Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Communication between S1N330 and a Region in S2 of Murine Coronavirus Spike Protein Is Important for Virus Entry into Cells Expressing CEACAM1b Receptor

Shutoku Matsuyama and Fumihiro Taguchi

National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan

Received November 9, 2001; accepted January 24, 2002

The soluble receptor-resistant (srr) mutants, srr7 and srr11, isolated from a murine coronavirus, mouse hepatitis virus (MHV) JHMV, have an amino acid mutation at positions 1114 (Leu to Phe) and 65 (Leu to His), respectively, in the spike (S) protein. These mutants failed to efficiently infect BHK cells expressing CEACAM1b (BHK-R2), due to their low entry into this cell line, although they infected cells expressing CEACAM1a (BHK-R1) in a manner similar to that of wild-type (wt) JHMV cl-2 (Matsuyama and Taguchi, Virology 273, 80–89, 2000). Following the repeated passage of these mutants through BHK-R2 cells, viruses were no longer isolated from srr11-infected cells, while two distinct mutants, srr7A and srr7B, were obtained from srr7-infected cells. Srr7A and srr7B grew 2 log10 higher than srr7 and induced fusion in BHK-R2 cells, being similar to wt virus. In addition to the amino acid change at position 1114 that stemmed from parental srr7, srr7A and srr7B had mutations around position 280, corresponding to the third region of the S1N330 receptor-binding site (S1N330-III) common to all MHV strains examined thus far. Srr7A and srr7B S proteins showed high fusogenicity in both BHK-R1 and BHK-R2 cells, like the wt virus, while srr7Aa and srr7Ba S proteins, which had mutations in S1N330-III but not at amino acid 1114, exhibited profoundly reduced fusion activity in these cell lines. These findings suggest that communication between S1N330-III and the amino acid at position 1114 is important for efficient fusion activity in BHK-R2 cells. S1N330-III is a possible region in the S1 involved in viral entry into cells. © 2002 Elsevier Science (USA)

INTRODUCTION

Mouse hepatitis virus (MHV), a member of the coronavirus family, causes a variety of diseases with different organ tropisms and virulence, depending upon MHV strains. The major target organs of MHV are the liver, central nervous system, and intestines. The MHV virion has a genome of single-stranded, positive-sense RNA of about 31 kb in length which is packaged by a nucleocapsid (N) protein of about 50 kDa within the viral envelope. The virion envelope contains an integral membrane (M) protein of 20–23 kDa, envelope (E) protein of about 8 kDa, and spike (S) protein of 170–200 kDa. Some strains of MHV have an optional protein, hemagglutinin-esterase protein (60–65 kDa) on the envelope as well. The S protein constitutes the projection or peplomer on the virion surface. After being synthesized and modified by glycosylation, the S protein is cleaved into N terminal S1 and C terminal S2 subunits by host cell-derived proteases (Sturman et al., 1985). S1 comprises the outermost, knob-like structure of the projection, and the membrane-anchored S2, the stem-like structure beneath the knob (De Groot et al., 1987). In the middle of S1, there is a highly variable region spanning about 150 amino acids, called the hypervariable region (HVR). A number of viruses and mutants have amino acid mutations or deletions of variable lengths in this region relative to the largest S protein of MHV-JHMV (La Monica et al., 1991; Parker et al., 1989; Taguchi et al., 1985; Wang et al., 1992).

S protein has several important biological functions. The N terminal region, consisting of 330 amino acids of S1 (S1N330), is responsible for the binding to the receptor, and the receptor-binding site is formed by a conformational structure (Kubo et al., 1994). In S1N330, there are three regions common to all MHV strains, S1N330-I, II, and III, of which I and II are involved in receptor-binding activity (Suzuki and Taguchi, 1996), while the function of S1N330-III is not yet clarified. Amino acid Thr at position 62, located in S1N330-I, and its neighboring residues, are assumed to be particularly important for receptor-binding activity (Suzuki and Taguchi, 1996). S protein is also involved in the viral entry into cells via fusion of the viral envelope and cell membrane (Collins et al., 1982; Taguchi et al., 1992). Several different regions in S2 are considered critical for this fusion activity (Gallagher, 1996; Luo and Weiss, 1998; Taguchi and Shimazaki, 2000). Cleavage events of S are not absolutely necessary for fusion activity (Stauber et al., 1993; Taguchi, 1993), and the S protein has epitopes for neutralizing antibodies and cytotoxic T cells (Castro and...
Perlman, 1995; Flory et al., 1993). The key to determining MHV virulence is believed to lie in the S protein (Dalziel et al., 1986; Fleming et al., 1986; Matsuyama et al., 2001; Phillip et al., 1999), and thus, it is critical to the early events of virus infection, as well as for the host animals to protect against MHV infection (Taguchi, 1999).

Several different proteins work as MHV receptors (Chen et al., 1995; Dveksler et al., 1991; Nedellec et al., 1994; Yokomori and Lai, 1992), among which CEACAM1 is the most functional receptor. Two allelic forms are known for CEACAM1, CEACAM1a (MHVR1) and CEACAM1b (MHVR2) (Beauchemin et al., 1999; Dveksler et al., 1993; Yokomori and Lai, 1992). MHVR1 is expressed in most mouse strains with a high susceptibility to MHV, while MHVR2 is derived from the SJL mouse strain resistant to MHV (Dveksler et al., 1993; Ohtsuka and Taguchi, 1997; Smith et al., 1984; Yokomori and Lai, 1992). MHVR1 is a 10- to 100-fold more functional receptor for MHV than is MHVR2 (Ohtsuka et al., 1996; Rao et al., 1997).

Mutant viruses resistant to neutralization by a soluble receptor (srr) have been isolated from various viruses. Generally, srr mutants fail to bind to a soluble receptor as efficiently as they bind to the membrane-anchored receptor (Colston and Racaniello, 1994; Kaplan et al., 1990; McKeating et al., 1991). However, some MHV srr mutants isolated in our lab bound to both soluble and membrane-anchored receptors as efficiently as the wt virus (Saeki et al., 1997). Thus, the mechanism of soluble receptor-resistance of those MHV mutants appeared to be different from that of other viruses.

