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Receptor-Induced Conformational Changes of Murine Coronavirus Spike Protein

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Although murine coronavirus mouse hepatitis virus (MHV) enters cells by virus-cell membrane fusion triggered by its spike (S) protein, it is not well known how the S protein participates in fusion events. We reported that the soluble form of MHV receptor (soMHVR) transformed a nonfusogenic S protein into a fusogenic one (F. Taguchi and S. Matsuyama, J. Virol. 76:950-958, 2002). In the present study, we demonstrate that soMHVR induces the conformational changes of the S protein, as shown by the proteinase digestion test. A cl-2 mutant, srr7, of the MHV JHM virus (JHMV) was digested with proteinase K after treatment with soMHVR, and the resultant S protein was analyzed by Western blotting using monoclonal antibody (MAb) 10G, specific for the membrane-anchored S2 subunit. A 58-kDa fragment, encompassing the two heptad repeats in S2, was detected when srr7 was digested after soMHVR treatment, while no band was seen when the virus was untreated. The appearance of the proteinase-resistant fragment was dependent on the temperature and time of srr7 incubation with soMHVR and also on the concentration of soMHVR. Coimmunoprecipitation indicated that the direct binding of soMHVR to srr7 S protein induced these conformational changes; this was also suggested by the inhibition of the changes following pretreatment of soMHVR with anti-MHVR MAb CC1. soMHVR induced conformational changes of the S proteins of wild-type (wt) JHMV cl-2, as well as revertants from srr7, srr7A and srr7B; however, a major proportion of these S proteins were resistant to proteinase K even without soMHVR treatment. The implications of this proteinase-resistant fraction are discussed. This is the first report on receptor-induced conformational changes of the membrane-anchored fragment of the coronavirus S protein.

The binding of virus to its receptor on the target cell is an initial step of infection. The surface glycoprotein comprising the virus spikes of enveloped viruses is responsible for this binding. Following binding, the surface glycoprotein mediates the fusion of the viral envelope and cell membrane. Either plasma or the endosomal membrane is fused with the viral envelope. Influenza virus virions are incorporated into the endosome by receptor-mediated endocytosis. Subsequently, the viral hemagglutinin (HA) is activated by the low-pH environment of the endosome and converted from a nonfusogenic form to a fusogenic form. This functional conversion is accompanied by conformational changes of the HA protein (51). In contrast, human immunodeficiency virus (HIV) is thought to enter the cell directly from the plasma membrane via a nonendosomal pathway. The HIV envelope protein is also converted from a nonfusogenic form to a fusogenic form by binding to its receptor and coreceptor. This is associated by conformational changes of the envelope protein (4, 40). Through the fusion of the viral envelope and cell membrane, the viral genome is released into the cell interior and replication is initiated. Murine coronavirus mouse hepatitis virus (MHV) induces syncytia in infected cultured cells in a pH-independent fashion, suggesting that MHV enters into cells by a nonendosomal pathway. However, the MHV entry pathway is still a matter of controversy (19, 28, 31, 32).

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MHV is an enveloped virus with a positive-stranded, nonsegmented genomic RNA of about 32 kb (23). MHV infects cells via MHV-specific receptor proteins. Several different molecules function as MHV receptors (MHVR) (1, 5, 12, 33), among which CEACAM1 is the most prevalent (12, 35). MHVR is an immunoglobulin superfamily protein with four or two ectodomains. Two allelic forms of MHVR are known, i.e., CEACAM1a (MHVR1) and CEACAM1b (MHVR2) (10, 35, 53). MHVR1 is derived from MHV-susceptible BALB/c mice, and MHVR2 is derived from resistant SJL mice; the former has a receptor function that is 10 to 100 times higher than that of the latter (34, 36). The N-terminal ectodomain of MHVR is sufficient for receptor function (9, 11).

The viral protein that interacts with MHVR is the spike (S) protein. The S protein is synthesized as a 180- to 200-kDa protein that is cleaved into two subunits by host-derived protease (41). The N-terminal subunit, called S1, forms the surface knob-like structure of the spike, and the C-terminal, membrane-anchored S2 subunit forms the stem-like structure beneath the knob (8). Each spike is composed of two molecules of the S1-S2 heterodimer (24). The S protein is a multifunctional protein (44). It is responsible for receptor binding, which is mediated by the N-terminal 330 amino acids of the S1 subunit (S1N330) (21, 42). Recently, it was shown by using soluble S1 that the dimeric conformation of S1N330 is important for receptor binding activity (24). Various different regions of the membrane-anchored S2 subunit are reported to be important for viral entry into cells (15, 16, 26, 48), though some regions in S1 affect the efficiency of entry (20, 30).

Wild type (wt) JHM virus (JHMV) is known to spread to

cells in an MHVR-independent fashion (14), while a soluble-receptor-resistant mutant (srr7), derived from wt virus, failed to spread in this manner (46, 47); wt infection spreads from DBT cells infected via MHVR to MHVR-deficient BHK cells, but srr7 infection failed to do so. Recently, we reported that soluble MHVR (soMHVR) potentiated an MHVR-independent infection of srr7 (46). This observation suggested that the binding of soMHVR to srr7 S protein converted a fusion-negative phenotype to a fusion-positive phenotype (46). This conversion is conceivably similar to that which takes place when the virion S protein binds to the cellular receptor. Thus, soMHVR behaves as a cellular receptor to activate the virion S protein to trigger viral envelope-cell membrane fusion.

