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Identification of Spike Protein Residues of Murine Coronavirus Responsible for Receptor-Binding Activity by Use of Soluble Receptor-Resistant Mutants

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We previously demonstrated by site-directed mutagenesis analysis that the amino acid residues at positions 62 and 214 to 216 in the N-terminal region of mouse hepatitis virus (MHV) spike (S) protein are important for receptor-binding activity (H. Suzuki and F. Taguchi, J. Virol. 70:2632–2636, 1996). To further identify the residues responsible for the activity, we isolated the mutant viruses that were not neutralized with the soluble form of MHV receptor proteins, since such mutants were expected to have mutations in amino acids responsible for receptor-binding activity. Five soluble-receptor-resistant (*srr*) mutants isolated had mutations in a single amino acid at three different positions: one was at position 65 (Leu to His) (*srr*11) in the S1 subunit and three were at position 1114 (Leu to Phe) (*srr*3, *srr*4, and *srr*7) and one was at position 1163 (Cys to Phe) (*srr*18) in the S2 subunit. The receptor-binding activity examined by a virus overlay protein blot assay and by a coimmunoprecipitation assay showed that *srr*11 S protein had extremely reduced binding activity, while the *srr*7 and *srr*18 proteins had binding activity similar to that of wild-type cl-2 protein. However, when cell surface receptors were used for the binding assay, all *srr* mutants showed activity similar to that of the wild type or only slightly reduced activity. These results, together with our previous observations, suggest that amino acids located at positions 62 to 65 of S1, a region conserved among the MHV strains examined, are important for receptor-binding activity. We also discuss the mechanism by which *srr* mutants with a mutation in S2 showed high resistance to neutralization by a soluble receptor, despite their sufficient level of binding to soluble receptors.

Mouse hepatitis virus (MHV) is a member of the coronaviruses, which are enveloped, positive-stranded RNA viruses associated with various diseases of economic importance in both animals and humans (26, 33, 34). MHV includes a variety of strains with different organ tropism and disease characteristics (46). Some of these strains serve as animal models for virus-induced neurological diseases and as models for various types of hepatitis (46).

The genome of MHV (about 32 kb) encodes four structural proteins: the 50- to 60-kDa nucleocapsid (N) protein, the 20- to 25-kDa integral membrane (M) glycoprotein, the 150- to 200-kDa spike (S) glycoprotein, and the envelope protein (26, 33, 34). Some strains of MHV have a fifth structural protein, the 65-kDa hemagglutinin-esterase glycoprotein. Several nonstructural proteins are encoded by the genome as well (26, 33, 34).

The S protein, which has a variety of important biological functions, forms the spike projecting from the virion surface (21, 34). The spike comprises two or three molecules of the S protein, each of which is a heterodimer consisting of two noncovalently bound subunits, S1 and S2 (34, 36). The S1 and S2 subunits are derived from the N-terminal and C-terminal halves cleaved from the precursor S protein by a host-cell-derived protease (36). The S1 and S2 subunits are believed to form the globular part of the petal-shaped spike and its stalk portion, respectively (6). Heterogeneity is evident within the S1 subunit, while the S2 subunit has a rather conserved structure among coronaviruses (34). There are two heptad repeats in S2, indicative of the coiled-coil structure of the S protein (6). One of the prominent biological functions of the MHV S protein is the fusion of cultured cells (3, 41, 44). Despite extensive studies on fusion activity, the region responsible for this activity has not been conclusively elucidated. Uncleaved S protein has fusion activity (35, 38), suggesting that the mechanism of MHV fusion differs from those of other fusogenic RNA viruses (47). However, the necessity of cleavage for MHV-2 fusion activity was recently reported (49). The S protein is the major target of the neutralizing antibodies induced in mice after infection with MHV. It also elicits cytotoxic T cells (13, 25). Furthermore, the S protein is suggested to be a major determinant of viral virulence in animals (5, 11, 12, 27, 45).

Another important biological function of the MHV S protein is binding to the virus-specific receptor protein, a member of the carcinoembryonic antigen gene family (9, 48). The receptor-binding domain on the S protein of MHV has been located on the S1 subunit (24, 37) but not on the S2 subunit (39), as expected from the topologies of these subunits (6). The receptor-binding site of the MHV S protein was found to be located in the N-terminal domain of S1, composed of 330 amino acids [S1N(330)] (24). Recently, we identified some amino acid residues responsible for the receptor-binding activity using various S1N(330) mutants generated by site-directed mutagenesis (37). However, it has not been confirmed yet whether these amino acid residues actually play crucial roles in the receptor-binding activity, because of the lack of viruses which have such mutations in the S protein.

In pursuit of the identification of amino acids responsible for

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receptor-binding activity in the S protein, we have utilized an alternative method, that is, the isolation and characterization of mutant viruses that escaped neutralization by a soluble receptor protein, as has been reported for poliovirus (4, 22). Since such resistant viruses are expected to carry the mutation in amino acids important for receptor-binding activity, they appeared to be most useful tools for identifying amino acids responsible for the activity. The findings for the soluble-receptor-resistant (*srr*) mutants in the present study support our previous findings on receptor-binding activity and provide new insights into the interaction between the MHV S protein and its receptor.

MATERIALS AND METHODS

Cells and viruses. All cell lines (DBT, RK-13, and BHK-21) used in the present study were grown and maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM; Nissui, Tokyo, Japan) supplemented with 5% fetal calf serum (FCS; GIBCO) under 5% CO_2 in air. MHV strain JHMV cl-2 (40, 42) was used as a wild-type virus for the isolation of *srr* mutant viruses. The cl-2 virus stocks were prepared from culture fluids of infected DBT cells (42). Virus titers were determined by a plaque assay with DBT cells as previously reported (20, 43). The recombinant vaccinia virus (VV) vTF7.3 harboring the T7 RNA polymerase gene (kindly provided by B. Moss) was used to express the soluble form of the MHV receptor protein in a VV transient expression system (14, 15). vTF7.3 was propagated and plaque assayed with RK-13 cells.