Recently, we found that our srr mutants failed to efficiently infect cells expressing MHVR2 (BHK-R2), while they infected cells expressing MHVR1 (BHK-R1) in a fashion similar to the wt virus (Matsuyama and Taguchi, 2000). Inefficient srr infection in BHK-R2 cells was demonstrated to be due to low fusionability, i.e., inefficient entry into cell (Matsuyama and Taguchi, 2000). From those srr mutants, we tried to isolate viruses that grow well in BHK-R2, revertants in terms of growth capability in BHK-R2 cells, to see whether any other region in the S protein compensates for the inefficient fusogenicity caused by the mutations in srr mutants. In the present study, we show revertants successfully isolated from srr7. Their characterization suggests the importance of the communication between S1N330-I and a region in S2 for the fusogenicity on BHK-R2 cells.

RESULTS

Isolation of mutant viruses from srr7

Srr7 and srr11 with a mutation at amino acid position 1114 (Leu to Phe) and 65 (Leu to His), respectively, failed to grow as efficiently as the wt virus in BHK-R2 cells that constitutively express MHVR2 receptor (Matsuyama and Taguchi, 2000). To see whether some other amino acids can compensate for the defects caused by these mutations, we tried to isolate viruses that grow in BHK-R2 cells as efficiently as the wt virus by repeated passage of these mutants through the cell line. These mutants, 5–10 × 10^6 PFU as examined using DBT cells, were inoculated onto BHK-R2 cells and the cells were incubated for 24 h after infection. Viruses grown in cells were subjected to a subsequent cycle of infection onto BHK-R2 cells. We repeated this passage 20 times. As for srr11, syncytia ceased to form after three to four passages, and no infectious viruses were recovered from cells in the fifth to sixth passage in two independent experiments. In contrast, srr7 was successfully adapted to grow in BHK-R2 cells. During the first two to four passage levels, no remarkable syncytia were observed; however, with repeated passages, syncytia became visible that were similar to those of wt virus. After 20 passages, we isolated viruses by plaque purification using DBT cells. In two independent experiments, we obtained two different viruses, srr7A and srr7B, that grew in BHK-R2 cells more efficiently and produced larger syncytia than did the parental srr7. These viruses were detected after the 10th to 15th passage in BHK-R2 cells.

Biological characterization of srr7A and srr7B

Two viruses, srr7A and srr7B, isolated after the 20th passage, were compared with the parental srr7, as well as wt cl-2 in terms of the growth in BHK-R2 and BHK-R1 cells. Following infection at a multiplicity of infection (m.o.i.) of 0.1 in BHK-R2 cells, progeny appeared in the cells at 12 h postinoculation (p.i.) irrespective of the type of viruses used. However, the titers of srr7A and srr7B grown in this cell line were about 2 log10 higher than that of parental srr7 throughout the course of infection, but slightly lower (1 in log10) than the wt cl-2 titer. All of these viruses reached a plateau at 18 to 24 h p.i. There was no significant difference in the growth kinetics in BHK-R1 cells among these viruses (Fig. 1). We compared the size of the syncytia produced by these viruses. In BHK-R1 and BHK-R2 cells infected and incubated at 37°C for 12 to 15 h, we analyzed syncytia formed with NIH image software. As shown in Fig. 2A and B, the syncytia formed on BHK-R2 cells by srr7A and srr7B were significantly larger (P < 0.001) than those produced by the wt virus (P < 0.001) (Fig. 2B). These findings indicated that the fusogenicity of srr7A and srr7B reverted, if not perfectly, into the wt-like feature in BHK-R2 cells, but not in BHK-R1 cells, and correlated well with the growth capacity in BHK-R2 cells as shown in Fig. 1.
as well as BHK-R1 cells, we examined the infectious centers and plaquing efficiencies of wt and mutant viruses. An infectious center assay demonstrated that srr7A and srr7B infected BHK-R2 cells with an efficiency similar to that of the wt virus rather than to srr7, whereas there was no remarkable difference in infection of BHK-R1 cells among these four viruses (Fig. 3). Also, the plaquing efficiency of those two mutants was similar to that of wt virus, but far higher than srr7 in BHK-R2 cells, although there was no difference in BHK-R1 cells (data not shown). These results demonstrated that srr7A and srr7B were more similar to the wt virus than to srr7 in terms of growth and cytopathic effects on BHK-R2 cells. Thus, those mutants were defined as phenotypical revertants of srr7, and those revertants were revealed by a virus overlay protein blot assay to bind to MHVR1 or MHVR2 as efficiently as wt cl-2 and srr7 (data not shown).

To compare the amino acid sequences of these revertant S proteins with those of wt and srr7, we directly sequenced the cDNA to the S genes of these viruses amplified by RT-PCR, as described in Materials and Methods. Amino acid sequences of these S proteins, as deduced from nucleotide sequences, contained a mutation at position 286 (Ser to Ile) in srr7B and two mutations at positions 278 (Ile to Leu) and 461 (Ser to Thr) in srr7A (Table 1). Relative to wt, these two revertants had a mutation as well at position 1114 (Leu to Phe), which stemmed from the parent srr7 (Table 1). Mutated amino acids 286 and 278, found in srr7B and srr7A, respectively, were revealed to lie in the third common region of S1N330 (S1N330-III), consisting of amino acids 278 to 288, which was shown to be one of three conserved regions at the receptor-binding site (Suzuki and Taguchi, 1996). The deduced sequence results suggested that amino acid mutations in S1N330-III found in both revertants are important for the phenotypic reversion of srr7 to the wt virus. They also suggested that inefficient infection of srr7 in BHK-R2 cells caused by the mutation at 1114 of the wt virus S protein could be compensated for by the additional mutation in S1N330-III.

