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N-Terminal Domain of the Murine Coronavirus Receptor CEACAM1 Is Responsible for Fusogenic Activation and Conformational Changes of the Spike Protein

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The mouse hepatitis virus (MHV) receptor (MHVR), CEACAM1, has two different functions for MHV entry into cells: binding to MHV spike protein (S protein) and activation of the S protein to execute virus-cell membrane fusion, the latter of which is accompanied by conformational changes of the S protein. The MHVR comprising the N-terminal and fourth domains [R1(1,4)] displays these two activities, and the N domain is thought to be critical for binding to MHV. In this study, we have addressed whether or not the N domain alone is sufficient for these activities. We examined three types of soluble form MHVR (soMHVR), one consisting of the N domain alone [soR1(1)], one with the N and second domains [soR1(1,2)], and one [soR1(1,4)] expressed by recombinant baculoviruses. We assessed the abilities of these three types of soMHVR to bind to MHV, activate fusogenicity, and induce conformational changes of the S protein. All three types of soMHVR similarly bound to MHV, as examined by a solid-phase binding assay and neutralized MHV infectivity. They also activated S protein fusogenicity and induced its conformational changes with similar levels of efficiency. However, R1(1) expressed on the BHK cell surface failed to serve as a receptor in spite of a sufficient level of expression. The inability of expressed R1(1) to work as a receptor was due to the inaccessibility of virions to R1(1); however, these were accessible using the MHVR-specific monoclonal antibody CC1. These results collectively indicated that the N domain retains all biological activities necessary for receptor function.

The viral receptor is a molecule expressed on susceptible cells to which a virus binds and initiates its infection (40). Following the binding to the receptor, a virus enters into the cell by different pathways. There are two major pathways for the entry of enveloped viruses, the endosomal and nonendosomal pathways. Influenza virus binds to a receptor on the cell surface and is internalized into the cell's endosome, where envelope protein is fusogenically activated by a low-pH environment and fusion between virus and cell membranes takes place. The receptor for this virus is thought to trigger the internalization of virions into the endosome but not to activate fusogenicity (40). A retrovirus uses a nonendosomal pathway (2, 31). In this case, the receptor binds to an envelope protein and also activates protein fusogenicity by inducing conformational changes, which permit viral entry directly from the plasma membrane into the cytoplasm. This implies that the receptor for a retrovirus has two distinct functions, binding to virus envelope protein and activation of its fusogenicity. Although some strains or mutants could take an endosomal pathway (12), mouse hepatitis virus (MHV) is supposed, in general, to enter into cells by a nonendosomal pathway, since cells infected with this virus are fused under a wide range of pH conditions. As expected, MHV S protein is fusogenically activated and transformed by receptor binding (24, 36).

MHV is classified into the second among four different coronavirus groups, of which one member, the virus causing severe acute respiratory syndrome (SARS) (4, 15), belongs to group IV. Coronaviruses are enveloped viruses with a single-stranded, positive-sense genomic RNA ca. 30 kb in length (19). Virions are formed of nucleocapsids consisting of a large genomic RNA with a nucleoprotein (N) of 50 to 60 kDa surrounded by an envelope in which three essential membrane proteins exist: an integral membrane protein (M protein) of ca. 23 kDa, an envelope protein (E protein) of 8 to 10 kDa, and a spike protein (S protein) of 180 to 200 kDa. One optional glycoprotein, hemagglutinin (HA), is found in the envelope of most, but not all, of the group II coronaviruses.

S protein is a component of a petal-shaped spike protruding from the virion surface. It is a type I glycoprotein. After being synthesized and glycosylated, the S protein is cleaved by cellular proteinase into two subunits, N-terminal S1 and C-terminal S2 (32). S1 constitutes an outermost knoblike structure of the spike, and S2 constitutes a stemlike part located beneath the knob. Two or three molecules of an S1-S2 heterodimer construct a spike (20). The S protein has a variety of important biological functions that MHV retains; it has receptor-binding activity, induces cell fusion, serves as a neutralizing epitope, and is a determinant of viral virulence for animals (11, 35). The N-terminal region of the S protein, consisting of 330 amino acids (S1N330), is responsible for binding to the MHV receptor (MHVR) (17). The tertiary structure in the S1N330 or its oligomerization is important for receptor-binding activity. There are at least two separated regions in S1N330 critical for virus binding (33). Various regions in the S2 have been reported to affect fusion activity, namely, viral entry into cells, though the fusion peptide has not yet been identified (21, 37). The combination of a region in S1N330 and a region in S2 is reported to affect viral entry activity (23).

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The major receptor protein for MHV is a cell adhesion molecule in a carcinoembryonic antigen called CEACAM1, which is classified in the immunoglobulin (Ig) superfamily (1, 9). Prototype CEACAM1 or MHVR is composed of four Ig-like ectodomains (in the order N, A1, B, and A2 or D1, D2, D3, and D4 from the N terminus), a transmembrane domain (TM), and a cytoplasmic tail (Cy). There are four isoforms: two have four Ig-like domains and the other two have two Ig-like domains (N and A2), one of which has either a long or a short Cy (1). Allelic forms are known for CEACAM1, CEACAM1^a, and CEACAM1^b; CEACAM1^a is expressed in most laboratory mouse strains susceptible to MHV, and CEACAM1^b is expressed in SJL mice that are relatively resistant to MHV (6, 43). CEACAM1 is expressed on various epithelial cells, endothelial cells, and hemopoietic cells (13, 25). It functions as a cell adhesion molecule (25), a signaling molecule (27), and an angiogenic factor (10).

The N domain of MHVR is responsible for the binding to MHV S protein (8). However, MHVR with N domain alone expressed on cells failed to work as a functional receptor (8), while the chimeric MHVR consisting of its N domain and mouse homolog of poliovirus receptor (PVR) deleted its N domain functions as an MHVR (5). These results imply that the N domain alone is not sufficient but that some other Ig-like domain, even one that is unrelated to MHVR, is necessary. When expressed in cells grown in culture, a soluble form of MHVR consisting of the N domain alone worked as a functional receptor by anchoring to the cell membrane via an unidentified molecule (7), hinting an importance of the unidentified molecule for MHVR function. These observations collectively suggest that the N domain binds to S protein but is not sufficient as a functional receptor; namely, N domain alone fails to transform MHV S protein from a fusion-negative to fusion-positive phenotype. The participation of molecules linked to the N domain could not be thoroughly ruled out in its activity as a functional MHVR.

In this study, we tested whether MHVR containing N domain alone is able to activate the fusogenicity of the S protein. The results indicate that the N domain has two different functions in that it binds to S protein as well as activates the fusogenicity of S protein concomitant with its conformational changes, suggesting that the N domain suffices for MHVR function.