Production of soluble receptor protein. The soluble form of the MHV receptor protein (soMHVR1) was expressed by a VV transient expression system as previously reported (30). The gene for mL900 (24) (designated as MHVR1 in this paper), which is identical to MHVR1 (2d) (8) or BgpC (28), was used for expression. The gene, originally cloned into the pT7 Blue Vector (Novagen, Madison, Wis.), was manipulated to delete the transmembrane and intracytoplasmic domains and to tag an epitope found in the hemagglutinin (HA) of influenza virus by the PCR as previously described (30). For preparation of soMHVR1, expression vector pT7-soMHVR1-HA was constructed and transfected into RK-13 cells by electroporation (30), and the treated cells were infected with vTF7.3 at a multiplicity of 2 to 5 PFU/cell (30). The infected cells were cultured with 90% DMEM–10% tryptose phosphate broth (TPB; Difco, Detroit, Mich.). At 24 to 48 h postinfection (p.i.), the culture fluid was collected and soMHVR1 was concentrated by ultrafiltration with Ultra-free PF or PFL (Millipore) after removal of VV by centrifuging at 20,000 rpm for 2 h.

Isolation of *srr* mutant viruses. Viruses resistant to neutralization by soMHVR1 were isolated from wild-type cl-2 virus. A mixture of 100 μl of cl-2 virus containing 2×10^5 PFU and 500 μl of concentrated soMHVR1 was incubated at room temperature (RT; 22 to 24°C) for 50 to 60 min. The mixture was inoculated onto confluent DBT cells, and the plaques produced were isolated 1 to 2 days later. The virus clones confirmed as showing resistance to soMHVR1 neutralization were further plaque purified three times. Such *srr* variants were inoculated onto confluent DBT cell monolayers, and culture fluids were collected after more than 95% of the cells were fused. After centrifugation at 3,500 rpm for 15 min, the clarified supernatants were divided into small aliquots and stored at −80°C as a stock virus.

Neutralization by soMHVR1. Neutralization of virus infectivity by soMHVR1 was done as follows. Concentrated soMHVR1 was twofold step diluted with DMEM containing 10% TPB. Each dilution (100 μl) was mixed with 100 μl of cl-2 virus containing 10^5 PFU, and the mixture was incubated at RT for 50 to 60 min. The remaining virus titers were determined by a plaque assay with DBT cells (43). The degree of neutralization of each dilution was estimated relative to that of virus mixed with DMEM containing 10% TPB and no soMHVR1.

Isolation and sequencing of the *srr* mutant S genes. Total RNA was extracted from DBT cells infected with virus by an acid guanidinium thiocyanate-phenol-chloroform procedure (2). The RNA (0.2 μg) was reverse transcribed in a 20-μl (final volume) reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 20 U of RNase inhibitor (Takara, Shiga, Japan), 50 U of cloned Moloney murine leukemia virus reverse transcriptase (U.S. Biochemical Corp., Cleveland, Ohio), and 2.5 mM oligo$(dT)_{16}$ primer. After incubation at 42°C for 20 min, 20 μl of the reaction sample was used for PCR with a 100-μl reaction cocktail containing 50 pmol of each primer (5′-CGCAAGCTTCTAA ACATGCTGTTCGTC-3′, corresponding to a region of cl-2 S gene initiation codon, and 5′-ATCTTGGGACCGATGAGGGCCAT-3′, corresponding to a region of the cl-2 S gene termination codon), 2.5 U of ExTaq DNA polymerase (Takara), 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 2 mM $MgCl_2$, and 0.2 mM each deoxynucleoside triphosphate. The S cDNAs were amplified for 35 cycles with a DNA thermal cycler (Perkin-Elmer Cetus). The PCRs consisted of 90 s at 95°C (denaturing), 60 s at 55°C (annealing), and 210 s at 72°C (extending). After purification of cDNA fragments, each sample was directly sequenced on both strands with a series of S gene-specific primers (41) labeled with fluorescein isothiocyanate and a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham, Arlington Heights, Ill.) in a DNA sequencer (Pharmacia, Uppsala, Sweden) (32). To avoid potential artifacts introduced by PCR (7), we sequenced two independent PCR samples for verification of the reaction results. For sequencing of both ends of the cDNAs, fragments were ligated into the pT7 Blue Vector and sequenced with primers specific for pT7 Blue Vector sequences.

Western blotting. All *srr* mutant and cl-2 S proteins were prepared from culture fluids of infected DBT cells or from infected DBT cells lysed with lysis buffer, consisting of phosphate-buffered saline (pH 7.2), 0.65% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (Sigma). Aliquots of culture fluids or cell lysates were electrophoresed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, and the proteins were electrically transferred onto Immobilon transfer membrane paper (Millipore). The paper with proteins was blocked with Block Ace (Yukijirushi, Sapporo, Japan) for 1 h at RT before reaction with antibodies. The membrane paper was then incubated with monoclonal antibody (MAb) 30B, specific for the S1 protein (31) and kindly provided by S. G. Siddell, for 1 h at RT. The paper was then washed three times with phosphate-buffered saline containing 0.1% Tween 20. The binding of the MAb to proteins was detected with horseradish peroxidase (HRPO)-conjugated anti-mouse immunoglobulin G (IgG) antibody (Cappel Organon Teknika, Durham, N.C.) as a secondary antibody. The bands were visualized on X-ray film with enhanced chemiluminescence (ECL) reagents (Amersham) according to the manufacturer's recommendations.

