEM containing 1% FBS in the presence (BHK) or the absence (BHK-R1) of 50 nM soMHVR. The number of plaque was counted after staining with crystal violet. Error bars represent standard deviations of the results of three independent experiments. (D) Competition of the srr7 attachment by heparin. 10^4 or 200 PFU of srr7 was mixed with heparin and incubated at 4 °C for 1 h and mixtures were inoculated onto BHK (■) and BHK-R1 (▲) cells, respectively. Cells were incubated for an additional 14 h at 37 °C in DMEM containing 1% FBS in the presence (BHK) or absence (BHK-R1) of 50 nM soMHVR. The number of plaque was counted after staining with crystal violet. Error bars represent standard deviations of the results of three independent experiments.

that about 50% of JHMV attached to target cells via HS, irrespective of the presence or absence of MHVR on the cell surface.

We further examined whether JHMV bound to cells via HS is infectious or not. BHK and BHK-R1 cells treated with heparinase I or III were inoculated with 2×10^4 and 200 PFU of srr7, respectively, and incubated for 15 h in the presence of soMHVR for BHK and in its absence for BHK-R1 cells to evaluate infectivity. As shown in Fig. 3C, virus infection of BHK cells was reduced by heparinase treatment in a heparinase concentration-dependent manner. At the highest concentration, a 70–90% reduction was observed, when compared to untreated cells. Together with the data shown in Fig. 3B that the binding of srr7 is reduced by heparinase treatment, the data in Fig. 3C suggest that srr7 bound HS in a physiologically active form since addition of soMHVR facilitated the infection to cells to which srr7 bound. The infection was not reduced in MHVR-positive BHK-R1 cells by heparinase treatment (Fig. 3C), indicating that HS does not influence the infection by srr7 via MHVR.

We have additionally examined the binding of srr7 with HS using heparin. The pretreatment of viruses with heparin with the same disaccharide-repeating units as HS can generally block virus infection when cell surface HS contributes to their attachment/infection (Liu and Thorp, 2002). We mixed srr7 with heparin and incubated the sample at 4 °C for 1 h before inoculation of BHK or BHK-R1 cells. Infection of BHK cells was examined in the presence of soMHVR. Heparin reduced soMHVR-mediated infection of BHK cells by srr7 at a concentration of 5 μg/ml or higher (Fig. 3D), suggesting that HS is the molecule that interacts with srr7 to attach it to the cell surface. There was no effect of heparin on virus infection of BHK-R1 cells. This suggested that the region of S protein responsible for heparin binding is different from that required for MHVR binding. In combination with the observation that normal BHK cells, untreated with heparinase, are not at all permissive to srr7 infection, the above data collectively suggest that HS is a binding molecule but does not function as a receptor for infection nor enhance MHVR-mediated infection.

HS as a functional receptor for MHV-rec1 but not for JHMV

MHV-rec1 contains the same S protein as the virus isolated from MHVR-positive 17cl-1 cells persistently infected with MHV-A59 (Schickli et al., 1997;) and it utilizes HS as a receptor (de Haan et al., 2005). We evaluated the requirement of HS for infection by MHV-rec1 and JHMV. Although both viruses could infect and form a large syncytium on MHVR-positive DBT cells