MATERIALS AND METHODS

Cells and viruses. DBT cells expressing MHVR (18), as well as MHVR-negative BHK 13 (BHK) cells, were grown in Dulbecco's minimal essential medium (DMEM; Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS) (DMEM-FBS; Gibco BRL, Grand Island, N.Y.). DBT cells were used for MHV infection, titration, and propagation. BHK cells nonpermissive for MHV were target cells for MHVR-independent fusion, as described previously (36). The wild-type (wt) virus, MHV-JHM strain of MHV (JHMV) cl-2 (38), its mutant, JHMV srr7 (30), and MHV-A59 were inoculated onto DBT cells at a multiplicity of infection (MOI) of 1, and the cells were harvested at 12 to 15 h, when syncytia occupied 80 to 90% of the total culture. For wt JHMV and srr7, those cells were ultrasonicated with a Bioruptor (Olympus, Tokyo, Japan) and sonicated cells were spun at 3,000 rpm for 10 min (CR 26H centrifuge; Hitachi, Tokyo, Japan). The supernatants were divided into small volumes and stored at −80°C as seed viruses for infection. Culture fluid isolated from MHV-A59-infected DBT cells was used as seed viruses. Compared with wt MHV-JHMV cl-2 virus, srr7 has a mutated amino acid at position 1114 (Leu to Phe) in the S protein.

Assay of virus infectivity. Virus infectivity was measured using DBT cells grown in 24-well plates (Iwaki, Tokyo, Japan) and expressed in PFU, as previously described (36).

Solid-phase virus-MHVR binding assay. The binding activity of soluble form MHVR (soMHVR) to MHV S protein was tested by a solid-phase binding assay. Various concentrations of soMHVRs dissolved in 40 μl of phosphate-buffered saline (PBS), pH 7.2, were distributed in 96-well ELISA plates (Sumitomo, Tokyo, Japan) and incubated at 4°C overnight or 37°C for 2 h. The plates were treated with Block-Ace (Yukijirushi, Sapporo, Japan) (250 μl per well) at 37°C for 45 min to prevent nonspecific binding. A total of 50 μl of wt JHMV cl-2 prepared as described above was adjusted to a protein concentration of 50 μg/ml in Block-Ace and was allowed to react to plate-adherent soMHVR at 37°C for 1 h. After washing vigorously with PBS containing 0.05% Tween 20, 50 μl of anti-MHV S monoclonal antibody (MAb) No. 7 that reacts with receptor-bound S1 subunit (16) was added and incubated at 37°C for 1 h. Then, 50 μl of anti-mouse IgG labeled with horseradish peroxidase (HRPO) (Cappel Organon Teknika, Durham, N.C.) was added and the mixture was incubated at 37°C for 40 min. The amounts of JHMV bound to soMHVRs were determined using *o*-phenylenediamine to measure HRPO levels (Sigma, St. Louis, Mo.). To test the specificity of MHV and soMHVR binding shown in this assay, we used the binding inhibition test with anti-MHVR MAb CC1 (41), kindly provided by Kathryn Holmes. Soluble R1(1,4) [soR1(1,4)] was prepared in 96-well plates as described above. After being blocked with Block-Ace, soR1(1,4) was reacted with different concentrations of CC1 at 37°C for 1 h. Then, 25 μg of wt JHMV/ml dissolved in Block-Ace was allowed to bind to soR1(1,4) in the plates at 37°C for 1 h. soR1(1,4)-bound JHMV was measured using biotinylated MAb no. 7 and HRPO-labeled avidin (Cappel) as described above.

Viral neutralization assay. The neutralization activity of each soMHVR was examined using wt JHMV, srr7, and MHV-A59 as previously described (36). wt JHMV and its mutant, srr7, (about 200 PFU in 25 μl) were mixed with 25 μl of diluted soR1 and incubated at room temperature (RT; 22 ± 2°C) and 37°C, respectively, for 50 min. MHV-A59 was incubated at 37°C for 50 min. The residual infectivity was examined with a plaque assay using DBT cells prepared in 24-well plates. The degree of neutralization was determined in comparison with virus titers of incubations in the absence of soMHVR.

Recombinant baculovirus for soMHVR expression. soMHVRs were expressed using recombinant baculoviruses, each of which contained the soMHVR gene. Methods of using baculovirus to express soR1(1,4) have already been reported (36). The genes to encode soMHVR containing the N domain alone [soR1(1)] and the N and second domains [soR1(1,2)] were amplified from a gene encoding MHVR with four ectodomains inserted in multiple cloning sites of commercial expression vector pTarget (Promega, Madison, WI), as reported previously (22). A pair of primers, primer MHVR5′ (28) and reverse primer soR1(1)-HA-rev (5′-TCTCCATATGATGTTCCAGATTATGCTGGGGTGTACATGAAA TCG-3′), and another pair of primers, primer MHVR5′ and reverse primer soR1(1,2)-HA-rev (5′-TCTCCATATGATGTTCCAGATTATGCTGGGGGA TATAATCGGGGT-3′), were used to amplify soR1(1) and soR1(1,2) genes, respectively, by PCR under conditions described previously (42). These two genes contain the nucleotides at the 3′ end encoding an influenza virus HA epitope (28).

With these amplified DNAs, we constructed genes containing HA, *myc*, and six-times-repeated histidine (6×His tag) at the 3′ end with MHVR5′ and HA-myc-His primers as described previously (36). These DNA fragments were inserted into a commercial vector pTarget downstream from the T7 promoter, and a clone with the right sequence inserted in a right orientation was selected; these vectors were designated pTarget soR1(1)-HA-myc-His and pTarget soR1(1,2)-HA-myc-His, respectively. From these vectors, soR1 genes with three tags at the 3′ end were cut out and inserted into a baculovirus transfer vector, pVL1392 (kindly provided by Y. Matsuura), in a right orientation. The recombinant baculoviruses harboring these genes were produced, using a commercial kit (BaculoGold DNA, PharMingen, San Diego, Calif.), in an Sf9 insect cell line according to the manufacturer's recommendation. Recombinant baculoviruses containing soR1(1) and soR1(1,2) [designated Bac-soR1(1)-HA-myc-His and Bac-soR1(1,2)-HA-myc-His, respectively] were plaque purified three times for the present study.