Conformational changes of the envelope protein in a number of viruses following binding to the receptor have been revealed by the appearance of a proteinase-resistant fragment or antigenic changes (4, 17, 39, 51). The conformational changes of the membrane-anchored fragment are thought to be directly associated with functional activation (4, 51). Recently, Lewicki and Gallagher reported conformational changes in the surface fragment of the MHV S protein, the S1 subunit, after binding to MHVR (24). However, the conformational changes of the membrane-anchored subunit S2 that may be responsible for fusion between viral and cell membranes have not been elucidated, though functional activation was clearly demonstrated (46).

In the present study, we attempt to detect such conformational changes and demonstrate by a proteinase digestion assay that the srr7 S protein undergoes conformational changes after binding to soMHVR. We also show that a large proportion of the wt JHMV S proteins have conformational changes without binding to soMHVR. The biological significance of these findings is also discussed.

MATERIALS AND METHODS

Cells and viruses. DBT cells expressing MHVR1 (22) and BHK 13 (BHK) cells devoid of MHVR were grown in Dulbecco's minimal essential medium (DMEM; Nissui, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS; Gibco BRL, Grand Island, N.Y.) (DMEM-FBS). DBT cells were used for MHV infection, titration, and propagation. BHK cells nonpermissive to MHV were target cells for MHVR-independent infection as described previously (46). wt MHV JHMV cl-2 (49) and its mutant, JHMV srr7 (38), were propagated in DBT cells, and the supernatants of culture fluids were used for infection as previously reported (50). Two revertant viruses from srr7, in terms of infectivity in cells expressing MHVR2, srr7A and srr7B (30), were also used in this study. srr7 has a mutated amino acid at position 1114 (Leu to Phe) in the S protein compared with wt virus. Relative to srr7, srr7A has two additional mutations in S1, at positions 278 (Ile to Leu) and 461 (Ser to Thr), while srr7B has one additional mutation at 286 (Ser to Ile) (30). Recombinant vaccinia virus vTF7.3, harboring the T7 RNA polymerase gene, was provided by B. Moss (13) and was used to express the MHV S protein as described previously (43).

Expression and purification of soMHVR. soMHVR with three different tags, influenza virus HA, myc, and six histidines (six-His) at its C terminus was expressed by recombinant baculovirus as previously reported (46). Insect Tn5 cells were infected with the recombinant virus at a multiplicity of infection of 1 or more and incubated at 26°C for 1 h. The cells were cultured with Ex-cell 405 medium (Gibco BRL) at 26°C for 3 days. The culture fluids were centrifuged at 12,000 rpm (ca. 12,000 × *g*) for 30 min, and the clarified culture fluids were mixed with polyethylene glycol 6000 at a final concentration of 20%. Following incubation at 4°C for 1 to 2 h, the mixture was centrifuged at 8,000 rpm (ca. 10,000 × *g*) for 30 min, and the resultant precipitate was dissolved in a small volume of a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). soMHVR with a six-His tag was purified by Ni-nitrilotriacetic acid (Qiagen, Hilden, Germany) affinity chromatography according to the manufacturer's instructions. The concentration and purity of the expressed soMHVR was examined by Western blot analysis as described previously (38).

Proteinase digestion assay. For the proteinase digestion assay, 8 μl of virus particles (5×10^7 PFU/ml) was incubated with 2 μl of 200 nM soMHVR or 200 nM bovine serum albumin (BSA; New England Biolabs, Beverly, Mass.) at 37°C for 30 min. Samples kept on ice for 5 min were mixed with 1 μl of a solution containing 20 mg of proteinase K (Wako, Tokyo, Japan), 15 μg of thermolysin (Sigma, St Louis, Mo.), or 100 μg of trypsin (Sigma)/ml and incubated at 4°C for 20 min. In some experiments using proteinase K, various concentrations (0.064 to 1,000 nM) of soMHVR were mixed with virus samples and incubated for different lengths of time and at different temperatures (4, 25, 37, and 42°C). In most of the experiments, proteinase K digestion was done at 4°C for 20 min. Reactions were stopped by treatment with 5 μl of electrophoresis sample buffer (0.125 M Tris [pH 6.8], 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate [SDS], 10% sucrose, 0.004% bromophenol blue) and subjected to SDS–10% polyacrylamide gel electrophoresis (SDS–10% PAGE), as described elsewhere (38). The digested S proteins were analyzed by Western blotting using anti-JHMV S2 monoclonal antibody (MAb) 10G (37), kindly provided by S. G. Siddell. In some experiments, anti-S2A rabbit serum (48) was also used to detect the digested S protein via enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.) as described previously (38). To neutralize the effect of soMHVR, 8 nM soMHVR1 was mixed with various concentrations (0.187 to 6.0 mg/ml) of anti-MHVR MAb CC1 (11, 52), kindly provided by K. V. Holmes, and incubated at 4°C for 20 min. Then, CC1-treated soMHVR was mixed with virus particles, and the mixture was subjected to digestion with proteinase K as described above.

MHVR-independent infection. MHVR-independent infection of BHK cells was done essentially as described previously (46). Confluent DBT cells in 24-well dishes (Iwaki, Tokyo, Japan) were infected with MHV at a multiplicity of infection of 1. After 1 h of incubation at 37°C, the cells were incubated with DMEM-FBS at 37°C for 3 to 4 h. Then, these cells were treated with trypsin to produce a single-cell suspension in DMEM-FBS. One hundred microliters of the suspension containing 10^4 DBT cells was overlaid onto confluent BHK cells (8×10^5 to 10×10^5) cultured in 200 μl of DMEM-FBS in collagen-treated 24-well plates. Mixed cells were incubated at 37°C for a further 12 to 24 h in the presence or absence of soMHVR (40 nM, final concentration). The mixed-cell culture was then fixed with 5% formalin and stained with 0.1% crystal violet dissolved in 50 mM boric acid. Syncytia were counted as plaques of MHVR-independent infection.