VOPBA. The virus overlay protein blot assay (VOPBA) was used to detect the binding of the S proteins to the receptor protein as described previously (24, 30). Briefly, soMHVR1 transferred onto membrane paper after electrophoresis on an SDS–10% polyacrylamide gel was incubated at RT for 1 h with culture fluids of DBT cells infected with wild-type cl-2 or *srr* mutants. The binding of the S proteins was monitored with MAb 7, specific for the S1 protein of cl-2 virus (23), and HRPO-labeled anti-mouse IgG by ECL as previously reported (24, 30, 38).

Coimmunoprecipitation of wild-type and *srr* mutant S proteins with soMHVR1. To investigate the interaction of soMHVR1 and the S proteins in the liquid phase, we used coimmunoprecipitation to precipitate soMHVR1-bound S proteins with an anti-HA MAb. We used viral particles in culture fluids of DBT cells infected with wild-type cl-2 or *srr* mutants. The concentrations of the S proteins in the samples were adjusted after estimation by Western blotting with anti-S1 MAb 30B. To precipitate S proteins with soMHVR1, soMHVR1 was incubated at RT for 1 h with anti-HA MAb (mouse MAb clone 12CA5; Boehringer, Mannheim, Germany) which had been previously bound to protein A–Sepharose CL-4B (Pharmacia) at 4°C overnight. The same amounts of S proteins of wild-type cl-2 and *srr* mutants in 100 μl of DMEM were then incubated with soMHVR1 bound to anti-HA MAb–Sepharose for 60 min at RT. Each sample was then washed five times in lysis buffer, resuspended in sample dissociation buffer (125 mM Tris-HCl [pH 6.8], 10% β-mercaptoethanol, 4% SDS, 20% glycerol, 0.2% bromophenol blue), boiled for 3 min, and electrophoresed on an SDS–10% polyacrylamide gel. Coimmunoprecipitated MHV S proteins were analyzed by Western blotting with MAbs 30B for S1 and 10G for S2 (31).

Establishment of a BHK-21 cell line expressing the MHVR1 protein. BHK-21 cells transfected with pKS336 harboring the MHVR1 receptor gene were kindly provided by Y. K. Yamada, National Institute of Infectious Diseases, Tokyo, Japan. For the cloning of cells, 20 to 100 live cells were cultured in a 10-cm petri dish (Falcon) with DMEM containing 5% FCS and Blastcydine (a gift from Y. K. Yamada) at a concentration of 3 μg/ml for 2 to 3 weeks. Colonies formed on dishes were individually isolated and maintained as cell clones. Among the cell clones obtained, BHK-mL-900 no. 6 (BHK-mL-6), the most sensitive to cl-2 infection, was used in the present study.

Assay for binding of *srr* mutants to MHVR1-expressing cell membranes. A cell line, BHK-mL-6, which continually expresses MHVR1, was used for analysis of binding of *srr* mutants to cellular MHVR1. The same amounts of S proteins of *srr* mutants and wild-type cl-2 adjusted by Western blotting in a 500-μl solution were overlaid onto BHK-mL-6 cells cultured on a 12-well plate (Falcon) or onto BHK-21 cells as negative controls to determine background binding of the S proteins. After incubation at RT for 1 h, cells were washed five times with DMEM to remove unbound viruses and lysed with lysis buffer. To estimate the amounts of S proteins bound to cellular MHVR1, we dotted twofold step-diluted lysates on membrane paper. The paper was then treated with Block Ace at RT for 1 h and incubated with 10,000-fold-diluted MAb 7. The paper was washed three times with phosphate-buffered saline–0.1% Tween 20 and then allowed to react with 50,000-fold-diluted anti-mouse IgG conjugated to HRPO (Cappel) as a secondary antibody. The MHV S proteins were visualized on X-ray film with ECL reagents as described above.

Comparison of growth of *srr* mutants and cl-2 in DBT cells. Confluent DBT cells in 3-cm-diameter culture dishes (Falcon) were infected at a multiplicity of infection (MOI) of 2 to 5 with either *srr* mutant virus or wild-type cl-2 virus. After 1 h of adsorption at 37°C, cells were washed three times with warm DMEM to remove unadsorbed viruses and incubated with DMEM containing 3% FCS at 37°C. At various intervals after virus inoculation, culture fluids and cells were harvested and frozen-thawed three times to destroy cells. After centrifugation at 3,000 rpm for 10 min, the virus titers in the supernatants were determined by a plaque assay with DBT cells as previously reported (43).

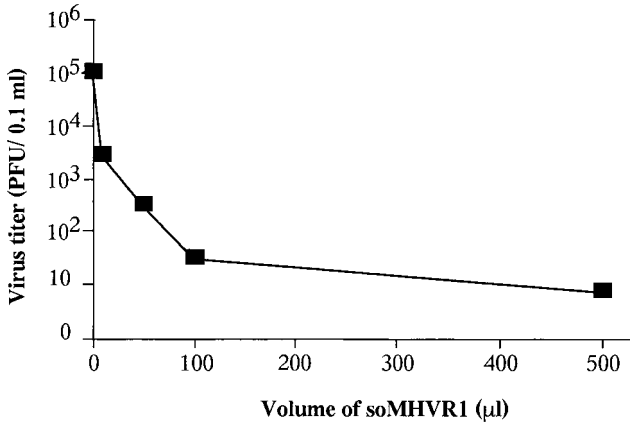

FIG. 1. Sensitivity of wild type cl-2 to soMHVR1. One hundred microliters of wild-type cl-2 (2×10^5 PFU) was mixed with 50, 100, or 500 μl of soMHVR1 and incubated at RT for 1 h. The surviving infectious viruses were determined by a plaque assay.