Expression and purification of soMHVR. SoR1s were expressed and purified as previously reported (36). Briefly, Tn*5* cells were infected with baculoviruses at an MOI of 1 or more, and infected cells were grown at 26°C for 3 days with Ex-cell 405 medium (Gibco BRL). The culture fluids were clarified by centrifugation at 13,000 × *g* for 30 min and then mixed with polyethylene glycol 6000 at a final concentration of 20%. Following incubation at 4°C for 2 h, the mixture was centrifuged at 13,000 × *g* for 30 min and the resultant precipitate was dissolved in a small volume of a lysis buffer for Ni-NTA (Qiagen, Hilden, Germany) affinity

chromatography. SoR1s with 6×His tag were purified by the affinity chromatography according to the manufacturer's instructions. The concentration of each soR1 was estimated using a commercial reagent (Bio-Rad protein assay; Bio-Rad, Richmond, Calif.). The purity of the expressed soMHVR was examined by silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34).

Activation of fusion by soMHVRs. Fusogenic activation of the S protein by soR1s was examined using MHVR-independent fusion and BHK cells as described previously (36). DBT cells infected with wt JHMV or srr7 at an MOI of 1 were dispersed with trypsin at 3 to 4 h after infection. DBT cells (10^4) were overlaid onto a BHK cell monolayer prepared in collagen-treated 24-well plates (Iwaki) in 200 μl of DMEM-FBS. Various concentrations of soMHVR were added into the medium and grown for a further 12 to 16 h. Cells were fixed with 5% formaldehyde and stained with crystal violet. Fusogenic activation was estimated according to the increase in syncytium number and size, as reported previously (36).

Proteinase digestion assay. Conformational changes of the S protein were examined by a proteinase digestion assay as previously described (24). srr7 (5×10^7 PFU/ml) was incubated in different concentrations of soR1 at 37°C for 30 min and stored on ice for 5 min. To this, proteinase K (Wako, Tokyo, Japan) was added at a concentration of 20 mg/ml and the mixture was incubated at 4°C for 20 min. Reactions were stopped by treatment with electrophoresis sample buffer (0.125 M Tris [pH 6.8], 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue) and subjected to SDS-PAGE. The digested S proteins were analyzed by Western blotting using anti-JHMV S2 MAb 10G (29), kindly provided by S. G. Siddell, and HRPO-conjugated anti-mouse IgG. The MHV S protein-specific bands were visualized on X-ray film by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.) as described previously (30).

Expression of MHVR on BHK cells. We expressed R1(1) and R1(1,4) linked with TM and Cy on BHK cells as previously reported (22). A pTarget expression vector containing R1(1) linked with TM, Cy, and HA epitope at the C terminus was constructed by PCR using pTargetR1(1,4) (22). The first cycle of PCR was done using pTarget 5′ forward primer ACTATAGGGCGAATTCGGAT and N-TM-rev AATGGCGCCATCTGAGAGGTGTACATGAAATCGCAC, which consists of the last 18 nucleotides of the N domain linked with 18 additional nucleotides corresponding to the first 18 nucleotides of TM. The other first cycle of PCR was done with primer N-TM-for CTCTCAGATGGCGCCATT, consisting of first 18 nucleotides of TM, and pTarget 3′ reverse primer CTCAAGCT TGGAATTCGCGG. The pTarget 5′ and pTarget 3′ primers correspond to the sequences upstream and downstream, respectively, of the multiple cloning site of the pTarget vector. PCR products amplified by a second cycle of reaction using pTarget 5′ and pTarget 3′ primers were inserted into the pTarget vector containing the neomycin-resistant gene. To express R1 proteins, these plasmids were transfected into BHK cells by electroporation (Gene Pulser; Bio-Rad) (34) and cells resistant to neomycin were selected by growing those cells in DMEM-FBS containing 200 μg of neomycin/ml. BHK cells constitutively expressing R1(1) and R1(1,4) were designated BHK-R1(1) and BHK-R1(1,4), respectively.

Flow cytometric analysis. R1(1,4) and R1(1) expressed on BHK cells established as described above were examined by flow cytometry. A single-cell suspension was prepared with Dispase (Rosche Diagnostics, Mannheim, Germany) from BHK, DBT, BHK-R1(1), or BHK-R1(1,4) cells according to the manufacturer's instructions. After washing, cells were resuspended in a tube in PBS supplemented with 1% FCS and 0.1% sodium azide at a concentration of 2×10^5 cells/100 μl. To prove that expression of R1 protein occurred, these cells were first incubated in 50 μl of MAb CC1 (IgG1) at a dilution of 1:100 (12 μg/ml) for 30 min at 4°C. After being washed three times with PBS, cells were then incubated in 50 μl of Alexa Fluor 488-labeled anti-mouse IgG goat antibody (Molecular Probes, Inc., Eugene, Oreg.) for 30 min at 4 C. To examine the amounts of JHMV cl-2 bound to R1 expressed on BHK cells, these cells were incubated with the virus (2×10^5 PFU/20 μl) for 30 min at 4 C. After being washed three times, they were then incubated with 50 μl of MAb no. 2 or 7 (IgG2a) against MHV followed by Alexa Fluor 488-labeled anti-mouse IgG goat antibody. IgG1 and IgG2a were used as isotype controls for nonspecific antibody binding of MAbs CC1 and no. 2 or 7, respectively. After staining, these cells were fixed in 1% paraformaldehyde and analyzed with a FACSCalibur apparatus on CellQuest software (Becton Dickinson, San Jose, Calif.), gating out cellular debris by forward and side scatters.

RESULTS

Binding of soMHVRs to MHV S protein. soMHVR consisting of the N and fourth domains [soR1(1,4)] has been shown to bind and activate the fusogenicity of the S protein of an srr7 mutant derived from MHV-JHMV cl-2 virus (36). To see whether both of these domains are important for those activities, we prepared soMHVR consisting of the N domain alone [soR1(1)]. We also made soMHVR consisting of the N and second domains [soR1(1,2)] to see whether the fourth domain in soR1(1,4) can be replaced by another Ig C2-like domain (Fig. 1). SoR1(1) is comprised of a leader region consisting of 34 amino acids and an N domain having 108 amino acids, while soR1(1,2) contains leader, N, and second (105 amino acids) domains and soR1(1,4) contains leader, N, and fourth (102 amino acids) domains. Mature soR1s are supposed to delete the leader region composed of 34 amino acids.