Coimmunoprecipitation. Coimmunoprecipitation was performed to see the direct binding of soMHVR and S protein. Eight microliters of virus particles (10^7 PFU/ml) was incubated with 2 μl of various concentrations (0.32 to 200 nM) of soMHVR or 200 nM BSA at 37°C for 30 min. Samples were incubated for 1 h at room temperature with anti-soMHVR (anti-HA MAb 12CA5) which had been coupled to protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). After being washed four times with phosphate-buffered saline, pH 7.2, each sample was treated with electrophoresis sample buffer and subjected to SDS-PAGE (38). Coimmunoprecipitated S proteins were analyzed by Western blotting using anti-JHMV S1 MAb 30B (37), a gift from S. G. Siddell. JHMV S proteins were detected by ECL as described previously (38).

Expression of MHV S proteins in BHK cells. MHV S proteins were expressed in BHK cells by a transient vaccinia virus expression system as previously reported (38, 43). BHK cells cultured in 60-mm-diameter dishes (Iwaki) were infected with vTF7.3, a recombinant vaccinia virus harboring the T7 RNA polymerase gene (13). After 1 h at 37°C, the cells were trypsinized and transfected with plasmids containing the JHMV wt (45), srr7, srr7A, or srr7B S gene under the control of the T7 promoter (30), i.e., pTarget cl-2S, pTarget srr7-S, pTarget srr7A-S, or pTarget srr7B-S, by electroporation with a Gene Pulser (Bio-Rad, Hercules, Calif.) (38). After incubation at 37°C for 12 h, cells were collected with a rubber policeman and lysed by 50 μl of buffer (phosphate-buffered saline containing 0.65% Nonidet P-40). The supernatants were used for the proteinase digestion assay.

RESULTS

Detection of srr7 S conformational changes by proteinase digestion assay. We have recently shown that the srr7 S protein is transformed from a nonfusogenic type to a fusogenic type by soMHVR, while the wt JHMV S protein is fusogenic without soMHVR treatment (46). This finding suggested that the srr7 S protein undergoes conformational changes by the interaction