RESULTS

Isolation of *srr* mutants resistant to neutralization by soMHVR1. *srr* mutants resistant to neutralization by the soluble receptor protein are supposed to contain mutated amino acid residues responsible for the receptor-binding activity. To identify those residues, we isolated *srr* mutants by using soMHVR1. To determine the appropriate volume of soMHVR1 for the selection of *srr* mutants, we assayed the sensitivity of the wild-type virus, JHMV cl-2, to soMHVR1. cl-2 at 2×10^5 PFU in 100 μl of culture fluid was incubated with different volumes of concentrated soMHVR1 at RT for 50 to 60 min, and the remaining infectious viruses were scored by a plaque assay with DBT cells. As shown in Fig. 1, virus infectivity was inactivated in a dose-dependent manner. With 500 μl of soMHVR1, the wild-type virus titer was reduced to <10 PFU. Nineteen neutralization-resistant virus clones were isolated in three independent experiments following a single round of soMHVR1 treatment. Of them, five clones (designated *srr*3, *srr*4, *srr*7, *srr*11, and *srr*18) were finally demonstrated to be highly resistant to neutralization by soMHVR1. *srr* mutants arose with a frequency of about 1 in 10^5 PFU of the wild-type cl-2 population. This value was comparable to that of poliovirus *srr* mutants (4) and variants resistant to neutralization with MAbs (19).

Comparison of S protein amino acid sequences of cl-2 and *srr* mutants. To investigate which mutations are present in *srr* mutants, we determined the nucleotide sequences of the entire S protein coding region of *srr* mutants and compared them with that of the S gene of wild-type cl-2. The viral S cDNAs were made by reverse transcription of total RNA prepared from infected DBT cells. Two synthetic oligonucleotide primers corresponding to the region of the S gene initiation codon and that of the termination codon (41) were used to amplify S cDNAs containing 4,131 nucleotides of the entire open reading frame by reverse transcription-PCR. Amplified S cDNA fragments were directly sequenced on both strands with a series of S gene-specific primers. Sequence analysis of S cDNA indicated that each *srr* mutant had a single nucleotide mutation which created an amino acid change in the S1 or S2 subunit (Table 1). *srr*11 had an amino acid change in the S1 subunit at position 65 (Leu to His), in an extremely conserved region among MHV strains (between positions 49 and 71) (37). Other alterations, at positions 1114 (Leu to Phe) (*srr*3, *srr*4, and *srr*7) and 1163 (Cys to Phe) (*srr*18), were present between two heptad repeat domains in the S2 subunit (6). We chose three *srr* mutants (*srr*7, *srr*11, and *srr*18) for further characterization because each of them had a single-amino-acid alteration at different positions of the S protein. The S proteins in lysates of DBT cells infected with these viruses and the S proteins on viral particles were compared with those of wild-type cl-2 by SDS-polyacrylamide gel electrophoresis and Western blotting. As shown in Fig. 2, both intracellular S proteins and mature S proteins on viral particles were not different among these variants and wild-type cl-2. The S proteins on viral particles were shown to be completely cleaved in all wild-type and *srr* mutant viruses. These results indicated that mutant S proteins were processed in a fashion similar to that of wild-type cl-2 S protein.

TABLE 1. Identification of S protein mutations in *srr* mutants

Virus	Substitution at the following position[a]:					
	Nucleotide			Amino acid		
	194	3340	3488	65	1114	1163
JMHV cl-2	T	C	G	Leu	Leu	Cys
*srr*3	—[b]	T	—	—	Phe	—
*srr*4	—	T	—	—	Phe	—
*srr*7	—	T	—	—	Phe	—
*srr*11	A	—	—	His	—	—
*srr*18	—	—	T	—	—	Phe

[a] Nucleotide and amino acid positions were numbered from the first ATG codon and methionine, respectively.
[b] —, identity with the wild-type sequence.

Resistance of *srr* mutants to neutralization by soMHVR1. To determine the level of resistance of *srr* mutants to neutralization with soMHVR1, we compared the neutralization kinetics of wild-type virus and *srr* mutant viruses. Viruses mixed with different concentrations of soMHVR1 were incubated at RT for 60 min, and the titers of remaining infectious virus were determined by a plaque assay with DBT cells. As shown in Fig. 3, the wild-type virus titer was reduced 1/100 and 1/1,000 by incubation with 5 and 50 μl of soMHVR1, respectively. In contrast, the three *srr* mutants were consistently resistant to these treatments, and there was no significant difference in the

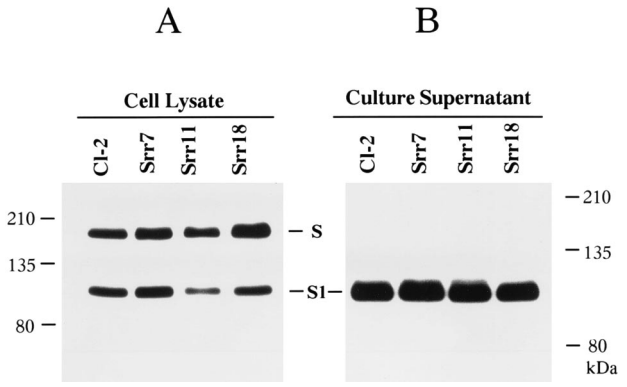

FIG. 2. Analysis of intracellular and viral particle S proteins by SDS-polyacrylamide gel electrophoresis and Western blotting. Lysates prepared from DBT cells infected with wild-type cl-2 or *srr* mutants (A) and viral particles prepared from culture supernatants of infected DBT cells (B) were electrophoresed on an SDS–10% polyacrylamide gel. After transfer to membrane paper, the S proteins on the paper were detected with S1-specific MAb 30B and then with HRPO-labeled anti-mouse IgG antibody by ECL.