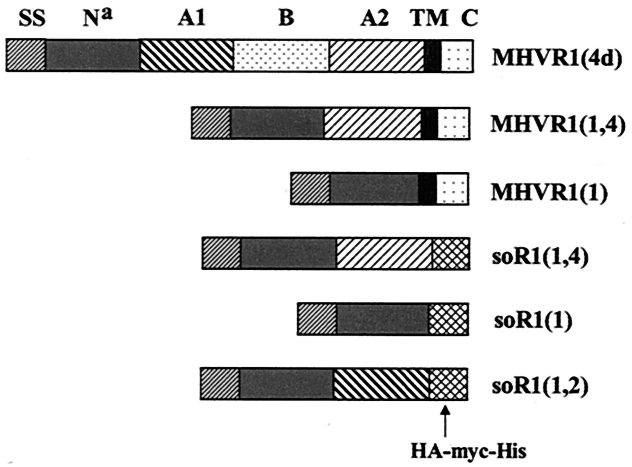

FIG. 1. Schematic diagram of the CEACAM1 used in the present study. Prototype CEACAM1 (MHVR) is composed of four ectodomains linked with TM and Cy, and genetically manipulated MHVRs lack some of those domains and regions. Three different soluble forms have three tags (HA, *myc*, and 6×His) that replace TM and Cy, and the other two MHVRs used for expression on cell membranes lack one or two ectodomains.

Since soR1(1,4) expressed by recombinant baculovirus was shown in our previous studies to be functionally active, exhibiting virus-binding and fusogenic activation activities, we expressed soR1(1) and soR1(1,2) in a baculovirus expression system. They were then purified by affinity chromatography using 6×His tags at their C terminus end and analyzed by Western blotting using anti-HA MAb as previously reported (36). As shown in Fig. 2, those soMHVRs showed different electrophoresis patterns (as expected from their calculated molecular masses). The molecular masses of soR1(1), soR1(1,2), and soR1(1,4) were calculated as 30, 47, and 44 kDa, respectively. The slightly broad bands seen using Western blotting could have been due to a different degree of glycosylation. These soMHVRs were subjected to binding to MHV S protein by a solid-phase binding assay. Three different soR1s (in dilutions of from 5 to 0.625 nM) was made adherent onto 96-well plates, and wt JHMV was incubated in the plates at 37°C for 1 h. The amounts of wt JHMV that bound to each soR1 were measured using anti-S protein MAb and HRPO-labeled anti-mouse IgG antibody. As shown in Fig. 3A, wt JHMV bound to the S protein prepared in 96-well plates in an soR1 dose-dependent fashion; there was no significant differ-

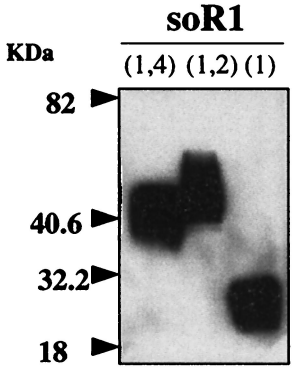

FIG. 2. Western blot analysis of the purified soMHVR. These soR1s were expressed by recombinant baculoviruses and purified by affinity chromatography using 6×His. After electrophoresis in an SDS–10% polyacrylamide gel, proteins were blotted onto nitrocellulose membrane and soR1s were detected with anti-HA MAb and anti-mouse IgG labeled with HRPO.

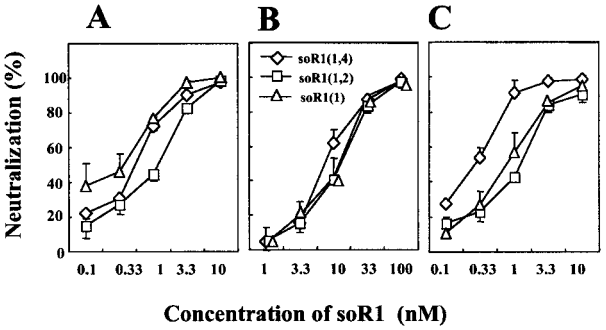

FIG. 4. Virus neutralization by soR1s. wt JHMV cl-2 (A), srr7 (B), or MHV-A59 (C) (about 100 PFU/20 μl) was mixed with an equal volume of soR1s and incubated at RT (A) or 37°C (B and C) for 50 min, and the residual infectivity was measured on DBT cells. Inhibition of fusion was estimated by comparing the infectivity after incubation in the absence of soR1s. Error bars represent standard deviations of the results for three independent samples.

ence in the levels of binding capacity among those three soR1 proteins.

To determine the specificity of this binding, we have examined whether the binding was inhibited by the pretreatment of adhered soR1(1,4) with anti-MHVR MAb CC1. soR1(1,4) (0.5 nM) was adhered to 96-well plates and incubated with different concentrations of CC1 at RT for 1 h. Then, wt JHMV was allowed to bind to the soR1(1,4). The amounts of JHMV bound to soR1s were determined using biotinylated anti-MHV-S MAb. The results showed that the binding of wt virus to soR1 was blocked by pretreatment with CC1 in an antibody concentration-dependent manner, indicating that this assay measured the specific binding of JHMV and its receptor (Fig. 3B). Though Dveksler et al. (7) have already shown that the N domain alone bound to MHV, we showed in this study that the N domain alone is capable of binding to virus as efficiently as an authentic receptor protein composed of two ectodomains.

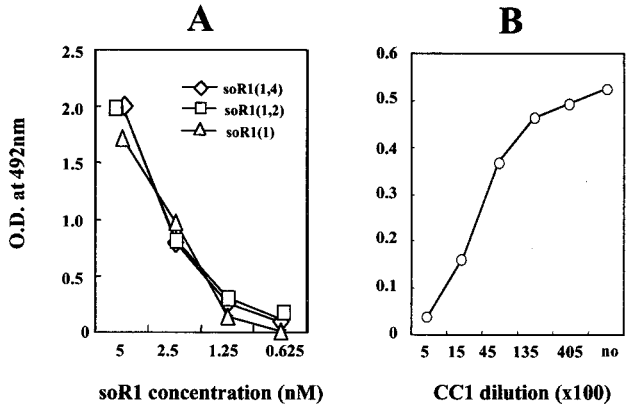

FIG. 3. Solid-phase virus-binding assay. (A) Various soR1s were prepared on 96-well plates by incubating at 4°C overnight or 37°C for 2 h. JHMV was added and incubated at 37°C for 1 h, and its binding was monitored using anti-JHMV S MAb and anti-mouse IgG labeled with HRPO. (B) To determine the levels of binding specificity, soR1(1,4) prepared in 96-well plates was treated with CC1 at 37°C for 1 h and then incubated with JHMV. JHMV binding to soR1(1,4) was monitored as described above. Results are representative of multiple independent experiments.