Fusogenicity of mutant S proteins in BHK-R2 cells

Wt S protein was demonstrated to induce large syncytia in BHK-R2 cells, while srr7 S protein failed to do so, which resulted in a difference in efficiency of infection between wt virus and srr7 in BHK-R2 cells (Matsuyama and Taguchi, 2000). We have compared the fusion activity of srr7A and srr7B in BHK-R2 cells with that of srr7 and the wt cl-2. Srr7A and srr7B S genes were inserted into a pTarget vector for expression, as described in Materials and Methods. These vectors and those containing wt S and srr7 S genes were transfected into BHK-R2 or BHK-R1 cells, and their fusogenicity was examined by using recombinant vaccinia virus vTF7.3 to express T7 RNA polymerase. Transfection efficiencies of these vectors were assessed by the activity of luciferase expressed from the cotransfected plasmid. As shown in Figs. 4A and 4C, the wt S showed strong fusion activity, while srr7 S failed to induce efficient fusion in BHK-R2 cells, as reported previously (Matsuyama and Taguchi, 2000). However, both srr7A and srr7B S proteins induced fusion in BHK-R2 cells similar to that induced by the wt virus rather than to that induced by srr7 (Figs. 4A and 4C). To see which of two mutations in the srr7A S protein is important for fusion activity, we have prepared vectors containing a single mutation found in srr7A. Srr7A S with a mutation in S1N330-III alone (srr7A-330III) displayed fusion activity similar to that of the original srr7A, while srr7A S protein with a mutation at position 461 (srr7A-HVR) had lost fusion activity (Fig. 4B), indicating that mutation in S1N330-III is responsible for fusion activity. The kinetics of fusion formation of srr7A and srr7B in BHK-R2 cells was also similar to that of wt virus; syncytia started to appear at 4 to 5 h after vTF infection and spread to most cells by 15 h. However, syncytia by srr7 were very restricted even at 15 h after infection. In contrast, all of these S proteins induced fusion extensively in BHK-R1 cells, and most cells were included into syncytia at 15 h after infection (Fig. 4A). These results indicated that the mutation in S1N330-III enhanced the fusogenicity of the S protein, which had been impaired by a mutation at amino acid 1114. This result suggested either that the mutation in S1N330-III enhances the fusion activity independent of amino acid 1114 or that the combination of these two regions located a long distance apart is critical for sufficient fusogenicity in BHK-R2 cells. To see which is correct, we have constructed srr7Aa and srr7Ba S genes without a mutation at amino acid 1114, designated them srr7Aa and srr7Ba, respectively, and examined their fusogenicity. Srr7Aa and srr7Ba S proteins were unable to induce fusion in BHK-R2 cells as
well as in BHK-R1 cells (Fig. 4A). Inability of srr7Aa and srr7Ba S proteins to induce syncytia was revealed to be due to defects in oligomerization (data not shown). The difference in fusogenicity in BHK-R1 and BHK-R2 cells shown in Fig. 4 did not appear to result from the difference in the amounts of S proteins expressed, because, as shown in Fig. 5, there was no apparent difference in the amounts expressed. It was noted that S2 with a mutation at amino acid 1114, as seen in srr7, srr7A, and srr7B S proteins, moved slightly faster than the S2 without mutation, although these S2 proteins did not differ in molecular weight (Fig. 5). All of these S proteins, irrespective of their fusogenicity, were similarly oligomerized and transported onto the cytoplasmic membrane (data

FIG. 2. Syncytia formed by wt cl-2 and srr mutants. (A) Syncytia produced by wt cl-2 and srr mutants in BHK-R2 cells. BHK-R2 cells prepared in 24-well plates were infected with about 100 PFU of either wt cl-2 or srr mutants and microscopically observed at 15 h after infection. (B) BHK-R1 and BHK-R2 cells cultured in 24-well plates were infected with about 100 PFU of virus, and syncytia were observed at 15 h after infection by microscopy. The size of more than 20 syncytia for each virus was analyzed by NIH image. The vertical line extending above each bar indicates the standard deviation.
not shown). These results suggested that the combination of these two regions played a critical role in fusion formation rather than that the mutation in S1N330-III enhanced the fusion activity irrespective of amino acid at 1114.

Taken together, the present study, which was summarized in Fig. 6, indicated that additional mutations in S1N330-III found in srr7A and srr7B enhanced the fusion activity of srr7 S protein to the level of the wt S protein. However, this enhancement was not simply caused by a mutation in S1N330-III alone, but was dependent on the combination of amino acids in S1N330-III and at 1114.

DISCUSSION

Two srr mutants, srr7 and srr11, showed heavily reduced growth in BHK-R2 cells, which originated from their reduced entry into the cell (Matsuyama and Taguchi, 2000). The former has a mutation in S2 and the latter in S1 (Saeki et al., 1997). To further analyze the region in the S protein important for fusion in BHK-R2 cells, we tried to isolate the revertant viruses from those two srr mutants in terms of growth in BHK-R2. After several passages through BHK-R2 cells, srr11 could no longer be detected. Srr11 could not adapt to grow in BHK-R2 cells, which implies that Leu at position 65 mutated to His in srr11 is critical for efficient infection in BHK-R2 cells. Because this amino acid is located within S1N330-1, one of the two most important regions for receptor-binding activity in S1N330 (Kubo et al., 1994; Suzuki and Taguchi, 1996), it could play a vital role in receptor binding.

As expected, the binding of srr11 to both BHK-R1 and BHK-R2 cells was slightly reduced relative to wt and srr7 (Matsuyama and Taguchi, 2000; Saeki et al., 1997). The inability of srr11 to adapt to BHK-R2 cells might be due to this low receptor-binding capacity, or to double defects in fusogenicity and receptor binding, as shown previously (Matsuyama and Taguchi, 2000; Saeki et al., 1997).