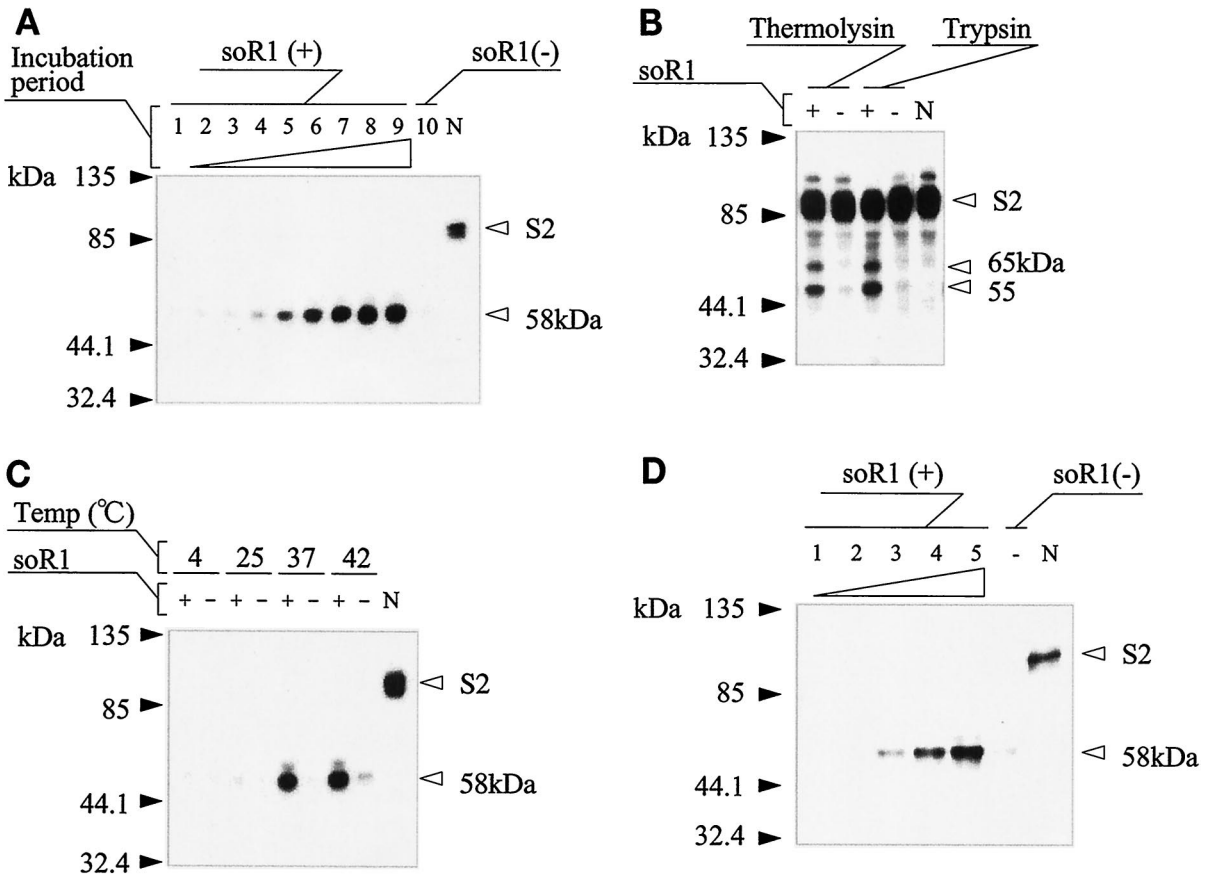

FIG. 1. soMHVR-induced conformational changes of the srr7 S protein detected by proteinase K digestion. (A) Incubation period-dependent conformational changes. srr7 virions were incubated with either 40 nM (final concentration) soMHVR [soR1(+)] or BSA [soR1(−)] at 37°C for various periods (lanes 1 to 10, 0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 64 min, respectively). Then, samples were digested with 2 mg of proteinase K/ml (final concentration) at 4°C for 20 min and subjected to SDS-PAGE. After transfer to nitrocellulose paper, a proteinase K-resistant fraction was detected by ECL with MAb 10G, specific for the S2 subunit. (B) Analysis of conformational changes due to thermolysin and trypsin. srr7 virions were incubated with either 40 nM soMHVR (+) or BSA (−) at 37°C for 30 min, and then samples were digested with 15 μg of thermolysin/ml or 100 μg of trypsin/ml at 4°C for 30 min. The fragment resistant to these proteinases was detected by SDS-PAGE and Western blotting as described for panel A. (C) Temperature-dependent conformational changes of the srr7 S protein. srr7 virions were incubated with either 40 nM soMHVR (+) or BSA (−) at the indicated temperature for 30 min. Then, the proteinase K digestion test was carried out as described for panel A. (D) soMHVR concentration-dependent conformational changes of the srr7 S protein. srr7 virions were incubated with various concentrations of soMHVR1 [soR1(+); lanes 1 to 5, 0.5, 1, 2, 4, and 8 nM, respectively] or BSA [soR1(−)] at 37°C for 30 min. Then, the samples were digested with proteinase K and analyzed by Western blotting as described for panel A. Lanes N (all panels), virion S2 protein not treated with soMHVR or proteinase K.

with soMHVR, as reported for various enveloped viruses (4, 6, 51). Thus, we examined by proteinase K digestion test whether soMHVR induced conformational changes of the srr7 S protein. Eight microliters of srr7 virus, 5×10^7 PFU/ml, was mixed with 2 μl of 200 nM soMHVR, incubated at 37°C for various periods of time, and then digested with proteinase K at a final concentration of 2 mg/ml at 4°C for 20 min. The treated virus S protein was analyzed by Western blotting with JHMV S2-specific MAb 10G after SDS-PAGE as described in Materials and Methods. As shown in Fig. 1A, a clear band of 58 kDa was detected when the virus was incubated with soMHVR but not when it was incubated without soMHVR. Occasionally, trace amounts of the 58-kDa band were detected even without soMHVR treatment (data not shown). The amounts of this band increased depending on the length of the incubation period. After 16 to 32 min, the band reached a peak. This result indicates that the srr7 S protein undergoes conformational changes after binding to soMHVR in an incubation time-dependent manner.

We have then examined whether other proteinases can also detect this difference. Srr7 virions treated with soMHVR at 37°C for 30 min were digested with either thermolysin (final concentration, 15 μg/ml) or trypsin (final concentration, 100 μg/ml) for 30 min at 4°C and analyzed by Western blotting in a manner similar to that in the proteinase K digestion test. As shown in Fig. 1B, two distinct bands of 65 and 55 kDa were clearly detected in the soMHVR-treated virions but not in the untreated virions. However, the majority of the S2 was not digested, and some minor bands were visible when virions were treated by these proteinases. All of these results suggested that soMHVR binding to the srr7 S protein converted S2 from a proteinase-susceptible form to a proteinase-resistant form. Since the most convincing results were obtained with proteinase K, we have further examined the nature of the soMHVR-

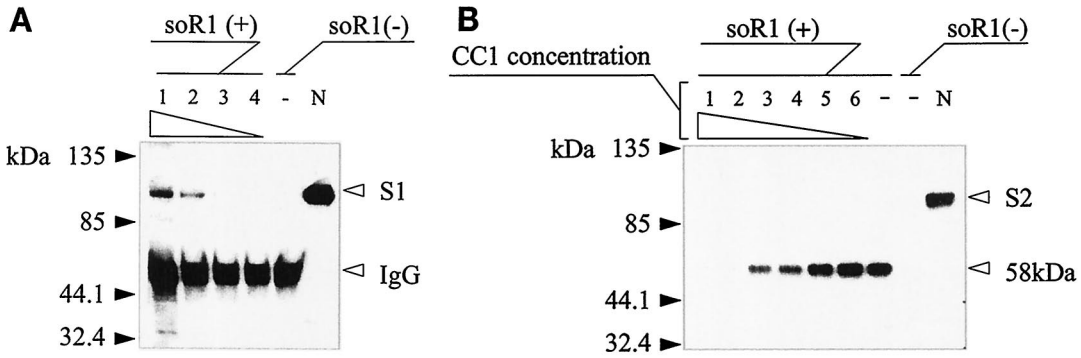

FIG. 2. Interaction between srr7 S protein and soMHVR1. (A) Direct binding of soMHVR to srr7 S protein. srr7 virions were incubated with soMHVR1 [soR1(+); lanes 1 to 4, 8, 1.6, 0.32, and 0.064 nM, respectively] or BSA [soR1(−)] at 37°C for 30 min. Then, the mixture was immunoprecipitated with anti-soMHVR, a MAb against the tagged HA peptide, and the precipitated materials were analyzed by SDS-PAGE and Western blotting using S1-specific MAb 30B. Lane N, native srr7 S1. IgG, immunoglobulin G. (B) Inhibition of soMHVR-induced conformational changes by the anti-MHVR antibody. soMHVR (8 nM) was incubated with anti-MHVR MAb CC1 [soR1(+); lanes 1 to 6, 6, 3, 1.5, 0.75, 0.38, and 0.19 mg/ml, respectively] or BSA [soR1(−)] at 4°C for 30 min. Then srr7 was added to the mixture, and the mixture was incubated at 37°C for 30 min and subjected to proteinase K digestion as described for Fig. 1A. Lane N, native srr7 S2.

induced conformational changes of the srr7 S protein with proteinase K.