Neutralization activity of soR1s. To alternatively see the binding of these soMHVRs, we have applied a neutralization test whose results we had shown to correspond to MHVR binding results as determined by a viral overlay protein blot assay (28). wt JHMV cl-2 was mixed with various concentrations of soR1s and incubated at RT (22 to 24°C) for 50 min, and residual infectivity was examined by a plaque assay. As shown in Fig. 4A, all of these soR1s neutralized wt virus in a concentration-dependent fashion; the 50% neutralization titer of each soR1 was roughly 1 nM. There was no significant difference in neutralization activity levels among these soR1s, indicating that these soR1s bind to wt S protein with similar levels of efficiency. We also used srr7 to examine neutralization. As srr7 is resistant to neutralization by soMHVR when incubated at RT, we incubated the mixture of virus and soR1 at 37°C for 50 min, as described previously (36). Also, there was no significant difference in the neutralization patterns of srr7 among three different soR1s; however, 50% neutralization required about a 10-times-higher concentration (10 nM) of soR1s (Fig. 4B). All of these results indicated that there were no significant differences among soR1s in binding to JHMV S protein.

Since Zelus et al. (44) described a difference in MHV-A59 neutralization activity between soR1(1,4) and soR1(1,2), we also used incubation of MHV-A59 at 37°C for 50 min to examine the neutralization activity. As shown in Fig. 4C, the neutralization patterns of soR1(1) and soR1(1,2) were slightly weaker than that of soR1(1,4).

Activation of MHV S protein fusogenicity by soMHVRs. The activation of MHV S protein fusogenicity was examined by the MHVR-independent fusion of wt and srr7 JHMV. wt cl-2-infected DBT cells induced fusion of MHVR-negative BHK cells when DBT cells were overlaid onto BHK cell monolayers (MHVR-independent fusion), while srr7 lacked this ability. However, the addition of soR1(1,4) to srr7-infected DBT cells induced BHK cell fusion, which resulted from the fusogenic activation of srr7 S protein (36). Addition of soR1(1,4) onto cl-2-infected DBT cells failed to increase syncytium number

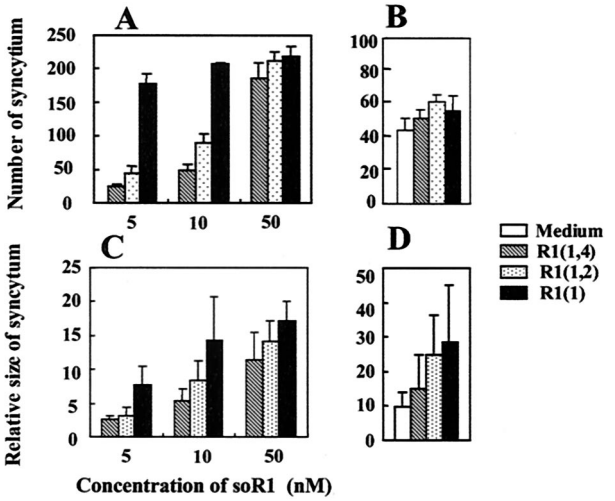

FIG. 5. Fusogenic activation of JHMV by soR1s. srr7 (A and C)- or wt JHM (B and D)-infected DBT cells (10^4) were overlaid onto BHK cell monolayers prepared in 24-well plates and incubated for 15 h in the presence or absence of soR1. The numbers (A and B) or sizes (C and D) of syncytia were measured after staining with crystal violet. Various soR1s were employed for the wt (B and D) at a concentration of 10 nM. Error bars represent standard deviations of the results for three independent samples.

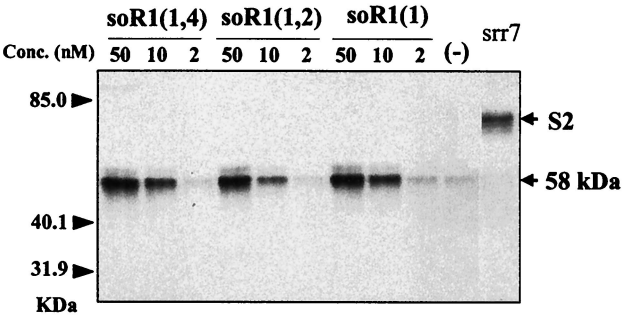

FIG. 6. Conformational changes of the S2 by soR1s. srr7 was mixed with soR1s or left untreated (−), incubated at 37°C for 30 min, and subsequently digested with proteinase K (20 mg/ml) at 4°C for 20 min. Following SDS-PAGE and blotting onto the nitrocellulose membrane, proteinase-resistant fractions of 58 kDa were detected using anti-S2 MAb 10G. Lane srr7, S protein not treated by soR1 and proteinase K.

but increased syncytium size. In this study, soR1(1) and soR1(1,2) were compared with soR1(1,4) to determine whether they activated the MHVR-independent fusion of MHV. Both the numbers and sizes of syncytia were compared in BHK cells overlaid with srr7-infected DBT cells. As shown in Fig. 5A, three soR1s induced syncytia formation with the same efficiency at 50 nM; at lower concentrations (5 and 10 nM), however, soR1(1) induced syncytia more efficiently than did soR1(1,2) and soR1(1,4). Also, this propensity was observed with respect to the sizes of syncytia induced by these soR1s (Fig. 5C). At lower concentrations, i.e., 1 nM, none of those soR1s activated the fusogenicity of srr7 (data not shown), which is in agreement with the previous results of Taguchi and Matsuyama (36). These results indicated that soR1(1) has the potential to convert a fusion-negative S protein to a fusion-positive one. Effects of soR1 on the size of the syncytia produced on BHK cells by wt JHMV-infected DBT cells were also examined. As shown in Fig. 5D, syncytia produced at an soR1(1) concentration of 10 nM were significantly larger than those produced by other types of soR1s. This observation was very similar to that of the fusion activation observed in srr7 and shown in Fig. 5C. However, syncytium numbers produced by wt JHMV did not significantly increase after treatment with various soR1s (Fig. 5B).

All of these results suggested that there were no apparent differences among the three soMHVRs in terms of both virus-binding activity and fusion activation, suggesting that the N domain alone works as a functional receptor, as does MHVR with two ectodomains.

Conformational changes of S protein induced by soMHVRs. Since fusogenic activation of S protein by soMHVR is accompanied by its conformational changes (36), we compared this activity of the soMHVRs. JHMV srr7 virus was mixed with different concentrations of soR1s and incubated at 37°C for 30 min. The mixture was then digested with proteinase K at 4°C for 30 min. The proteinase K-resistant fraction of 58 kDa was detected using anti-S2 MAb and Western blotting. As shown in Fig. 6, conformational changes were induced by either of those soR1s in a concentration-dependent fashion and there was no significant difference in the degrees of the changes induced by those three soR1s. The conformational changes were detected at concentrations greater than 10 nM, but not with 2 nM, in agreement with the previous results of Matsuyama and Taguchi for soR1(1,4) (24). These results indicate that the N domain alone is sufficient for the induction of S protein conformational changes.