Srr7 could successfully be adapted to grow in BHK-R2 cells by repeated passage through this cell line. This biological change was accompanied by an amino acid change in S1. Srr7B had only one additional mutation in S1N330-III; however, srr7A contained two, one in S1N330-III and the other in HVR of S1. The mutation in the S1N330-III, but not that in the HVR, was revealed to be responsible for the acquisition of fusion activity by srr7A. Thus, the mutation in S1N330-III must be critical for conversion from the srr7-type virus, which is inefficient in infecting in BHK-R2 cells into the wt-like virus with efficient infection and fusion activity. However, the amino acid changes in S1N330-III alone did not enhance fusion activity, as seen for srr7Aa or srr7Ba, but a simultaneous mutation at position 1114 was inevitable. These findings strongly suggest that the combination of S1N330-III and the amino acid at 1114, presumably 1114 and its neighbors common to various MHV strains (Kunita et al., 1995; Luytjes et al., 1987; Parker et al., 1989; Taguchi et al., 1992; Yamada et al., 1997; Yamada and Yabe, 2000), determines the fusogenic feature of the S protein. It is not evident at present how S1 and S2

### TABLE 1

| Virus          | Substitution at the following position | Nucleotide | Amino acid |
|---------------|----------------------------------------|------------|------------|
|               | 832  | 857  | 1382  | 3340  | 278  | 286  | 461  | 1114  |
| wt (JHMV cl-2)| A    | G    | G     | C     | Ile  | Ser  | Ser  | Leu   |
| srr7          | —    | —    | —     | —     | —    | —    | —    | —     |
| srr7A         | C    | —    | C     | T     | Leu  | —    | —    | Thr   |
| srr7B         | —    | T    | —     | T     | —    | Ile  | —    | Phe   |

* Nucleotide and amino acid positions were numbered from the first ATG codon and methionine, respectively.

b —, Identity with the wild-type sequence.
FIG. 4. (A) and (B) Fusion activity of wt cl-2 and srr mutant S proteins in BHK-R1 and BHK-R2 cells. BHK-R1 and BHK-R2 cells were transfected with plasmids containing various S genes or plasmid alone and infected with vTF7.3. After 15 h incubation, cells were fixed with formaldehyde and stained with hematoxylin and eosin. (C) Fusion activity of the various S proteins as accessed by β-galactosidase activity. Target cells, either BHK-R1 or BHK-R2 cells, were infected with wt vaccinia virus, then transfected with plasmid pG1NT7β-gal and cultured in 96-well plates for 15 h. Onto these cells, effector cells, from the same cell line infected with vTF7.3 and transfected with pTarget vector containing either wt or mutant S genes, were overlaid and cultured for 7 h. The β-galactosidase activity expressed as a result of fusion of effector and target cells was measured.
interact to execute fusion, but it is not surprising in coronavirus S protein that two regions a long distance apart in primary structure cooperatively interact to execute an important biological function. Interaction or proximal localization in the tertiary structure of S1 and S2 in the native S protein was suggested by Grosse and Siddell (1994), who isolated a MAb-resistant mutant with a mutation in S2 after neutralization by S1-specific MAb.

The present study demonstrated that a combination of S1N330-III and amino acid 1114 is important for MHV entry to cells expressing MHVR2. However, such a strict combination was not necessary for entry into cells expressing MHVR1. This difference could be accounted for by the difference in virus-binding ability between MHVR1 and MHVR2. The former has more than at least 100-fold higher binding ability than the latter, as examined by virus overlay protein blot assay and neutralization tests using soluble forms of these MHV receptors (Ohtsuka et al., 1996; Zelus et al., 1998). Tight binding of MHV to the receptor could compensate for the combination undesirable for efficient entry into cells found in sr77 S protein.

S1N330-III, consisting of 11 amino acids at positions 278 to 288 in S1, has been demonstrated to be perfectly conserved among the seven MHV strains examined (Suzuki and Taguchi, 1996). Furthermore, this region is also conserved in three newly isolated MHVs (Kunita et al., 1995; Yamada and Yabe, 2000), and such conservation is suggestive that S1N330-III retains some biological function critical for MHV replication. We suspected it to be involved in receptor binding, because it was located in S1N330, the receptor-binding domain. Site-directed mutagenesis analysis showed, however, that S1N330-III was the least involved in receptor-binding activity in the three conserved regions in S1N330 (Suzuki and Taguchi, 1996). S1N330-III of sr77A and sr77B contained a mutation at positions 278 and 286, respectively, relative to the wt or sr77 S protein; nevertheless, those S proteins equally bound and infected both BHK-R1 and BHK-R2 cells. This is also suggestive that S1N330-III is not important for receptor-binding activity. One may argue that sr77A and sr77B with a mutation in S1N330-III could have

| S1       | S2       | Fusion |
|----------|----------|--------|
| wt       | 278 286 461 | +      |
| sr7      |          | +      |
| sr7A     |          | + +    |
| sr7Aa    |          | +      |
| sr7A-330III | ↑  | + +    |
| sr7A-HVR |          | +      |
| sr7B     |          | + +    |
| sr7Ba    |          | - -    |

a.a. 278 : O→● (Ile→Leu)  a.a 461 : △→▲ (Ser→Thr)
a.a. 286 : O→● (Ser→Ile)  a.a 1114 : □→■ (Leu→Phe)

FIG. 5. Western blot analysis of the expressed S proteins. BHK-R1 and BHK-R2 cells transfected with plasmids harboring various S genes and infected with vTF7.3 as described above were lysed at 15 h after transfection. The amounts of S proteins in each lysate were examined by Western blotting using MAb specific to the S2 subunit and ECL.

FIG. 6. Summary of JHMV S protein structure and their fusion activity in BHK-R1 and BHK-R2 cells. Mutated amino acids in mutant S proteins were shown by closed marks, while original amino acids in the wt virus were displayed by open ones.
receptor-binding activity due to a second mutation at 1114 in S2, suggesting the possibility that S1N330-III is important for receptor binding, but the latter is unlikely, because the binding of the S protein to the receptor takes place with S1N330 alone, without the cooperation of the S2 subunit or other parts of S1 (Kubo et al., 1994). All of these factors could suggest that S1N330-III plays an important role not in receptor binding, but rather in viral entry into cells.

Several regions or amino acids in the S protein have been reported to be important for fusion activity (Gallagher, 1996; Gallagher et al., 1991; Luo and Weiss, 1998; Luo et al., 1999; Taguchi and Shimazaki, 2000). All of those, such as heptad repeats and amino acid position 1114, are located in the S2 subunit. Alterations from Leu to Arg at position 1114 resulted in the reduced fusion activity in DBT cells expressing MHVR1, suggesting the importance of this amino acid for fusion activity (Gallagher et al., 1991). We also described in the present study that srr7 with an alteration of the same amino acid from Leu to His has reduced fusion activity in BHK-R1, which strengthened the importance of 1114 Leu for fusion. A variant virus S protein of MHV-4 (JHVM) containing a mutation at position 1114, as well as two other mutations, showed pH-dependent fusion activity (Gallagher et al., 1991). The pH-independent fusion activity of srr7 and its revertants suggests no or minor involvement of 1114 Leu in the pH dependence of fusion activity.