To determine the temperature necessary for conformational changes, we mixed srr7 virus with soMHVR at a final concentration of 40 nM and incubated the mixture at 4, 25, 37, or 42°C for 30 min and then subjected it to proteinase K digestion as described above. Figure 1C shows that conformational changes took place at 37 and 42°C, while they were not observed at temperatures under 25°C. The finding of a low level of conformational change or none at lower temperatures was not due to the reduced binding of soMHVR to the S protein, since it is evident that srr7 S bound to MHVR efficiently at 25°C as previously reported (29, 38). Binding at 4°C was also confirmed (data not shown). These results, taken together, suggested that conformational changes of the srr7 S protein due to soMHVR occurred at 37°C or higher. This result is compatible with the observation that srr7 is not neutralized at room temperature (22 to 24°C), while it is neutralized at 37°C (46).

We also examined the soMHVR concentration-dependent activation of the srr7 S protein. srr7 virions were mixed with various concentrations of soMHVR and incubated at 37°C for 30 min. The mixture was then digested with proteinase K as described above. As shown in Fig. 1D, a band of 58 kDa, which became stronger with increasing amounts of soMHVR mixed with srr7, was seen. The band was detected when srr7 was mixed with 2 to 8 nM soMHVR but was not detected at concentrations lower than 1 nM soMHVR. For the S protein conformational changes a slightly higher concentration of soMHVR than that required for neutralization activity was necessary (1 neutralization unit is about 1 nM [46]). One neutralization unit corresponds to the concentration necessary to neutralize 50% of MHV plaques, as previously reported (50).

Evidence that the direct binding of soMHVR to the srr7 S protein induces conformational changes. To learn whether soMHVR binds to the virion S protein under the conditions we have employed to see the conformational changes, coimmunoprecipitation was done. srr7 virions were mixed with various concentrations of soMHVR and incubated at 37°C for 30 min. This mixture was immunoprecipitated by an anti-soMHVR antibody, the anti-HA antibody recognizing the HA epitope tag at the C terminus of soMHVR. Precipitants were analyzed for the presence of the S protein by SDS-PAGE and Western blotting using anti-MHV S1 MAb 30B. As shown in Fig. 2A, the MHV S protein was coimmunoprecipitated in an soMHVR concentration-dependent fashion, indicating the direct binding soMHVR with the srr7 S protein. We further examined whether anti-MHVR antibody CC1, which prevents MHVR binding to the S protein (11, 52), was able to prevent soMHVR-induced conformational changes. Serially diluted CC1 was mixed with 8 nM soMHVR and incubated at 4°C for 30 min. To this mixture, srr7 was added, the mixture was further incubated at 37°C for 30 min, and the resistance of srr7 S to proteinase K digestion was examined. As shown in Fig. 2B, conversion to a proteinase K-resistant form was prevented in a CC1 concentration-dependent fashion, suggesting that the binding of soMHVR to the srr7 S protein is critical for the induction of conformational changes.

Conformational changes of wt JHMV and revertant S proteins. We examined whether the S proteins of wt JHMV cl-2 and revertants derived from srr7 undergo conformational changes similar to those of srr7 following the interaction with soMHVR. srr7 has a unique feature in that it infects cells expressing MHVR1 as efficiently as does wt virus, while it fails to infect cells expressing MHVR2. By serial passages of srr7 through MHVR2 cells, we obtained two revertants, srr7A and srr7B, that infect MHVR2 cells as efficiently as does wt JHMV. These revertants have mutations in S1N330-III, in addition to a mutation at amino acid 1114 that stemmed from srr7 (30). The wt and revertant viruses were treated with various concentrations of soMHVR, and the S protein resistant to proteinase K digestion was examined as described above. As shown in Fig. 3, a 58-kDa band was visible even in the viruses not treated with soMHVR, indicating that the S proteins of these viruses were resistant to proteinase K digestion without binding to soMHVR. However, treatment with soMHVR had a propensity to result in increased density of the 58-kDa band; this suggests that a proportion of the S proteins of these viruses were conformationally changed by soMHVR. These observa-