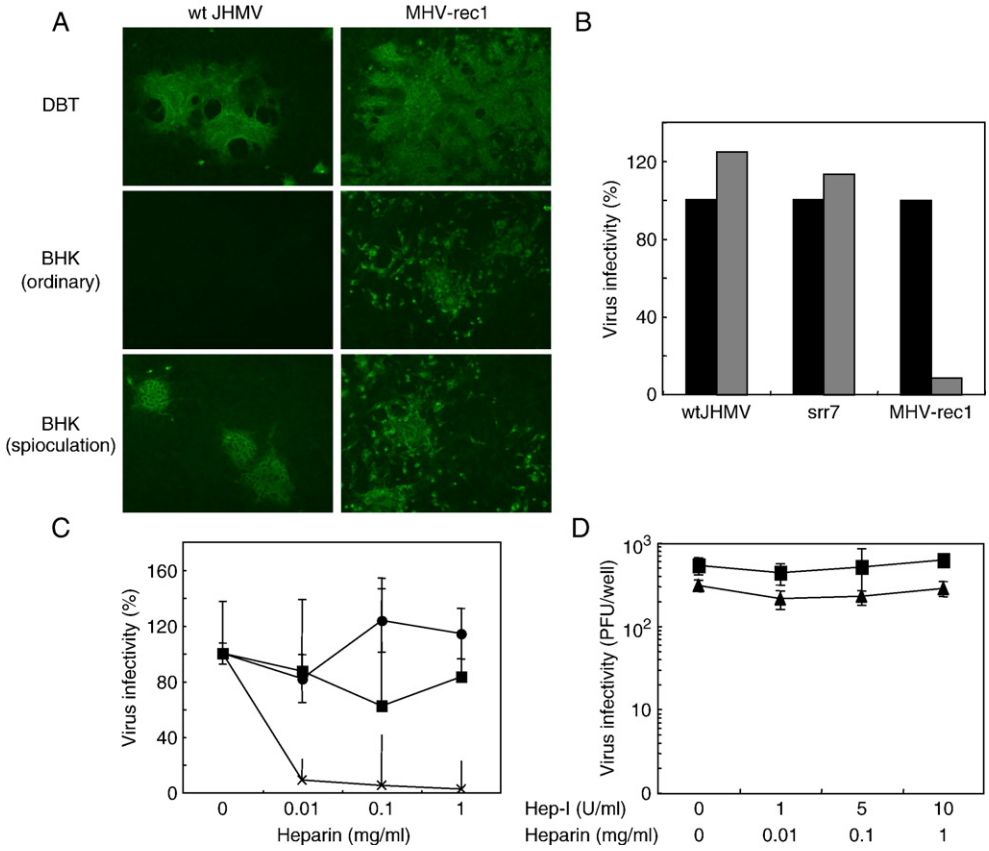

Fig. 4. HS-mediated infection by JHMV or MHV-rec1. (A) Comparative infection of wt JHMV and MHV-rec1 onto MHVR-positive DBT or MHVR-negative BHK cells. 100 and 1000 PFU of each virus were infected onto DBT (DBT) and BHK cells, respectively, with an ordinary infection method; 37 °C for 1 h adsorption (BHK ordinary). 1000 PFU of each virus was spinoculated onto BHK cells (BHK spinoculation). Those infected cells were further incubated at 37 °C for 15 h. Infection was evaluated by immunofluorescence using the mixture of anti-JHMV-S MAbs followed by anti-mouse antibodies conjugated with FITC. (B) DBT cells were treated (gray column) or untreated (black column) with 5 U/ml of heparinase I, inoculated with 100 PFU of wt JHMV, srr7 or MHV-rec1 and incubated at 4 °C for 1 h. After 15 h of incubation at 37 °C, cells were stained with crystal violet. Virus titers are represented as the relative percentage of the virus titer obtained after infection of untreated cells. The result is representative of three independent experiments. (C) Effect of heparin on the infection of JHMV and MHV-rec1 on DBT cells. Two hundred plaque-forming units of wt JHMV (■), srr7 (●) or MHV-rec1 (X) in 50 μl was mixed with an equal volume of heparin at a different concentration and the mixture was incubated at 4 °C for 1 h. The mixture was inoculated onto DBT cells, and cells were incubated at 4 °C for 1 h for virus adsorption. Cells were further incubated at 37 °C for 15 h and the number of plaque was counted after staining with crystal violet. Infectivity after treatment with heparin was shown as a percentage of the virus titer without heparin treatment. Error bars represent standard deviations of the results of three independent experiments. (D) Irrelevance of HS on MHVR-independent infection of wt JHMV. BHK cells were treated with various concentrations of heparinase I (Hep-1, U/ml) as described in the legend to Fig. 3C and then spinoculated with 10^4 PFU of wt JHMV. After 15 h of incubation at 37 °C, the number of plaque was counted (▲). 10^4 PFU of wt JHMV was mixed with various concentrations of heparin (mg/ml), as described in the legend to Fig. 3D, and the mixture was spinoculated onto BHK cells. The number of plaque was counted at 15 h after infection (■). Error bars represent standard deviations of the results of three independent experiments.

(Fig. 4A), there was a clear difference between MHV-rec1 and wt JHMV in the infection of MHVR-negative BHK cells. MHV-rec1 could infect BHK cells when they were inoculated by the ordinary infection method, whereas wt JHMV required spinoculation to infect efficiently (Fig. 4A).