Whereas a number of reports suggest the importance of S2 for fusion activity for viral entry into cells, there is little to suggest the importance of S1. Tsai et al. (1999) reported that a stretch of 12 amino acids in HVR is important for fusion activity. They compared the S protein of fusion-negative MHV-2 with that of fusion-positive MHV-JHM (Schmidt et al., 1987), and 12 amino acids deleted in MHV-2, but not in MHV-JHM, were identified as being responsible for fusion activity. However, this stretch is not important for virus entry into cells, because MHV-2 is able to infect cells. Recently, Krueger et al. (2001) reported that mutant JHVMs containing a deletion in the HVR have reduced fusion activity in MHV-dependent and -independent infections. We also described that JHMV sp-4 virus, with a 141-amino acid deletion in the HVR, has a reduced fusion activity on DBT cells with MHVR1, which was revealed by the slightly smaller plaques relative to wt cl-2 (Matsubara et al., 1991). Sp-4 also showed reduced fusion activity in MHV-independent infection (Taguchi et al., 1999). However, these viruses can infect and grow in cells expressing MHVR1 with an efficiency higher than or similar to wt virus, in spite of their low fusogenicity (Krueger et al., 2001; Taguchi et al., 1985; Matsubara et al., 1991). This clearly indicates that HVR is not a vital region in viral entry into cells. Taken together, HVR may influence fusion activity of MHV but it does not affect the potential for virus entry into cells.

In addition to the differing abilities of wt cl-2 and srr7 to infect BHK-R2 cells, we previously revealed another biological difference between them that infection spreads from wt-infected DBT cells to BHK cells deficient in MHVR (called MHVR-independent infection), while srr7 failed to spread in this fashion (Taguchi et al., 1999; Taguchi and Matsuyama, 2002). Srr7A and srr7B, revertants in terms of infective ability to BHK-R2 cells, are able to spread to BHK cells by MHVR-independent infection (data not shown), indicating that srr7A and srr7B are reverted in terms of MHVR-independent infection as well. These findings suggest that the mechanism to infect cells with MHVR2 has some similarity to that of MHVR-independent infection. We are currently studying the mechanism underlying MHVR-independent infection using wt, srr7 as well as two revertants from srr7.

The present study showed that double mutations, one in S1N330-III and the other at amino acid 1114, were important for fusogenicity in BHK-R2 cells. This suggests that these two regions interact with each other and cooperatively execute fusion formation, namely viral entry into cells. How the combination of S1N330-III and amino acid 1114 determines the fusion activity was not addressed in this study. It is reported for retroviruses and other viruses that the receptor-binding protein undergoes conformational change after binding to the receptor (Damico et al., 1998; Ikeda et al., 2000), which is an essential step for virus entry into cells, via virus–cell fusion. MHV S protein supposedly undergoes conformational change after binding to the receptor, which converts the S protein to fusion active phenotype. Reduced fusion activity of srr7 S protein in BHK-R2 cells could be due to the lack of conformational change after binding to the receptor. In contrast, the combination of these two regions in wt virus, srr7A and srr7B, may permit or promote the conformational changes of the S protein. Studies are currently in progress to examine these possibilities.

**MATERIALS AND METHODS**

**Cells and viruses**

The highly neurovirulent wt MHV-JHMV cl-2 (Taguchi et al., 1985), as well as srr7 and srr11 derived from cl-2 (Saeki et al., 1997), were propagated on DBT cells (Kumanishi, 1967). Newly isolated mutants, srr7A and srr7B, were propagated on DBT cells as well. DBT cells were also used to estimate the infectivity of MHV. BHK cells as well as BHK cells constitutively expressing MHV receptor MHVR1 (BHK-R1) or MHVR2 (BHK-R2) (Matsuyama and Taguchi, 2000) were used for MHV infection and expression of S proteins. BHK cells lack the MHV receptor and are thus nonpermissive to MHV infection. Recombinant vaccinia virus, vTF7.3, harboring the T7 RNA polymerase gene (Fuerst et al., 1986), kindly provided by B. Moss, was propagated and plaque-assayed on RK 13...
cells. All cell lines used in this study were grown in Dulbecco’s minimal essential medium (DMEM, Nissui, Tokyo) supplemented with 5% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY).

Isolation of mutant viruses

To select the mutants that grow efficiently in BHK-R2 cells, srr7 and srr11, 5–10 × 10^5 PFU as examined using DBT cells, were inoculated onto confluent BHK-R2 cells prepared in a 35-mm dish (Costar, Cambridge, MA). Both supernatants and cells were harvested at 24 h p.i. and subjected to three rounds of freeze–thawing. After centrifugation at 3000 rpm for 10 min, the clarified supernatants and cells were harvested at 24 h p.i. and prepared in a 35-mm dish (Costar, Cambridge, MA). Both DBT cells, srr7 and srr11, 5–10^5 PFU as examined using DBT cells, were inoculated onto confluent BHK-R2 cells prepared in 35-mm dishes. After culture for 15 h at 37°C in DMEM supplemented with 0.5% methylcellulose, the cells were stained with neutral red and plaques were counted.