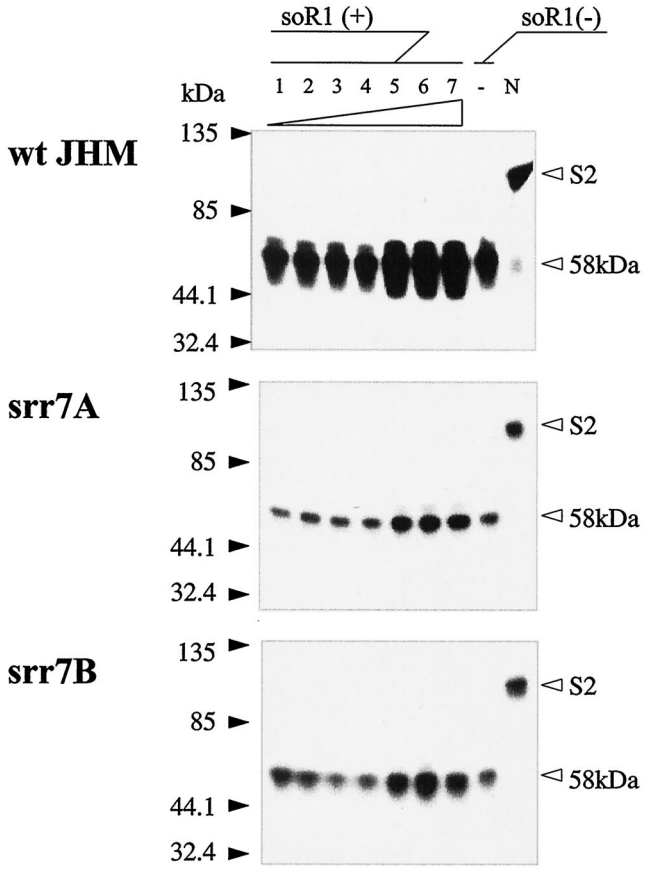

FIG. 3. Conformational changes of wt JHMV and revertant S proteins induced by soMHVR. The wt JHMV, srr7A, and srr7B virions were incubated with soMHVR1 [soR1(+); lanes 1 to 7, 0.0128, 0.064, 0.32, 1.6, 8, 40, and 200 nM, respectively] or BSA [soR1(−)] at 37°C for 30 min. Then, the proteinase K digestion test was carried out for these viruses as described in the legend to Fig. 1A. Lane N (each panel), native virion S2.

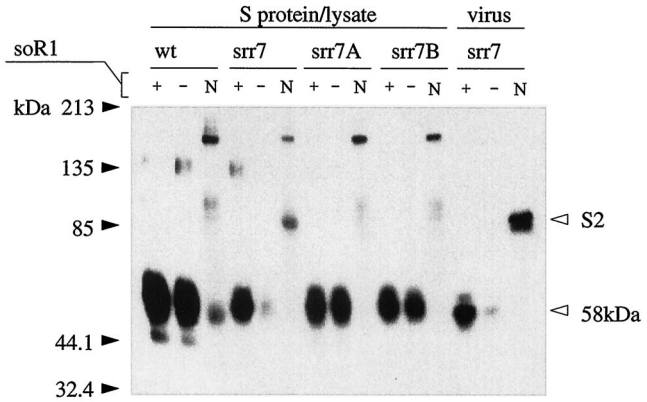

FIG. 4. Proteinase K digestion assay of S proteins expressed in BHK cells. Lysates of BHK cells expressing various S proteins were incubated with either 40 nM soMHVR (+) or BSA (−) at 37°C for 30 min. The samples were then digested with proteinase K as described in the Fig. 1A legend. As a control (virus lanes), srr7 virions propagated in MHVR-positive DBT cells were treated with soMHVR and proteinase K as described for Fig. 1A. Lanes N, native S2 protein of each virus.

tions suggested that JHMV S proteins, with the exception of srr7, have a conformationally changed S protein without interaction with soMHVR. Since the conformational changes of S proteins of these viruses could result from the previous interaction with MHVR that is expressed within or on the cells in which the viruses replicate, we performed a proteinase K digestion assay using the S proteins expressed in MHVR-deficient BHK cells. Those S proteins were expressed by a vaccinia virus expression system using vTF7.3 as described in Materials and Methods. Cell lysates were mixed with soMHVR (final concentration, 40 nM), incubated at 37°C for 30 min, and subjected to proteinase K digestion. The results are shown in Fig. 4. The srr7 S protein expressed in BHK cells was almost thoroughly digested with proteinase K when not treated with soMHVR, whereas a 58-kDa band was visible when it was treated with soMHVR; these results are very similar to the protease K digestion results obtained by using virions produced in MHVR-positive cells. The S proteins of the wt, srr7A, and srr7B were resistant to protease K digestion, as shown by the existence of the 58-kDa protein even without treatment with soMHVR. These results were compatible to those shown in Fig. 3, in which viruses were propagated in MHVR-positive DBT cells, suggesting that the proteinase-resistant forms of wt, srr7A, and srr7B S proteins did not result from the previous interaction with MHVR. In Fig. 4, a strong band of about 175 kDa, corresponding to the uncleaved S, was also seen in all samples not treated with proteinase K. Moreover, the amounts of S2 were not large. These features are different from those obtained by using viruses prepared from DBT cells (Fig. 3). A difference in the cleavability of S proteins in these cells could account for the difference in S protein pattern.

MHVR-independent infection of JHMV and its mutants. We reported previously that wt JHMV cl-2 spread from infected DBT cells to MHVR-deficient BHK cells (MHVR-independent infection), while srr7 failed to spread by this mode of infection (46). In this study we showed that the wt virus has a proteinase-resistant form of S protein, while srr7 has no such form. These findings collectively suggest a correlation between MHVR-independent infection and the presence of a proteinase-resistant form of S2. Then, we examined whether or not srr7A and srr7B, which retained a proteinase-resistant form of the S protein similar to that of wt S, were able to spread in an MHVR-independent fashion. DBT cells infected with these viruses were overlaid onto BHK cells and cultured overnight in the presence or absence of soMHVR (final concentration, 40 nM). The syncytia produced were counted after staining with crystal violet. As shown in Fig. 5, wt virus produced syncytia even in the absence of soMHVR, while srr7 produced syncytia only in the presence of soMHVR, confirming our previous observation (46). Treatment with soMHVR did not increase the number of syncytia produced by wt virus. srr7A and srr7B produced syncytia in a fashion similar to that of wt JHMV but different from that of srr7. These two revertants from srr7 produced syncytia even in the absence of soMHVR, showing that S proteins of these viruses are functionally active without being bound by MHVR. These results indicated that the ability of the wt and srr mutants to infect cells in an MHVR-inde-