The major difference among MHV-rec1, wt JHMV and srr7 in the use of HS for infection became apparent with the use of heparinase-treated DBT cells. DBT cells treated with heparinase had reduced amounts of HS by FACS analysis as described above (data not shown). Heparinase-treated or untreated DBT cells were infected with MHV-rec1, wt JHMV and srr7. Then, their infectivity was evaluated by counting the number of plaque that was formed 15 h after infection. As shown in Fig. 4B, wt JHMV and srr7 infection of DBT cells was not affected by the heparinase pretreatment, whereas MHV-rec1 infection was ca. 90% suppressed, confirming that HS serves as a fully functional receptor for MHV-rec1 infection but it does not for either wt JHMV or srr7.

To support the result obtained above, the infection-interference assay with heparin was performed. Two hundred plaque-forming units of wt JHMV, srr7 and MHV-rec1 was mixed with various concentrations of heparin and incubated at 4 °C for 1 h. The mixture was inoculated onto DBT cells and the number of plaque was counted after incubation for 15 h. As shown in Fig. 4C, almost 90% of MHV-rec1 infectivity was blocked by the heparin at a concentration of 0.01 mg/ml, whereas the infectivity of both wt JHMV and srr7 was not inhibited by heparin at a concentration of 1 mg/ml. These results clearly show the major difference between JHMV and MHV-rec1 on the usage of HS as a receptor for entry into cells.

Irrelevance of HS on MHMR-independent infection of wt JHMV

We also addressed whether HS works as an entry receptor for MHVR-independent infection by wt JHMV. BHK cells were treated with heparinase I and spinoculated with 10^4 PFU of wt JHMV. Then, the infection was monitored by plaque formation. As shown in Fig. 4D, no reduction of MHVR-independent infection was observed. We also confirmed this result by using heparin interference assay. 10^4 PFU of wt JHMV was treated with heparin at 4 °C for 1 h, and the infectivity was measured by spinoculation. The results also show no reduction in MHVR-independent infection by wt JHMV after its treatment with heparin. These two different approaches clearly indicated that HS is not involved in the MHVR-independent infection of wt JHMV, which is highly neurovirulent.

Discussion

In the present study, we showed that wt JHMV, and a mutant derived from this strain by selection for being resistant to inactivation by soMHVR, interacts with HS on the cell surface, but fails to utilize this molecule as an entry receptor. It was further shown that HS does not work as a receptor for MHVR-independent infection by wt JHMV, namely infection by spinoculation. We have also confirmed the previous observation that MHV-rec1, another MHV with MHVR-independent infection activity, is able to use HS as a functional receptor. The remarkable biological difference between wt JHMV and MHV-rec1 (Schickli et al., 1997) is that the latter infects cells without MHVR by the standard protocol of infection; however, the former fails to infect under such conditions. Wt JHMV must be forced to attach to cells by spinoculation when it executes the infection of MHVR-negative cells. This difference could be attributed to the nature of S protein in terms of its process of binding to HS; MHV-rec1 has three copies of the HS-binding motif, while wt JHMV S contains only one copy (Fig. 1). This difference could affect the strength of binding between HS and the S protein. It is possible that tight binding of MHV-rec1 with HS could trigger the conformational changes of the S protein and facilitate its infection, while weak binding of the wt JHMV S protein with HS fails to trigger the conformational changes and also, therefore, entry. In fact, de Haan et al. (2006) reported that cooperative involvement of two regions containing HS consensus sequences is important for the utilization of HS as an entry receptor by MHV-rec1.

Wt JHMV as well as the MHV-rec1, both of which infect in an MHVR-independent fashion, must have been selected under an environment with strong selection pressure. Before permissive cell lines become available for virus propagation, JHM strains of MHV have been maintained over the years by passage through mouse brains, where only microglia cells are positive for MHVR among the various types of cells (Nakagaki et al., 2005; Ramakrishna et al., 2004). To survive in the brain, JHMV would have to have been selected during passage through the mouse brains because of its unique ability to spread to a variety of MHVR-negative cells. Thus, the original virus may have been more like srr7 that infects truly in an MHVR-dependent fashion. Also, MHV-A59 mutant viruses that have a wide range of hosts were selected from unusual infection environments: some were forced to infect cells without MHVR (Baric et al., 1999; Baric et al., 1997) and the others were isolated from persistently infected cells with profoundly reduced MHVR expression (Sawicki et al., 1995; Schickli et al., 1997, 2004). Thus, the viruses that survived to grow in an environment of reduced or no receptor expression could have adapted to use another molecule as a receptor, as did MHV-rec1, while some others, like wt JHMV, established unique features to allow them to survive in the environment. Comparative studies on the S proteins of these viruses that infect in an MHVR-independent fashion will be of interest in terms of the molecular mechanism of receptor independence in viral infection.