Isolation and expression of S genes

DBT cells prepared in 60-mm dishes were infected with srr7A or srr7B at an m.o.i. of 1. Total RNA was isolated from those cells at 15 h after inoculation with an isogenic RNA extraction kit (Nippon Gene, Tokyo, Japan). Srr7A and srr7B S cDNAs were made by reverse-transcription (RT)-PCR from isolated RNA with a pair of primers as previously described (Matsuyama and Taguchi, 2000), and the PCR products were directly sequenced (Sanger et al., 1977). To obtain the mutant S genes for expression, the amplified DNAs were cloned into a commercial expression vector, pTarget (Promega, Madison, WI). As the S genes integrated into the vector contained some nucleotide differences relative to authentic mutant S genes, we have constructed the mutant S genes by replacing srr7 S gene with DNA fragments containing mutations specific for srr7A or srr7B. Because the wt and srr7 S genes previously constructed had three amino acid mutations (Matsuyama and Taguchi, 2000), compared to the originally reported S genes (Taguchi et al., 1992, 1995), we newly prepared the vectors that contain the authentic S genes, pTargetcl-2S and pTargetsrr7S. The pTargetsrr7S plasmid harboring the srr7 S gene downstream from the T7 promoter (Matsuyama and Taguchi, 2000) was cut with PfiM I at nucleotide 749 and Apa I at 1548 calculated from the first nucleotide of the S gene initiation codon. The resulting 800-bp fragment was replaced with the corresponding fragment of srr7A and srr7B. Srr7A and srr7B S genes without a mutation at amino acid 1114 were also constructed by replacing the fragment containing the mutation with the fragment of wt virus as described previously (Matsuyama and Taguchi, 2000). They were named pTargetsrr7Aa and pTargetsrr7Ba. We constructed srr7A containing only one mutation in S1N330-III, srr7A-330III, and that with a mutation at position 465, srr7A-HVR, by exchanging the srr7 and srr7A fragments cut with Sca I. Sca I cuts pTarget vectors at position 8412. Thus, digestion by Sca I results in two fragments; one is JHMV S gene 5’ fragment containing a mutated region in S1N330-II, srr7A-330II, and the other in pTarget vector at position 8412. Digestion by Sca I results in two fragments; one is JHMV S gene 5’ fragment containing a mutated region in S1N330-II alone (3.5 kb) and the other JHMVS 3’ fragment containing mutated HVR alone (6.3 kb). Srr7A-330II S is a chimera consisting of srr7A S 5’ 3.5 kb and srr7 3’ 6.3 kb fragments. The Srr7A-HVR S gene is composed of fragments of srr7 5’ 3.5 kb and srr7A 3’ 6.3 kb. The constructed plasmids were confirmed by sequencing (Sanger et al., 1977). S proteins of wt and mutants were statistically compared using Student’s t test.
expressed using recombinant vaccinia virus vTF7.3 harboring the T7 RNA polymerase gene as previously described (Saeki et al., 1997).

Western blot

S proteins expressed in BHK-R1 and BHK-R2 cells after transfection with vectors containing various S genes were analyzed by Western blotting using anti-S MAb kindly provided by S. G. Siddell (Routledge et al., 1991) by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) as previously reported (Matsumura and Taguchi, 2000).

Fusion activity

Fusion activities of wt and mutant S proteins were examined principally as reported by Nussbaum et al. (1994). BHK-R1 and BHK-R2 cells were used for this assay. The same type of cells were used for target and effector cells. Target cells were infected with wt vaccinia virus WR strain (m.o.i. = 10) and incubated at 37°C for 1 h. Those cells (1 × 10⁷ cells) were then transfected with 10 μg of pG1NT7β-gal harboring β-galactosidase gene downstream of the T7 promoter, kindly provided by Dr. E. Berger (Nussbaum et al., 1994), by electroporation as described previously (Ohtsuka et al., 1996). These cells were distributed in collagen-coated, 96-well plates (lwaki), 5 × 10⁴ cells/well, in DMEM supplemented with 5% FBS and incubated at 37°C for 15 h before being overlaid by effector cells. Effector cells were infected with vTF7.3 (m.o.i. = 10) and incubated at 37°C for 1 h and they (10⁵ cells) were transfected with 5 μg of pTarget vectors containing either wt or mutant S genes together with 0.5 μg of plasmid containing firefly luciferase gene pTM-luc (Aoki et al., 1998), kindly provided by Dr. Y. Matsuura, by electroporation. The pTM-luc was used to measure the transfection efficiencies of each vector to express various S proteins. Those treated cells, 5 × 10⁴ cells/well, were overlaid onto target cells prepared in 96-well plates and mixed cells were further cultured at 37°C for 6 to 8 h. The activity of β-galactosidase expressed as a result of fusion of effector and target cells by expressed S protein was measured using a commercial kit (Promega, β-galactosidase enzyme assay system) as recommended by manufacturer.

ACKNOWLEDGMENTS

We thank Drs. S. Siddell, B. Moss, E. Berger, and Y. Matsuura for MAb specific for the S protein, recombinant vaccinia virus vTF7.3, and expression vectors pG1NT7β-gal and pTM-luc, respectively. This work was partly supported by a Grant-in-Aid (11460148) from the Ministry of Education, Culture, Sports and Science of Japan.

REFERENCES

Aoki, Y., Aizaki, H., Shimoike, T., Tani, H., Ishii, K., Saito, I., Matsuura, Y., and Miyamura, T. (1998). A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. Virology 250, 140–150.

Beauchemin et al. (1999). Nomenclature announcement. Redefined nomenclature for members of the carcinoembryonic antigen family. Exp. Cell Res. 252, 243–249.

Castro, R. F., and Pertman, S. (1996). CD8+ T cell epitopes within the surface glycoprotein of a neurotropic coronavirus and correlation with pathogenicity. J. Virol. 69, 8127–8131.

Chen, D. S., Asanaka, M., Yokomori, K., Wang, F., Hwang, S. B., Li, H., and Lai, M. M. C. (1995). A pregnancy-specific glycoprotein is expressed in the brain and serves as a receptor for mouse hepatitis virus. Proc. Natl. Acad. Sci. USA 92, 12095–12099.

Collins, A. R., Knobler, R. L., Powell, H., and Buchmeier, M. J. (1982). Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell fusion. Virology 119, 358–371.

Colston, E., and Racaniello, V. R. (1994). Soluble receptor-resistant poliovirus mutants identify surface and internal capsid residues that control interaction with the cell receptor. EMBO J. 13, 5855–5862.

Dalziel, R. G., Lampert, P. W., Talbot, R. J., and Buchmeier, M. J. (1986). Site-specific alteration of murine hepatitis virus type 4 peplomer glycoprotein E2 results in reduced neurovirulence. J. Virol. 59, 463–471.