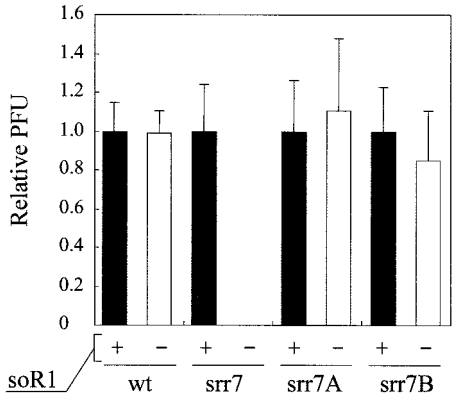

FIG. 5. Effect of soMHVR on MHVR-independent infection. Confluent BHK cells were overlaid with 10^4 DBT cells infected with wt JHMV or srr mutants and incubated at 37°C for 12 h in the presence (+) or absence (−) of 40 nM soMHVR. The syncytia were counted by staining with crystal violet. The relative number of syncytia in each virus was determined by the following formula; relative syncytium number = syncytium number obtained/syncytium number obtained in the presence of soMHVR. Error bars, standard deviations of three independent samples.

pendent manner correlated well with the presence of a proteinase-resistant form of S protein on the virions.

Mapping of a proteinase K-resistant fragment in the S2 subunit. A 58-kDa fragment resistant to proteinase K digestion was recognized by MAb 10G, whose epitope was mapped between amino acids 1264 and 1278 in the JHMV cl-2 S protein in a total of 1,376 amino acids (37). The epitope is inside heptad repeat 2 (HR2) and about 40 amino acids upstream from the transmembrane domain (Fig. 6A). To find the precise location of the 58-kDa fragment, we examined whether it was recognized by the antibody specific for another epitope, S2A (47), mapped about 370 amino acids upstream from the 10G epitope, between amino acids 899 and 908 (27) (Fig. 6A). An S protein treated with soMHVR and subsequently digested with proteinase K, as described above, was examined for its reactivity to the anti-S2A antibody by Western blotting. As shown in Fig. 6B, the native S2 protein was recognized by both anti-S2A and 10G, while the 58-kDa fragment, detectable by 10G, was not detected by anti-S2A. Judging from the size of the 58-kDa fragment, it consists of ca. 400 amino acids in JHMV S2. Moreover, it was found that this fragment does not include S2A. This fragment should consist of a region including, at least, HR1 and most of HR2. The appearance of a proteinase-resistant fragment in the HRs of MHV S2 following receptor binding is very similar to that observed in other viruses (25).

DISCUSSION

The surface glycoproteins of a number of enveloped viruses have been shown to bind to their specific receptors on the cell surface, which is a critical, initial step of virus infection. The receptor binding induces surface protein conformational changes, as revealed by proteinase resistance or antigenic changes (17, 39, 40, 51). These conformational changes are thought to be essential for the functional activation of the protein from a fusion-negative form to a fusion-positive form, which mediates viral entry into the cell (6, 40, 46, 51). The activated glycoprotein also develops affinity for the liposome, which is thought to be mediated by a fusion peptide (7, 18). In murine coronavirus MHV, we recently showed that the binding of soMHVR to the surface S glycoprotein functionally activates the S protein from a fusion-negative type to a fusion-positive type (46). In the present study, we attempted to detect the conformational changes of the activated S protein. We have tried in vain to detect changes in the antigenicity of the soMHVR-bound S protein by using different MAbs. However, we succeeded in detecting the difference between S proteins treated and not treated with soMHVR in terms of resistance to three different proteases, among which proteinase K displayed the most prominent results. The proteinase K-resistant fragment was induced in a membrane-anchored S2 subunit by soMHVR. The fragment was revealed to consist roughly of the

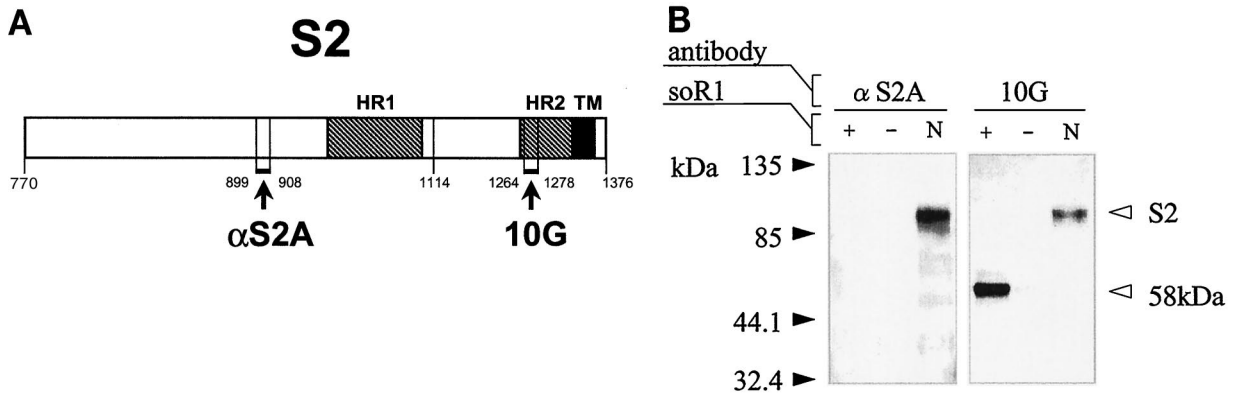

FIG. 6. Mapping of a 58-kDa fragment resistant to proteinase K digestion. (A) Schematic diagram of JHMV cl-2 S2 subunit. The locations of two HRs, the transmembrane domain (TM), and epitopes recognized by two antibodies (10G and anti-S2A) are depicted. Amino acid position 1114 is mutated in srr7S (L to F) relative to wt JHMV. (B) Western blot analysis of 58-kDa protein using anti-S2A and 10G. srr7 virions were treated (+) or not treated (−) with soMHVR and subsequently digested with proteinase K as described in the Fig. 1A legend. After SDS-PAGE and Western blotting, a 58-kDa band was examined for its reactivity to the anti-S2A antibody as well as MAb 10G. The untreated virion S2 protein (lanes N) was also examined as a control.

region encompassing two HRs. This is the first report in coronaviruses that a surface glycoprotein membrane-anchored subunit undergoes conformational changes after binding to soluble receptor that are accompanied by functional conversion (46).