Receptor function of R1(1) expressed on cell surface. The findings described above (i.e., that the N domain has two different functions that are important components of a viral receptor) suggested that it works as a functional receptor, though Dveksler et al. (8) reported that when expressed on the cell surface, the R1 composed of the N domain linked with TM and Cy failed to function as a receptor for MHV. We tried to confirm their observations and, in addition, to examine whether or not R1(1) is able to bind to JHMV when expressed in MHV-nonpermissive cells. We have used pTarget-R1(1)-HA (containing the N domain, TM, and Cy) for expression of R1(1) in BHK cells. We have also prepared BHK cells expressing R1(1,4) and shown to be susceptible to MHV (22). Transfected cells were grown in the presence of neomycin, and neomycin-resistant cells were examined for their susceptibility to JHMV cl-2. Those cells, as well as MHVR-negative BHK cells, were infected with wt JHMV at an MOI of 0.1, and their levels of susceptibility were examined by estimating virus titers in the culture fluids. As shown in Fig. 7, virus multiplied in cells expressing R1(1,4) [BHK-R1(1,4)]; however, no replication was detected in cells expressing R1(1) [BHK-R1(1)] or BHK cells without these receptor proteins. Nonsusceptibility of BHK-R1(1) cells to MHV infection was also confirmed by the findings that whole cultures of BHK-R1(1) cells or BHK cells were intact at 24 h after infection and that whole cultures of BHK-R1(1,4) were included in syncytia (data not shown).

We then examined the expression of R1(1) and R1(1,4) on BHK cells and the capacity of binding to JHMV by flow cy-

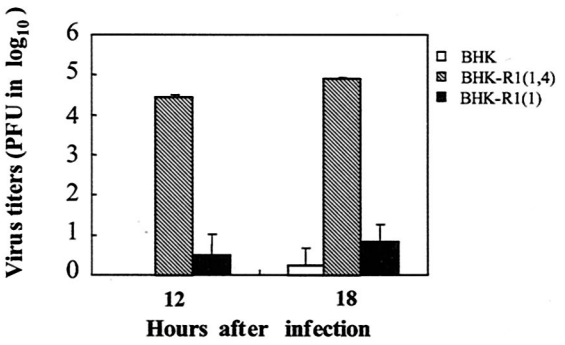

FIG. 7. Virus growth in BHK cells expressing various R1s. BHK cells expressing R1(1,4), R1(1), or no R1 and designated BHK-R1(1,4), BHK-R1(1), or BHK cells, respectively, were infected with wt JHMV cl-2 at an MOI of 0.1 and incubated for 12 or 18 h. Virus titers in the culture were measured using DBT cells. Bars show the standard deviations of the results for three independent samples.

tometry. DBT cells expressing MHVR and MHVR-negative BHK cells as controls were also examined. These cells were incubated with anti-R1 MAb CC1, the epitope of which is located in the N domain of MHVR, and Alexa Fluor 488-labeled anti-mouse IgG. As shown in Fig. 8A, B, C, and D, BHK-R1(1,4) cells were revealed to react strongly with CC1 and BHK-R1(1) and DBT cells reacted weakly, but significantly, with CC1, indicating that R1(1,4) was expressed in large amounts on the BHK cell surface, while the expression level of R1(1) was fairly low but was equivalent to the amounts of MHVR expressed on highly susceptible DBT cells. We then examined whether JHMV is able to bind to those R1s expressed on the cell surface. Those cells expressing various R1s were incubated with wt JHMV, and cell-bound JHMV was examined using anti-S protein MAb and flow cytometry. As shown in Fig. 8E, F, G, and H, large amounts of the virus bound to R1(1.4) expressed on BHK cells but no significant amounts of virus bound to R1(1). Virus binding to MHVR expressed on DBT cells was seen in low amounts, but significant binding was detectable. No significant binding of the virus was observed with MHVR-negative BHK cells. These results indicated that R1(1) was expressed on BHK cells but failed to bind to viruses. The amounts of R1(1) expressed on cells were supposed to be sufficient to allow them to serve as MHVRs, since MHVR were expressed on DBT cells in amounts similar to those of R1(1) expressed on BHK cells. It was likely that the R1(1) molecule was not accessible by the virion when expressed on the cell surface, probably because it is buried among plenty of molecules on the cell surface, as discussed previously (8).

DISCUSSION

Various proteins categorized in the Ig superfamily are shown to work as a receptor for viruses. CD4 is the first protein among the members of the Ig superfamily to be recognized as a receptor for a virus, namely, the human immunodeficiency virus (3). PVR is also a member of this superfamily (26), and the measles virus receptor was identified recently as SLAM, a member of the Ig superfamily (39). Finally, the MHVR CEACAM1 is also a member of the Ig family (9). Among these proteins, the N-terminal domain resembling the Ig variable region plays an essential role for receptor function, i.e., binding to virions. Also, the N domain of MHVR has been demonstrated to bind to virions (though it has not yet been shown to induce MHV S protein fusion activity, another important activity of the virus receptor). A line of evidence suggests that the N domain of MHVR is sufficient for the MHVR. The N domain linked with 24 amino acids downstream bound to MHV, and that linked with PVR, deleting its N domain responsible for poliovirus binding, and acted as an MHVR (6, 8). Soluble MHVR with an N domain works alone as a receptor by cell surface expression, which is due to the interaction with another molecule on the cell surface (7). However, MHVR composed of the N domain alone failed to function as a receptor for MHV when it was expressed on MHVR-negative cells (8). These observations collectively suggest that an Ig C2-like do-