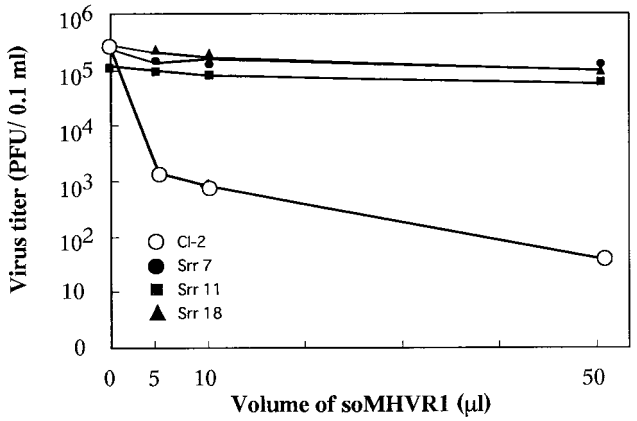

FIG. 3. Virus neutralization kinetics for soMHVR1. Various concentrations of soMHVR1 in 100 μl of DMEM were mixed with an equal volume of wild-type cl-2 or *srr* mutants (10^5 PFU) and left at RT for 1 h. The surviving viruses were determined by a plaque assay.

level of resistance among these three *srr* mutants. With even 500 μl of soMHVR1, there was no difference in resistance among these mutants (data not shown).

The resistance of *srr* mutants to soMHVR1 might be due to the utilization of other MHV receptors, such as Bgp2 (29) or bCEA (1), possibly expressed on DBT cells. To test such a possibility, we examined whether *srr* mutants infect cells expressing only MHVR1 and not other functional MHV receptors (BHK-mL-6 cells) and whether their infectivity is neutralized with soMHVR1 on these cells. Wild-type cl-2 or *srr* mutants mixed with 50 μl of soMHVR1 or DMEM and incubated at RT for 60 min were plaque assayed for infectivity on BHK-mL-6 cells. As shown in Table 2, all *srr* mutants produced plaques as efficiently as the wild type, yet they were not neutralized by soMHVR1, while wild-type cl-2 was efficiently neutralized by soMHVR1. Moreover, the plaquing efficiency and growth of *srr* mutants on BHK-mL-6 cells were very similar to those on DBT cells (data not shown). The results obtained with BHK-mL-6 cells were not different from those obtained with DBT cells, suggesting strongly that *srr* mutants utilize MHVR1 as a major receptor and that they were selected because of their high level of resistance to neutralization by soMHVR1.

Analysis of binding of *srr* mutant S proteins to soMHVR1 by a VOPBA and coimmunoprecipitation. To compare the receptor-binding activities of wild-type and *srr* S proteins, we assessed the amounts of S proteins that bound to soMHVR1 by a VOPBA, an assay of the binding of S protein to soMHVR1 prepared on membrane paper by Western blotting. For quantitation of binding activity, twofold dilutions of viral particles of the wild type and *srr* mutants whose S protein concentrations had been adjusted after Western blotting as described in Fig. 2B were allowed to react with soMHVR1. As shown in Fig. 4, wild-type cl-2, *srr*7, and *srr*18 showed similar receptor-binding activities, while the binding of *srr*11 was remarkably reduced, to less than 1/64. This result indicated that *srr*7 and *srr*18 had capacities to bind to the soluble receptor protein to the same extent as wild-type virus. To confirm this result, we employed another method of coimmunoprecipitation in which we examined the binding ability of each *srr* mutant and wild-type S protein with a native soluble receptor protein.

The same amounts of *srr* and wild-type S proteins (Fig. 2B) were mixed with soMHVR1 bound to protein A beads via an anti-HA MAb. The S proteins bound to soMHVR1 were precipitated and analyzed by Western blotting with S1-specific MAb 30B. As shown in Fig. 5A, the S1 subunit of wild-type cl-2 was coimmunoprecipitated by this procedure, demonstrating a tight interaction between soMHVR1 and the cl-2 S protein. The binding of *srr*11 S protein was as low as 1/64 that in the control, while the binding of *srr*7 and *srr*18 S proteins was not significantly different from that of the wild-type cl-2 S protein. To ensure the specificity of this reaction, we tried to precipitate cl-2 S protein without soMHVR1 in the reaction mixture but failed to precipitate the S1 subunit (Fig. 5A). This finding suggested that the S1 subunit was specifically precipitated by an interaction with soMHVR1.

We also assayed the binding activities of S proteins from cell lysates or S proteins solubilized from viral particles by changing the reaction time (from 10 to 60 min). However, the binding pattern for the *srr*7 and *srr*18 S proteins again was similar to that for the wild-type S protein (data not shown). These results suggested that an alteration, particularly at position 65 (Leu to His), in the S1 subunit resulted in a remarkable loss of the capacity to bind to soMHVR1. However, the alteration at position 1114 (Leu to Phe) or 1163 (Cys to Phe) in the S2 subunit did not adversely affect binding to soMHVR1. This result implied that the *srr*7 and *srr*18 S proteins were capable of binding to the cellular MHV receptor even after they had been fully bound to soMHVR1, since they were not neutralized by soMHVR1.