Damico, R. L., Crane, J., and Bates, P. (1998). Receptor-triggered membrane association of a model retroviral glycoprotein. Proc. Natl. Acad. Sci. USA 95, 2580–2585.

De Groot, R. J., Luytjes, W., Horzinek, M. C., Van der Zeijst, B. A. M., Spaan, W. J. M., and Lenstra, J. A. (1987). Evidence for a coiled-coil structure in the spike of coronaviruses. J. Mol. Biol. 196, 963–966.

Dveksler, G. S., Difffenbach, C. W., Cardellichio, C. B., Mccuaig, K., Pensiero, M. N., Jiang, G., Saito, I., Beauchemin, N., and Holmes, K. V. (1993). Several members of the mouse carcinoembryonic antigen-related glycoprotein family are functional receptors for the coronavirus mouse hepatitis virus-A59. J. Virol. 67, 1–8.

Dveksler, G. S., Pensiero, M. N., Cardellichio, C. B., Williams, R. K., Jiang, G., Holmes, K. V., and Difffenbach, C. W. (1991). Cloning of the mouse hepatitis virus (MHV) receptor: Expression in human and hamster cell lines confers susceptibility to MHV. J. Virol. 65, 6881–6891.

Fleming, J. O., Trousdale, M. D., El-Zaatari, F. A. K., Stohlman, S. A., and Weiner, L. P. (1986). Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. J. Virol. 58, 869–875.

Flory, E., Pfleiderer, M., Stuhler, A., and Wege, H. (1993). Induction of protective immunity against coronavirus-induced encephalomyelitis: Evidence for an important role of CD8+ T cells in vivo. Eur. J. Immunol. 23, 1757–1761.

Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986). Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes T7 RNA polymerase. Proc. Natl. Acad. Sci. USA 83, 8122–8126.

Gallagher, T. M. (1996). Murine coronavirus membrane fusion is blocked by modification of thiol buried within the spike protein. J. Virol. 70, 4683–4690.

Gallagher, T. M., Escarmis, C., and Buchmeier, M. J. (1991). Alteration of the pH dependence of coronavirus-induced cell fusion: Effect of mutations in the spike glycoprotein. J. Virol. 65, 1916–1938.

Grosse, B., and Siddell, S. G. (1994). Single amino acid changes in the pH dependence of coronavirus-induced cell fusion: Effect of mutations in the spike glycoprotein. J. Virol. 65, 1916–1938.

Ikeda, H., Kato, K., Suzuki, T., Kitani, H., Matsubara, Y., Watanabe, R., Kitagawa, M., and Aizawa, S. (2000). Properties of the naturally occurring soluble surface glycoprotein of ectropic murine...
leukemia virus: Binding specificity and possible conformational change after binding to receptor. J. Virol. 74, 1815–1826.

Kaplan, G., Peters, D., and Racaniello, V. R. (1990). Poliovirus mutants resistant to neutralization with soluble receptors. Science. 250, 1596–1599.

Krueger, D. K., Kelly, S. M., Lewicki, D. N., Ruffolo, R., and Gallagher, T. M. (2001). Variations in disparate regions of the murine coronavirus spike protein impact the initiation of membrane fusion. J. Virol. 75, 2792–2802.

Kubo, H., Yoden-Takase, S., and Taguchi, F. (1993). Neutralization and fusion inhibition activities of monoclonal antibodies specific for the S1 subunit of spike protein of neurovirulent JHMV cl-2 variant. J. Gen. Virol. 74, 1421–1425.

Kubo, H., Yamada, Y. K., and Taguchi, F. (1994). Localization of neutralizing epitopes and the receptor-binding site within the amino-termini 330 amino acids of the murine coronavirus spike protein. J. Virol. 68, 5403–5410.

Kumanishi, T. (1967). Brain tumors induced with Rous sarcoma virus, Schmidt-Ruppin strain. 1. Induction of brain tumors in adult mice with Rous chicken sarcoma cells. Ipn. J. Exp. Med. 37, 461–474.

Kunita, S., Zhang, L., Homberger, F. R., and Compton, S. R. (1995). Molecular characterization of the S proteins of two enterotropic murine coronavirus strains. Virus Res. 35, 277–289.

La Monica, N., Banner, L. R., Morris, V. L., and Lai, M. M. C. (1991). Localization of extensive deletions in the structural genes of two neurotropic variants of murine coronavirus JHM. Virology 182, 883–888.

Luo, Z., Matthews, A. M., and Weiss, S. R. (1999). Amino acid substitutions within the leucine zipper domain of the murine coronavirus spike protein cause defects in oligomerization and the ability to induce cell-to-cell fusion. J. Virol. 73, 8152–8159.

Luo, Z., and Weiss, S. (1998). Roles in cell-to-cell fusion of two conserved hydrophobic regions in the murine coronavirus spike protein. Virology 244, 483–494.

Luytjes, W., Sturman, L. S., Bredenbeek, P. J., Charite, J., van der Zeust, B. A. M., Horzinek, M. C., and Spaan, W. J. M. (1987). Primary structure of the glycoprotein E2 of coronavirus MHV-A59 and identification of the trypsin cleavage site. Virology 161, 479–487.

Matsubara, Y., Watanabe, R., and Taguchi, F. (1991). Neurovirulence of six different murine coronavirus JHMV variants for rats. Virus Res. 20, 45–58.

Matsuyama, S., and Taguchi, F. (2000). Impaired entry of soluble receptor-resistant mutants of mouse hepatitis virus into cells expressing MHVR2 receptor. Virology 272, 80–89.

Matsuyama, S., Watanabe, R., and Taguchi, F. (2001). Neurovirulence in mice of soluble receptor-resistant (srr) mutants of mouse hepatitis virus. Intensive apoptosis caused by less virulent srr mutants. Arch. Virol. 146, 1643–1654.

McKeating, J., Balfe, P., Clapham, P., and Weiss, R. A. (1991). Recombinant CD4-selected human immunodeficiency virus type 1 variants with reduced gp120 affinity for CD4 and increased cell fusion capacity. J. Virol. 65, 4777–4785.