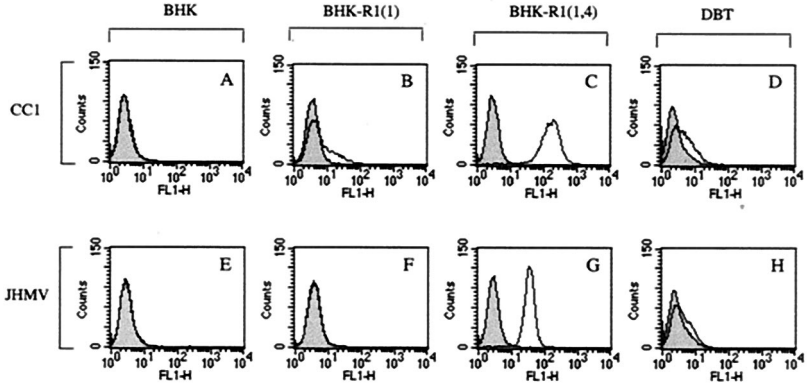

FIG. 8. Flow cytometric analysis of MHVRs expressed on cell membrane, as examined using MAb CC1 (A to D), and of JHMV that bound to those MHVRs (E to H). (A to D) Cells expressing various MHVRs were dispersed by proteinase and reacted with anti-MHVR MAb CC1 (open histograms) at 4°C for 30 min and subsequently with anti-mouse IgG labeled with Alexa Fluor 488 at 4°C for 30 min. Mouse IgG1 was used as an isotype control (shaded histograms). After fixation in 1% paraformaldehyde, cells were analyzed on a FACSCalibur apparatus using CellQuest software. (E to H) Cells (dispersed as described above) were mixed with 2×10^5 PFU of JHMV (open histograms) and incubated at 4°C for 30 min. Then, the cells were reacted with anti-MHV S protein MAb no. 2 or 7 at 4°C for 30 min and subsequently with the above-described anti-mouse IgG. Finally, the cells were analyzed as described above. IgG2a was used as an isotype control (shaded histograms). FL1-H, log intensity of fluorescence.

main or some other molecules are essential for functional MHVR.

Since Taguchi and Matsuyama have developed a system to test one of the most important receptor functions, fusogenic activation of the S protein (36), we have addressed in this study whether the N domain retains this function. As controls for MHVR with the N domain alone, we have used two different receptor proteins, R1(1,4) (which is a splice variant of MHVR and functions as MHVR) and R1(1,2) (which contains the N-terminal and second Ig C2-like domains). The latter receptor is used to determine whether another C2-like domain corresponding to the fourth domain of R1(1,4) can replace it. The present study showed that all three different types of soMHVR displayed similar levels of fusogenic activation and induction of S protein conformational changes. We also used a direct binding assay and neutralization test to show that the N domain is sufficient for binding to MHV S protein. Thus, the present study strongly suggests that the N domain suffices for MHVR function.

Although this study used soR1(1) to indicate that the N domain is sufficient for receptor function, it actually did not work as a functional receptor when expressed in MHVR-negative BHK cells. This observation is in close agreement with the previous studies reported by Dveksler et al. (8). The failure of R1(1) expressed on BHK cells to work as an MHVR should not be attributed to a depressed expression on the cell surface, since the amounts of R1(1) detected on BHK cells were similar to the amounts of MHVR expressed on highly MHV-susceptible DBT cells. The failure of cell membrane R1 to work as a receptor could be due to the short structure of R1(1), as discussed by Dveksler et al. (8). R1(1) molecules on the cell surface may be buried among various large molecules expressed on BHK cells; thus, virions might not be accessible to R1(1) molecules. Likewise, R1(1) linked with a C2-like domain of the MHVR molecule or another molecule unrelated to MHVR (such as PVR with its N domain deleted) can recover receptor function when expressed on BHK cells. However, R1(1) is not totally buried, since CC1 is able to bind it, and CC1's epitope is reportedly not identical to the virus-binding site (6). Alternatively, TM and cytoplasmic domains directly linked to the N domain could affect the N domain structure, resulting in the reduced receptor functionality.

We have shown in the present study that three different soR1s neutralized wt JHMV and srr7 with similar levels of efficiency, but soR1(1,4) showed a higher neutralization activity for the MHV-A59 strain than did the other two. Previously, Zelus et al. (44) also showed that soMHVR corresponding to soR1(1,2) has about 10-times-less neutralizing activity than soR1(1,4). They further described another allelic form of MHVR, CEACAM1^b, having four domains and functioning as a receptor for A59 but not at all for JHMV (44). These findings suggest a difference between JHMV and A59 in their interactions with MHVR. Collectively, we interpret these data to indicate that the N domain is the major determinant of activity as a receptor for JHMV but that some other regions could affect the interaction between MHVR and the MHV-A59 strain. Unfortunately, our system designed for the observation of S protein activation by soR1s turned out not to work for A59; A59 fusion was not efficiently induced, not even by soR1(1,4) (data not shown).

The N domain has three glycosylation sites; however, none is reported to be critical for MHVR function (5). In conjunction with the present observations, it can be speculated that the nonglycosylated N domain functions alone as a receptor for MHV. If so, the bacterially expressed N domain could also work as a receptor. Moreover, a synthetic peptide composed of amino acids essential for receptor function could serve as a functional receptor; nominally, such a peptide could neutralize and execute the fusogenic activation of MHV. Recently, Knauss and Young (14) reported that a synthetic peptide containing 15 amino acids of cognate receptor neutralized and fusogenically activated avian leukosis and sarcoma virus. A possibility also exists that a synthetic peptide covering the receptor-active site of the N domain works to neutralize and fusogenically activate MHV. Identification of the critical amino acid sequence for receptor function could be very useful for the development of anti-MHV compounds, which provide an excellent, general model for antiviral strategies for various virus infections, especially for the recently emerging human coronavirus causing SARS (4, 15).

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REFERENCES

1. **Beauchemin, N., et al.** 1999. Redefined nomenclature for members of the carcinoembryonic antigen family. Exp. Cell Res. **252:**243–249.
2. **Chen, D. C., and P. S. Kim.** 1998. HIV entry and its inhibition. Cell **93:**681–684.
3. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312:**763–766.
4. **Drosten, C., et al.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
5. **Dveksler, G. S., A. A. Basile, C. B. Cardellichio, and K. V. Holmes.** 1995. Mouse hepatitis virus receptor activities of an MHVR/mph chimera and MHVR mutants lacking N-linked glycosylation of the N-terminal domain. J. Virol. **69:**543–546.
6. **Dveksler, G. S., C. W. Diffenbach, C. B. Cardellichio, K. McCuaig, M. N. Pensiero, G. S. Jiang, N. Beauchemin, and K. V. Holmes.** 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.
7. **Dveksler, G. S., S. E. Gagneten, C. A. Scanga, C. B. Cardellichio, and K. V. Holmes.** 1996. Expression of the recombinant anchorless N-terminal domain of mouse hepatitis virus (MHV) receptor makes hamster or human cells susceptible to MHV infection. J. Virol. **70:**4142–4145.
8. **Dveksler, G. S., M. N. Pensiero, C. W. Diffenbach, C. B. Cardellichio, A. A. Basile, P. E. Elia, and K. V. Holmes.** 1993b. Mouse hepatitis virus strain A59 and blocking antireceptor monoclonal antibody bind to the N-terminal domain of cellular receptor. Proc. Natl. Acad. Sci. USA **90:**1716–1720.
9. **Dveksler, G. S., M. N. Pensiero, C. B. Cardellichio, R. K. Williams, G. Jiang, K. V. Holmes, and C. W. Diffenbach.** 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.
10. **Ergun, S., N. Kilik, G. Ziegeler, A. Hansen, P. Nollau, J. Gotze, J. H. Wurmbach, A. Horst, J. Weil, M. Fernando, and C. Wagener.** 2000. CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. Mol. Cell **5:**311–320.
11. **Gallagher, T. M., and M. Buchmeier.** 2001. Coronavirus spike proteins in viral entry and pathogenesis. Virology **297:**371–374.
12. **Gallagher, T. M., C. Escarmis, and M. J. Buchmeier.** 1991. Alteration of the pH dependence of coronavirus-induced cell fusion: effect of mutations in the spike glycoprotein. J. Virol. **65:**1916–1938.
13. **Godfraind, C., S. G. Langreth, C. B. Cardellichio, R. Knobler, J. P. Coutelier, M. Dubois-Dalcq, and K. V. Holmes.** 1995. Tissue and cellular distri-