By using the same membrane paper as that used for Fig. 5A, we compared the amounts of S2 found in precipitates with S2-specific MAb 10G. As shown in Fig. 5B, large amounts of S2 from *srr*7 and *srr*18 were detected, while S2 of wild-type cl-2 was hardly detectable. In Fig. 5B, the S1 bands were also visible because of the reaction of HRPO-labeled anti-mouse IgG to S1-specific MAb 30B previously bound to S1. In precipitates of *srr*7 and *srr*18, N and M proteins were detected by anti-MHV-2 rabbit serum; however, such viral components were hardly detectable in the cl-2 precipitate (data not shown). These results implied that cl-2, *srr*7, and *srr*18 bound to soMHVR1 in a similar manner; however, most of cl-2 S1 was dissociated from S2, while such dissociation was minimally limited in *srr*7 and moderately limited in *srr*18. These observations are in good accordance with the findings recently reported by Gallagher (17) that S1 is easily dissociated from S2 after binding to the receptor for MHV-4, whose S gene is very similar to the cl-2 S gene (41), while JHMX S1, containing a large deletion, associates tightly with S2. *srr*11 S2 was also detected and was more abundant than wild-type S2 (Fig. 5B), although the *srr*11 S1 protein was only present in trace amounts (Fig. 5A). This result may have been due to a difference in the affinities of the MAbs used to detect S1 (30B) and S2 (10G). This result also suggests that *srr*11 S1 associates tightly with S2, as in other *srr* mutants.

Binding of wild-type and *srr* mutant S proteins to cellular receptor proteins. In order to compare the binding capacities

TABLE 2. Resistance of *srr* mutants to soMHVR1 neutralization in BHK-mL-6 cells[a]

soMHVR1	Titer (PFU) of:			
	cl-2	*srr*7	*srr*11	*srr*18
Present	1.2×10^1	4.5×10^4	2.0×10^4	2.3×10^4
Absent	1.9×10^4	4.6×10^4	2.2×10^4	2.4×10^4

[a] Viruses were incubated with or without equal volumes of soMHVR1 for 1 h at RT. The mixtures were inoculated onto monolayer cultures of BHK-mL-6 cells. Virus plaques were scored after 36 h of incubation at 37°C. Plaque assays were also performed with BHK cells lacking MHVR1; in all cases, the titer was <1.0 PFU.

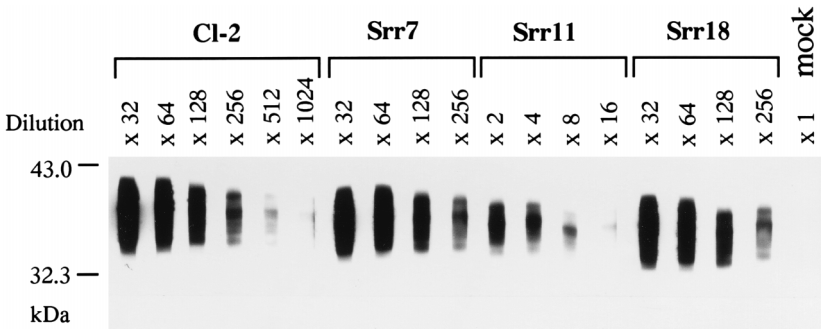

FIG. 4. Analysis of receptor-binding activities by VOPBA. Twofold dilutions of the wild type and *srr* mutants were incubated at RT for 1 h with soMHVR1 on membrane paper prepared by electrophoresis on an SDS–10% polyacrylamide gel and Western blotting. The binding of viruses was evaluated with S1-specific MAb 7 and HRPO-labeled anti-mouse IgG antibody by ECL.

of wild-type and *srr* mutant S proteins to cellular receptors, the same amounts of wild-type and *srr* mutant viruses, as judged by Western blotting (Fig. 2B), were incubated with BHK-mL-6 cells, which stably express MHVR1. The amounts of S proteins bound to cellular MHVR1 were compared by dot blotting with a mixture of S protein-specific MAbs 7 and 30B. As shown in Fig. 6, all *srr* mutants bound to the cellular receptor to almost the same extent as the wild type. The binding capacity of *srr*7 and *srr*18 was decreased to one-half that of the wild type and was equivalent to the receptor-binding ability determined by the VOPBA and coimmunoprecipitation. The binding capacity of *srr*11 was decreased to one-quarter that of the wild type and was remarkably higher than the receptor-binding ability determined by the VOPBA and coimmunoprecipitation.

Growth of *srr* mutants in DBT cells. The growth in DBT cells of *srr* mutants *srr*7, *srr*11, and *srr*18 was compared with that of wild-type cl-2. DBT cells were infected at an MOI of 2 to 5, and virus titers in cultures collected at intervals after inoculation were determined by a plaque assay. As shown in Fig. 7, there was no significant difference in the growth of the wild type and *srr* mutants, although the virus titers of *srr* mutants were slightly higher than wild-type virus titers. The progeny viruses were first detected at 6 h p.i., and the titers reached a peak at 12 to 15 h p.i. Polykaryocyte formation (cytopathic effect) was also observed in DBT cells infected with either of these viruses. Such cytological changes were first detected at 6 h, and almost all of the cells were included in polykaryocytes at 10 to 12 h p.i. No significant difference in polykaryocyte formation was found between the *srr* mutants and the wild type.