Nedellec, P., Dveksler, G. S., Daniels, E., Turbide, E., Chow, B., Basile, A. A., Holmes, K. V., and Beauchemin, N. (1994). Bgp2, a new member of the caruncinembryonic antigen-related gene family, encodes an alternative receptor for mouse hepatitis viruses. J. Virol. 68, 4525–4537.

Nussbaum, O., Broder, C. C., and Berger, E. A. (1994). Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. J. Virol. 68, 5411–5422.

Ohtsuka, N., and Taguchi, F. (1997). Mouse susceptibility to mouse hepatitis virus infection links with viral receptor genotype. J. Virol. 71, 8860–8863.

Ohtsuka, N., Yamada, Y. K., and Taguchi, F. (1996). Difference of virus-binding activity of two receptor proteins for mouse hepatitis virus. J. Gen. Virol. 77, 1683–1692.

Parker, S. E., Gallagher, T. M., and Buchmeier, M. J. (1989). Sequence analysis reveals extensive polymorphism and evidence of deletions within the E2 glycoprotein gene of several strains of murine hepatitis virus. Virology 173, 664–673.

Phillips, J. J., Chua, M. M., Lavi, E., and Weiss, S. (1999). Pathogenesis of chimeric MHV4/MHV-A59 recombinant viruses: The murine coronavirus spike protein is a major determinant of neurovirulence. J. Virol. 73, 7752–7760.

Rao, P. V., Kumari, S., and Gallagher, T. M. (1997). Identification of a contiguous 6-residue determinant in the MHV receptor that controls the level of virion binding to cells. Virology 229, 336–348.

Routledge, E., Stauber, R., Pfeiferder, M., and Siddell, S. G. (1991). Analysis of murine coronavirus surface glycoprotein functions by using monoclonal antibodies. J. Virol. 65, 254–262.

Saeki, K., Ohtsuka, N., and Taguchi, F. (1997). Identification of spike protein residues of murine coronavirus responsible for receptor-binding activity by use of soluble receptor-resistant mutants. J. Virol. 71, 9024–9031.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Schmidt, I., Skinner, M., and Siddell, S. (1987). Nucleotide sequence of the gene encoding the surface projection glycoprotein of coronavirus MHV-JHM. J. Gen. Virol. 68, 47–56.

Smith, M. S., Click, R. E., and Plagemann, P. G. W. (1984). Control of mouse hepatitis virus replication in macrophages by a recessive gene on chromosome 7. J. Immunol. 133, 428–432.

Stauber, R., Pfeiferder, M., and Siddell, S. (1993). Proteolytic cleavage of the murine coronavirus surface glycoprotein is not required for fusion activity. J. Gen. Virol. 74, 183–191.

Sturman, L. S., Ricard, C. S., and Holmes, K. V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell fusing activity of virions by trypsin and separation of two different 90k cleavage fragments. J. Virol. 56, 904–911.

Suzuki, H., and Taguchi, F. (1996). Analysis of the receptor binding site of murine coronavirus spike glycoprotein. J. Virol. 70, 2632–2636.

Taguchi, F. (1993). Fusion formation by uncleaved spike protein of murine coronavirus JHMV variant cl-2. J. Virol. 67, 1195–1202.

Taguchi, F. (1999). Biological functions of mouse hepatitis virus (MHV) spike (S) protein and implication of S protein-MHV receptor interaction in virus virulence. Curr. Topics Virol. 1, 245–252.

Taguchi, F., Ikeda, T., and Shida, H. (1992). Molecular cloning and expression of a spike protein of neurovirulent murine coronavirus JHMV variant cl-2. J. Gen. Virol. 73, 1065–1072.

Taguchi, F., Kubo, H., Takahashi, H., and Suzuki, H. (1995). Localization of neurovirulence determinant for rats on the S1 subunit of murine coronavirus JHMV. Virology 208, 67–74.

Taguchi, F., and Matsuyama, S. (2002). Soluble receptor potentiates receptor-independent infection by mouse coronavirus. J. Virol. 76, 950–958.

Taguchi, F., Matsuyama, S., and Saeki, K. (1999). Difference in Bgp-independent fusion activity among mouse hepatitis viruses. Arch. Virol. 144, 2041–2049.

Taguchi, F., and Shimazaki, Y. K. (2000). Functional analysis of an epitope in the S2 subunit of murine coronavirus spike protein: Involvement in fusion activity. J. Gen. Virol. 81, 2867–2877.

Taguchi, F., Siddell, S. G., Wege, H., and ter Meulen, V. (1989). Characterization of a variant virus selected in rat brain after infection by coronavirus mouse hepatitis virus JHM. J. Virol. 54, 429–435.

Tsai, C. W., Chang, S. C., and Chang, M. F. (1999). A 12-amino acid stretch in the hypervariable region of the spike protein S1 subunit is critical for fusion activity of mouse hepatitis virus. J. Biol. Chem. 274, 26085–26090.

Wang, F.-I., Hilton, D. R., Gilmore, W., Trousdale, M. D., and Fleming, J. O. (1992). Sequential infection of glial cells by the mouse hepatitis virus
JHM strain (MHV-4) leads to a characteristic distribution of demyelination. *Lab. Invest.* **66**, 744–754.

Yamada, Y. K., Takimoto, K., Yabe, M., and Taguchi, F. (1997). Acquired fusion activity of a murine coronavirus MHV-2 variant with mutations in the proteolytic cleavage site and the signal sequence of the S protein. *Virology* **227**, 215–219.

Yamada, Y. K., and Yabe, M. (2000). Sequence analysis of major structural proteins of newly isolated mouse hepatitis virus. *Exp. Anim.* **49**, 61–66.

Yokomori, K., and Lai, M. M. C. (1992). The receptor for mouse hepatitis virus in the resistant mouse strain SJL is functional: Implication for the requirement of a second factor for virus infection. *J. Virol.* **66**, 6931–6938.

Zelus, B. D., Wessner, D. R., Williams, R. K., Pensiero, M. N., Philbe, F. T., deSouza, M., Dveksler, G. S., and Holmes, K. V. (1998). Purified, soluble recombinant mouse hepatitis virus receptor, Bgp1b, and Bgp2 murine coronavirus receptors differ in mouse hepatitis virus binding and neutralizing activities. *J. Virol.* **72**, 7237–7244.