bution of an adhesion molecule in the carcinoembryonic antigen family that serves as a receptor for mouse hepatitis virus. Lab. Investig. **73:**615–627.
14. **Knauss, D. J., and J. A. T. Young.** 2002. A fifteen-amino-acid TVB peptide serves as a minimal soluble receptor for subgroup B avian leukosis and sarcoma viruses. J. Virol. **76:**5404–5410.
15. **Ksiazek, T. G., et al.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
16. **Kubo, H., S. Yoden-Takase, and F. Taguchi.** 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.
17. **Kubo, H., Y. K. Yamada, and F. Taguchi.** 1994. Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino acids of the murine coronavirus spike protein. J. Virol. **68:**5403–5410.
18. **Kumanishi, T.** 1967. Brain tumors induced with Rous sarcoma virus, Schmidt-Ruppin strain. I. Induction of brain tumors in adult mice with Rous chicken sarcoma cells. Japan. J. Exp. Med. **37:**461–474.
19. **Lai, M. M. C., and D. Cavanagh.** 1997. The molecular biology of coronaviruses. Adv. Virus Res. **48:**1–100.
20. **Lewicki, D. N., and T. M. Gallagher.** 2002. Quaternary structure of coronavirus spikes in complex with CEACAM cellular receptors. J. Biol. Chem. **277:**19727–19734.
21. **Luo, Z., and S. Weiss.** 1998. Roles in cell-to-cell fusion of two conserved hydrophobic regions in the murine coronavirus spike protein. Virology **244:**483–494.
22. **Matsuyama, S., and F. Taguchi.** 2000. Impaired entry of soluble receptor-resistant mutants of mouse hepatitis virus into cells expressing MHVR2 receptor. Virology **273:**80–89.
23. **Matsuyama, S., and F. Taguchi.** 2002. Combination between S1N330 and a region in S2 of murine coronavirus spike protein is important for virus entry into cells expressing CEACAM1b receptor. Virology **295:**160–171.
24. **Matsuyama, S., and F. Taguchi.** 2002. Receptor-induced conformational changes of murine coronavirus spike protein. J. Virol. **76:**11819–11826.
25. **McCuaig, K., C. Turbide, and N. Beauchemin.** 1992. MmCGM1a: a mouse carcinoembryonic antigen gene family member, generated by alternative splicing, functions as an adhesion molecule. Cell Growth Differ. **3:**165–174.
26. **Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56:**855–865.
27. **Obrink, B.** 1997. CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. Curr. Opin. Cell Biol. **9:**616–626.
28. **Ohtsuka, N., Y. K. Yamada, and F. Taguchi.** 1996. Difference of virus-binding activity of two receptor proteins for mouse hepatitis virus. J. Gen. Virol. **77:**1683–1692.
29. **Routledge, E., R. Stauber, M. Pfleiderer, and S. G. Siddell.** 1991. Analysis of murine coronavirus surface glycoprotein functions by using monoclonal antibodies. J. Virol. **65:**254–262.
30. **Saeki, K., N. Ohtsuka, and F. Taguchi.** 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.
31. **Sodroski, J. G.** 1999. HIV-1 entry inhibitors in the side pocket. Cell **99:**243–246.
32. **Sturman, L. S., Ricard, C. S., and K. V. Holmes.** 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.
33. **Suzuki, H., and F. Taguchi.** 1996. Analysis of the receptor binding site of murine coronavirus spike glycoprotein. J. Virol. **70:**2632–2636.
34. **Taguchi, F.** 1993. Fusion formation by uncleaved spike protein of murine coronavirus JHMV variant cl-2. J. Virol. **67:**1195–1202.
35. **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. Top. Virol. **1:**245–252.
36. **Taguchi, F., and S. Matsuyama.** 2002. Soluble receptor potentiates receptor-independent infection by murine coronavirus. J. Virol. **76:**950–958.
37. **Taguchi, F., and Y. K. Shimazaki.** 2000. Functional analysis of an epitope in the S2 subunit of murine coronavirus spike protein: involvement in fusion activity. J. Gen. Virol. **81:**2867–2871.
38. **Taguchi, F., S. G. Siddell, H. Wege, and V. ter Meulen.** 1985. Characterization of a variant virus selected in rat brains after infection by coronavirus mouse hepatitis virus JHM. J. Virol. **54:**429–435.
39. **Tatsuo, H., N. Ono, K. Tanaka, and Y. Yanagi.** 2000. SLAM (CDw150) is a cellular receptor for measles virus. Nature **406:**893–897.
40. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Ann. Rev. Physiol. **52:**675–697.
41. **Williams, R. K., G. S. Jiang, and K. V. Holmes.** 1991. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc. Natl. Acad. Sci. USA **88:**5533–5536.
42. **Yamada, Y. K., M. Yabe, A. Yamada, and F. Taguchi.** 1993. Detection of mouse hepatitis virus by the polymerase chain reaction and its application to the rapid diagnosis of infection. Lab. Anim. Sci. **43:**285–290.
43. **Yokomori, K., and M. M. C. Lai.** 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.
44. **Zelus, B. D., D. R. Wessner, R. K. Williams, M. N. Pensiero, F. T. Phibbs, M. deSouza, G. S. Dveksler, and K. V. Holmes.** 1998. Purified, soluble recombinant mouse hepatitis virus receptor, $Bgp1^b$, and Bgp2 murine coronavirus receptors differ in mouse hepatitis virus binding and neutralizing activities. J. Virol. **72:**7237–7244.