DISCUSSION

S1N(330), containing 330 amino acids of the N terminus of the MHV S protein, was demonstrated to have receptor-binding activity (24). From an analysis of mutants of S1N330 that were generated by site-directed mutagenesis and that contained amino acid substitutions in a few regions conserved in all MHV strains examined, we revealed that the amino acid at position 62 and a region consisting of amino acids 212 to 216 are important for receptor-binding activity (37). In the present study, we employed an alternative procedure to define the region involved in the receptor-binding activity. *srr* mutants resistant to neutralization by the soluble receptor protein are supposed to contain the changes in amino acids responsible for the receptor-binding activity, as studied in detail for poliovirus

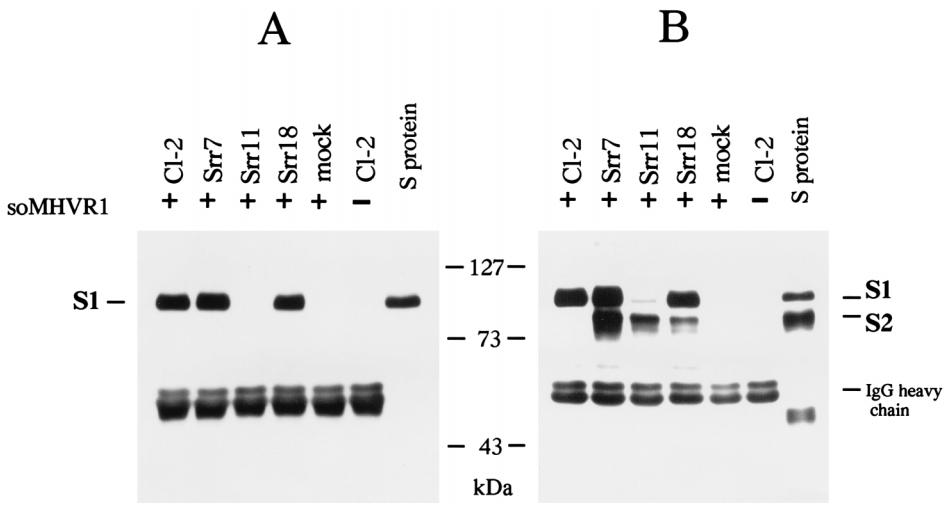

FIG. 5. Analysis of receptor-binding activity in the liquid phase by coimmunoprecipitation. Wild-type and *srr* mutant viruses containing the same amounts of S proteins in 100 μl of DMEM were mixed and incubated at RT for 1 h with soMHVR1 bound to protein A–Sepharose via an anti-HA MAb. The S proteins bound to soMHVR1 were denatured with sample buffer and electrophoresed on an SDS–10% polyacrylamide gel. After transfer to membrane paper, the S proteins were detected with S1-specific MAb 30B (A) or S2-specific MAb 10G (B). The binding of MAbs was examined with HRPO-labeled anti-mouse IgG antibody by ECL. The S protein shown in the rightmost lane indicates the bands containing the cl-2 S proteins in the viral particles.

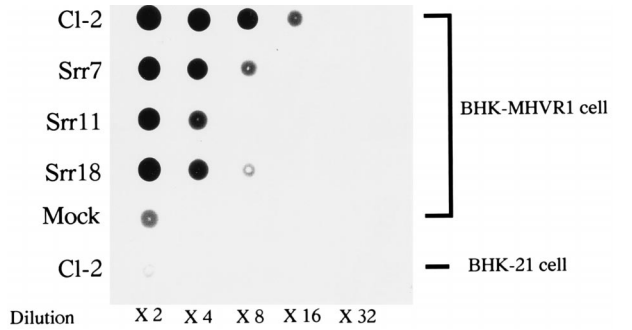

FIG. 6. Analysis of binding to cellular MHVR1 by dot blotting. BHK-mL-6 cells expressing MHVR1 on the cell surface were prepared in 12-well plates and incubated with wild-type and *srr* mutant viruses containing the same concentrations of S proteins at RT for 1 h. The cells were washed five times, and the viruses bound to membrane MHVR1 were harvested by lysing cells. Twofold dilutions of lysates were prepared on membrane paper by dot blotting, and the paper was incubated with S1-specific MAb 7. MAb binding was estimated by ECL with HRPO-labeled anti-mouse IgG antibody.

(4, 22). We obtained three different *srr* mutants, each of which contained only one amino acid change in the S protein. One of them, *srr*11, contained the mutation at amino acid position 65 (Leu to His) in the S1 subunit. This mutant showed a high level of resistance to neutralization by the soluble receptor. Its binding to the soluble receptor was drastically decreased. Such reduced receptor-binding capacity could not have resulted from mutations in other viral structural proteins, since viral particles treated with detergents which dissociated S proteins from other viral proteins showed the same reduced receptor-binding capacity (data not shown). Thus, this particular amino acid, at position 65, appeared to be responsible for the receptor-binding activity. This amino acid is located in the vicinity of amino acid 62, which was previously identified to be important for receptor binding (37). These facts suggest that a stretch of amino acids from positions 62 to 65 is critical for receptor-binding activity. Our previous study showed that mutations at different amino acids in a region between positions 49 and 70 of the S protein, a region conserved in all MHV strains examined, did not influence receptor-binding activity, with the exception of the amino acid at position 62 (37). These data indicate that amino acids at positions 62 and 65 are important and that not necessarily all of the amino acids in this region are involved in receptor-binding activity. However, it is not clear whether this region is the actual receptor-binding site or whether mutations at these amino acids alter the conformation of the protein, thus preventing receptor binding.

Our site-directed mutagenesis analysis also suggested that amino acids at positions 212 to 216 as well as at position 62 are important for receptor-binding activity (37). The region composed of amino acids 33 to 40, recognized by MAb 11F, may also be involved in receptor binding, since 11F failed to bind to the receptor-bound S protein (24). Since denatured S protein has completely lost receptor-binding ability, secondary or tertiary structure must be critical for this activity (24). A structure composed of the three putative regions mentioned above may participate in an interaction with the N domain of the MHV receptor protein, which contains the virus-binding site (9, 10).