Receptor-binding and subsequent conformational changes in the envelope protein in two different viruses, influenza virus and HIV, have been extensively studied (40, 51). The conditions triggering conformational changes in these virus envelope proteins are different; influenza virus HA protein conformational changes are induced by a low-pH environment in the endosomes (40), while receptor and coreceptor binding to the HIV Env protein induces its conformational changes (51). In spite of this difference, the transmembrane fragments of those envelope glycoproteins display a great similarity in structure in that they have two HRs that are important for their conformation and for viral entry into cells (2, 3). The MHV transmembrane S2 protein also shares this feature. Of two HRs found in HIV, HR1 is located at the N-terminal region relative to HR2 in the ectodomain. Receptor binding to surface fragment gp120 induces conformational changes in gp41, which result in the formation of alpha-helical bundles consisting of three HR1s and three HR2s (4). These structural changes play a critical role in viral and cell membrane fusion by placing envelope and cell membranes in close proximity. As an analogy of conformational changes in HIV gp41, the HR1 and HR2 of MHV could form the bundle following interaction with MHVR. The 58-kDa fragment detected after treatment with proteinase K in this study, derived from the S2 region consisting of HR1 and HR2, could consist of bundles similar to those found in the HIV gp41 protein.

Although receptor-induced conformational changes were detected for srr7, wt virus and revertants from srr7 were revealed to have a large proportion of proteinase-resistant S proteins without receptor interaction. The viruses with a proteinase-resistant form of the S protein displayed MHVR-independent infection (46) (Fig. 5). Strain MHV-A59 has also been reported to have the ability to spread in an MHVR-independent fashion, although its ability was not as strong as that of JHMV (14, 47). This virus contained a proteinase-resistant form of S protein as well (data not shown). Thus, it seems evident that viruses that spread in an MHVR-independent fashion have a proportion of S proteins with conformational changes. However, the proteinase-resistant S proteins found in the wt and revertants presumably do not execute a fusion of viral and cell membranes, since the process of conformational changes itself must be critical for fusion events, as indicated by HIV gp41 (4). Then how do these viruses spread in an MHVR-independent fashion to MHVR-negative cells? We assume that the conformational changes in the S proteins of these viruses that occur spontaneously, the mechanism of which is not yet understood, will induce fusion of the cell membrane when S proteins expressed on cells are in close contact with MHVR-negative cells.

The proteinase-resistant S proteins of wt JHMV and other viruses did not result from their interaction with receptor proteins in cells in which those viruses multiplied, because S proteins expressed in MHVR-deficient BHK cells displayed the proteinase-resistant form as well (Fig. 6). Whether the S proteins of those viruses are converted to the proteinase-resistant form in the modification process of the protein or are cotranslationally formed as proteinase resistant has not yet been addressed. However, it is evident that the combination of amino acid 1114 in S2, which is mutated in srr mutants, and a region called S1N330-III, which contains mutations of srr7A and srr7B (30), plays an important role in determining the nature of the S protein, namely, whether the S protein remains in its native form or is easily converted to the proteinase-resistant form. Only the combination found in the srr7 S protein does not allow the transition to a proteinase-resistant S2 protein. We recently proposed the importance of this combination between the region in S1 and that in S2 for efficient viral entry into cells expressing MHVR2 (30).

Recently, Lewicki and Gallagher reported that a spike of MHV is composed of two molecules of the S1-S2 heterodimer (24). They also showed that S protein oligomerization was formed between the two molecules of S1 and that the S2 fragments were not maintained as oligomers after S1 was stripped away, suggesting that S1 has a major determinant for the oligomerization of the entire S protein. However, a number of structural studies concerning membrane-anchored fragments suggested an intermolecular interaction through HRs of the membrane-anchored subunit (2, 3). Our present results showed that the conformation of HRs in S2 is changed after MHVR binds to the surface S1 subunit and that this change is very similar to the conformational changes found in HIV and influenza virus (4, 51). Thus, we prefer the idea that S2 plays a major role in the oligomerization of the entire S protein, though there is no evidence to support this idea thus far.

srr7 was selected as a mutant virus resistant to neutralization by soMHVR (38). However, this resistance is conditional, since srr7 is resistant when incubated with soMHVR at room temperature (22 to 25°C) but not resistant when incubated at 37°C (46). wt JHMV cl-2 is highly susceptible to neutralization by soMHVR even when incubated at room temperature. It is also observed in this paper that soMHVR-induced conformational changes of srr7 take place at 37°C or higher but not at 25°C (Fig. 1C). These findings suggest that neutralization of srr7 by soMHVR is not due to the blockade of binding to cellular receptors but rather due to receptor-induced conformational changes of the S protein. The binding of soMHVR to the S protein may be reversible, since soMHVR-treated srr7, whose MHVR binding capacity is the at the same level as that of wt virus (29, 38), can infect cells via cellular receptors; however, receptor-induced conformational changes of the S protein could be irreversible.

In the present study, we have shown the conformational changes of the MHV S protein for the first time. This is clearly observed in mutant virus srr7, but not as clearly seen in wt virus, since a proportion of the wt S is already in a proteinase-resistant form. These findings indicate that srr7 is an ideal virus for further study of the conformational changes of the MHV S protein.

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