The present study showed that HS works as a functional receptor for MHV-rec1, but not for wt JHMV, both of which infect in an MHVR-independent fashion. These findings suggest that HS does not play a role to make mice susceptible to MHVR-independent infection by wt JHMV. However, pathogenic studies on MHV-rec1 are very limited and little information is available on the participation of HS in MHV pathogenesis. Such studies will possibly provide new insights into MHV pathogenicity.

Materials and methods

Cells and viruses

BHK cells, BHK-R1 cells stably expressing MHVR (Matsuyama and Taguchi, 2000) and DBT cells were maintained in Dulbecco's minimum essential medium (DMEM: Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS, Sigma, St. Louis, MO) as previously reported (Taguchi and Matsuyama, 2002). A highly neurotropic JHMV cl-2 (defined as wt JHMV) (Taguchi et al., 1985), and a soluble-receptor-resistant mutant derived from wt JHMV, srr7 (Saeki et al., 1997), as well as MHV-rec1 derived from MHV-A59 (Schickli et al., 2004), were propagated and assayed on DBT cells. Viral infectivity is shown as plaque-forming units (PFU). Srr7 has a single amino acid mutation at position 1114 (Leu to Phe) of the S2 subunit of the S protein relative to wt JHMV (Saeki et al., 1997).

Heparinase treatment

Heparinase treatment was performed mostly as described previously (Klimstra et al., 1998). BHK and BHK-R1 cells were prepared in a 24-well culture plate (Falcon, Franklin Lakes, NJ) and were treated with Heparinase I (Sigma) and III (Sigma) dissolved in a buffer (10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% glucose, 1% FBS and 0.5% bovine serum albumin) for 1 h at 37 °C. Then BHK and BHK-R1 cells were chilled on ice and inoculated with 2×10^4 and 200 PFU of viruses, respectively, and further incubated for 1 h at 4 °C. After washing three times with phosphate buffered saline, pH 7.2 (PBS), cells

were incubated with DMEM containing 1% FBS for 14 h at 37 °C. Cells were fixed and stained with crystal violet, and the number of plaque was counted under light microscopy.

To infect the BHK cells, the culture was supplemented with soMHVR (50 nM in final concentration). The soMHVR used for this purpose consisted of only the N domain from the MHVR (Miura et al., 2004), which was expressed by recombinant baculovirus and purified by using its tag as described previously (Taguchi and Matsuyama, 2002).

Heparin competition assay

The heparin competition assay was performed as described previously (Klimstra et al., 1998). Viruses at 2×10^4 PFU (for BHK) or 200 PFU (for BHK-R1) in 50 µl were mixed with an equal volume of heparin (Sigma) and incubated for 1 h at 4 °C. BHK or BHK-R1 cells prepared as described above were inoculated with those mixtures and incubated for 1 h at 4 °C. Cells were washed in ice-cold PBS and incubated with DMEM supplemented with 1% FBS for a further 14 h at 37 °C. The number of plaque was obtained as described above. To confirm the infection of the BHK cells, soMHVR was added at 50 nM in a final concentration.

Flow cytometry

The level of HS or MHVR on the cell surface was evaluated by flow cytometry analysis as previously described (de Parseval and Elder, 2001). Cells were incubated with anti-HS MAb F58-10E4 (Seikagaku corporation, Tokyo, Japan) alone or in combination with anti-MHVR MAb CC1, a gift of Dr. K. Holmes. FITC-conjugated anti-mouse IgM (BD Pharmingen, San Diego, CA) and phycoerythrin (PE)-conjugated anti-mouse IgG1 (Jackson ImmunoResearch, West Grove, PA) were used to detect F58-10E4 and CC1, respectively. The fluorescence intensity was measured using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) and analyzed by CellQuest software.

Quantitative estimation of viral RNA by real-time PCR

Attachment of inoculated viruses onto cells treated with heparinase I, heparinase III or untreated cells was estimated by real-time PCR as described previously using a LightCycler RNA Master mix (Loche Diagnostics, Mannheim, Germany) (Watanabe et al., 2006).

Spinoculation

Spinoculation was done as described previously (Watanabe et al., 2006). Cells prepared in a 24-well plate were inoculated with viruses in 300 µl DMEM, centrifuged at 3000 rpm (1750×*g*) for 2 h at 4 °C and incubated with DMEM supplemented with 1% FBS for an additional 14 h at 37 °C. soMHVR was added onto the cells infected with srr7. The number of plaque was counted after staining with crystal violet as described above.

Acknowledgments

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