Two *srr* mutants, *srr*7 and *srr*18, contained amino acid mutations in the S2 subunit at positions 1114 (Leu to Phe) and 1163 (Cys to Phe), respectively. Since mutations in the S2 subunit have been thought to influence the conformation of the S1 subunit (19), we first speculated that the mutations at positions 1114 and 1163 reduced the receptor-binding activity of S1. However, that was not the case. Although these two mutants showed resistance to neutralization by the soluble receptor protein, their receptor-binding capacity was not different from that of the wild type. This result implies that the S proteins of these mutants bind to the soluble receptor protein as efficiently as the wild-type S protein and that such soluble receptor-bound viral particles are still infectious. At present, we have no experimental data to account for this phenomenon. However, a difference was found between the mutant S proteins and the wild-type S protein in the stability of the S1-S2 association. Wild-type S1 protein could easily be dissociated from S2 protein after binding to the receptor protein, but this dissociation did not occur in *srr*7 and *srr*18, as shown in Fig. 5B. Such a stable association between S1 and S2 after binding to the receptor protein may account for the characteristics of the two *srr* mutant viruses. Recently, Gallagher (17) reported that S1 of wild-type JHM, which has a large S protein, can easily be dissociated from S2 after binding to the receptor but that JHMX, which contains a large amino acid deletion in the S1 subunit, shows a stable association of S1 and S2. We have found that JHMX was resistant to neutralization by soMHVR1, that is, JHMX has characteristics similar to our *srr* viruses (data not shown). Taken together, these data suggest that the stable association of S1 and S2 subunits after binding to the receptor protein may implicate the *srr* feature. The dissociation of S1 from S2 may trigger an irreversible processing in viral particle conformation, so that wild-type cl-2 bound to the soluble receptor loses infectivity, while such processing does not take place for *srr*7 and *srr*18, so that these viruses can bind to the cellular receptor with S1 unoccupied with soluble receptor or with S1 from which soluble receptor is dissociated. Experiments are currently in progress test such a possibility.

A mutant of virus JHM with mutations at positions 1067

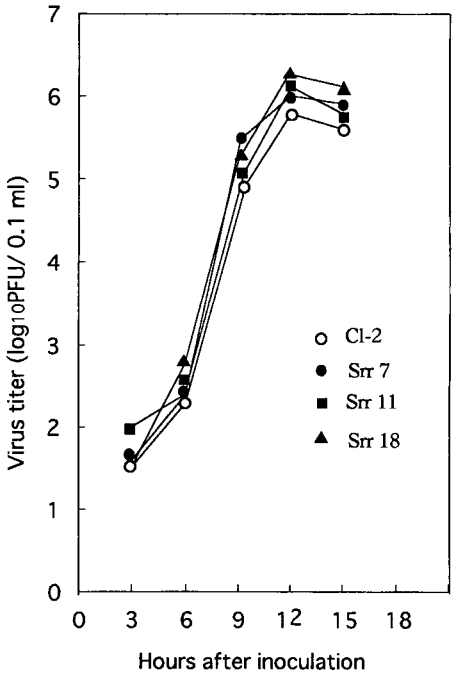

FIG. 7. Growth of the wild type and *srr* mutants in DBT cells. DBT cells prepared in 3-cm dishes were infected with the wild type or *srr* mutants at an MOI of 2 to 5. At various intervals, cells and culture fluids were harvested and frozen-thawed three times. After centrifugation at 3,000 rpm for 10 min, the virus titers in the supernatants were determined by a plaque assay.

(Gln to His), 1094 (Gln to His), and 1114 (Leu to Arg) has been reported to show fusion activity only under acidic conditions, while wild-type JHMV fuses over a wide range of pHs (18). These mutations are located in or close to the heptad repeat in S2, which is supposed to be important for fusion activity (18). *srr*7 contained a single mutation at amino acid 1114 (Leu to His), located close to the first heptad repeat, and was not different from wild-type JHMV in fusion activity. This result shows that the single mutation in S2 at amino acid position 1114 alone is not enough to destroy the fusogenicity of the S protein. It has also been reported that the cysteine residue at position 1163, located between two heptad repeats, plays an important role in fusion activity, as examined with the Ellman reagent to modify the exposed cysteine residue on the cell membrane (16). MHV A-59 does not contain this cysteine residue and is resistant to the Ellman reagent (16). The importance of the cysteine residue at position 1163 for fusion activity could be confirmed by use of the *srr*18 mutant virus, which contains a mutation at position 1163 (Cys to Phe).

Many poliovirus *srr* mutants have been isolated and characterized (4, 22). All of these reported *srr* mutants fail to bind to soluble receptors or have reduced binding activity (4, 22). This characteristic is very similar to that of our *srr*11 mutant of JHMV. Poliovirus *srr* mutants fail to bind to the soluble receptor but bind to the cellular receptor with almost the same efficiency as wild-type poliovirus (4). This difference has been explained by a higher valency of the virus-cellular receptor interaction than of the virus-soluble receptor interaction (4). This could also be the case for JHMV mutant *srr*11. The fluidity of the cellular membrane may enhance the valency of the virus-cellular receptor interaction. This idea is suggested by the fact that the level of binding of poliovirus *srr* mutants to the cellular receptor is low at 4°C, at which cellular membranes are not as fluid as they are at 37°C. The *srr*11 mutant of JMHV, however, showed no significant difference in binding to the cellular receptor at 4 and 37°C, as judged by virus adsorption (data not shown). This finding indicates that *srr*11 is different from poliovirus *srr* mutants in binding to the cellular receptor or that coronavirus behaves differently from poliovirus in the stages of adsorption to cells and penetration.

In the present study, we have clarified the S protein amino acid residues which could be important for receptor-binding activity. We have also obtained *srr* mutants with unexpected features. We believe that a detailed study of such *srr* mutants and soluble receptor interactions would delineate important aspects of coronavirus processing after adsorption to the receptor.

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