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Analysis of the Receptor-Binding Site of Murine Coronavirus Spike Protein

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It has been found that a domain composed of 330 amino acids of the N terminus of murine coronavirus spike protein [S1N(330)] is involved in receptor-binding activity (H. Kubo, Y. K. Yamada, and F. Taguchi, J. Virol. 68:5403–5410, 1994). To delineate the amino acid sequences involved in receptor-binding activity, we have compared the S1N(330) proteins of seven different mouse hepatitis virus MHV strains that are able to utilize the MHV receptor protein. Three conserved regions (sites I, II, and III) were found to consist of more than 10 identical amino acids, and they were analyzed for receptor-binding activity by site-directed mutagenesis. S1N(330) with a substitution at position 62 from the N terminus of S1 in region I and that with substitutions at positions 212, 214, and 216 in region II showed no receptor-binding activity. The S1N(330) mutants without receptor-binding activity were not able to prevent virus binding to the receptor. These results suggest that the receptor-binding site on S1N(330) is composed of regions located apart from each other in the protein's primary structure, in which Thr at position 62 as well as amino acids located at positions 212, 214, and 216 are particularly important.

Mouse hepatitis virus (MHV) is a member of coronaviruses, which are enveloped, positive-stranded RNA viruses associated with various diseases of economic importance in both animals and humans (16, 17, 26). The genome of MHV, with about 31 kb, encodes four structural proteins: the 50- to 60-kDa nucleocapsid protein, the 20- to 25-kDa integral membrane glycoprotein, the 150- to 200-kDa spike (S) glycoprotein, and the small membrane protein (16, 17).

The S protein, which has a variety of important biological activities, forms the spike on the virion surface (16, 17). 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 (17). These subunits derive from the N-terminal and C-terminal halves of the S protein after cleavage of the precursor S protein by a host cell-derived protease (18). It is believed that S1 forms the globular part of the spike and S2 forms the stalk portion (1). One of important biological activities of the MHV S proteins is binding to the virus-specific receptor proteins (11), which initiates virus infection. The receptor protein of MHV is mmCGM or biliary glycoprotein (Bgp) (3, 4, 13, 27, 29), a member of carcinoembryonic antigen group of the immunoglobulin superfamily (27). The N-terminal, immunoglobulin V region-like domain of mmCGM has been found to function as a receptor of MHV (5). On the other hand, the receptor-binding domain on the S protein of MHV has been found to be located in the S1 subunit (11) but not in the S2 subunit (20), as deduced from the topologies of these subunits (1). The receptor-binding site of MHV S protein was found to be located in the N-terminal domain of S1, composed of 330 amino acids (aa) [S1N(330)] (11). However, precise amino acid sequences involved in receptor-binding activity have not yet been identified. In this study, experiments were undertaken to identify the amino acid sequences forming the receptor-binding sites.

We have compared the S1N(330) proteins of various MHV strains that utilized Bgp C as a receptor by assuming that the receptor-binding site is conserved in the S1N(330) proteins of such strains. We first tested which strains of MHV could utilize the receptor. We expressed the receptor protein, mL900 protein (11), which is identical to MHVR1 (2d) (3) or Bgp C (13), on BHK-21 cells by using a transient expression system using eukaryotic expression vector pcDL-SRα296 (25) containing the receptor gene mL900 (11) (SRαmL900). We introduced the recombinant plasmid into cells by electroporation with a Gene Pulser as previously reported (20). We then infected seven different MHV strains, MHV-1, -2, -3, -A59, -S, and -NuU (8, 14) and variant JHMV cl-2 (23), onto BHK cells 2 days after gene transfection at a multiplicity of 0.1 to 1 PFU per cell, and virus growth as well as cytopathic changes were examined as described previously (24). As shown in Table 1, BHK cells expressing the receptor protein and infected with any strain of MHV except the nonfusogenic MHV-2 strain showed polykaryocyte formation (fusion of cells), while no changes were observed in mock-transfected BHK cells infected with MHV strains. The culture fluids isolated when fusion cov-

TABLE 1. Fusion formation and growth of various MHV strains in BHK-21 cells transfected with SRαmL900

Virus[a]	Transfected BHK-21 cells		Virus titer in mock-transfected BHK-21 cells ($\log_{10}$ PFU/0.1 ml)[b]
	Fusion[c]	Virus titer[d] ($\log_{10}$ PFU/0.1 ml)	
MHV-1	+	4.6	<1.0
MHV-2	−	5.1	2.0
MHV-3	+	5.4	2.1
MHV-A59	+	6.0	2.9
MHV-S	+	4.9	1.9
MHV-U	+	4.6	<1.0
JHMV cl-2	+	5.1	<1.0
JHMV sp-4	+	4.2	<1.0

[a] BHK-21 cells mock transfected or transfected with SRαmL900 were infected with various MHV strains at a multiplicity of 0.1 to 1 PFU per cell.
[b] Focus formation was absent in all cases.
[c] Fusion formation was microscopically checked at 12 to 24 h postinoculation.
[d] In supernatants of infected BHK-21 cells collected at 12 to 24 postinoculation.

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TABLE 2. Sequences of oligonucleotides used

Primer name	Sequence	Position	Amino acid mutation
PL-3	5′-GCGTCCGTACGTACCCTCTCTACTCTAAAA-3′	Leader 1–30	
S1N 990N	5′-TTATTTACAATCAGGTAGGT-3′	984–1000 + TC[a]	
S1N 990N(MHV-S)	5′-TTATCTACAATCAGGCAAATT-3′	983–1000 + TC	
S1N 1056	5′-CTTACACTCCCAGTTGAGAGG-3′	1036–1056	
MCS-Hd-Pst(−)	5′-GGAAAGCTCGCATGCCGGCAGGTCG-3′	pT7Blue(R) 42–66	
S1N 159nt-P	5′-CTTACTCTTGTTTAGATCATGTTTACTTAACTGCCTCG-3′	159–196	Y-52→S, R-56→H, N-60→T, T-62→S
S1N 170nt-P	5′-TTAGATCATGTTTACTTAACTGCCACGTTAGTGCTT-3′	170–205	R-56→H, N-60→T, L-64→V
S1N 195nt-P	5′-CGTTAGTGCTTACTGGTTCTTATCCTGTGGAAGGT-3′	195–229	L-64→V, Y-68→S, D-72→E
S1N 640nt-P	5′-GGTGGTTCTTTTTCTGCGTCCTATGCGGAT-3′	638–667	T-212→S, Y-214→S, Y-216→S
S1N 840nt-P	5′-ATTACTACTGCTGCTGATTGCGTCAGCAGC-3′	842–871	S-280→T, V-282→A, A-285→V
S1N 52aaF-P	5′-GGTCTGGGCACTTACTTTGT-3′	149–168	Y-52→F
S1N 52aaS-P	5′-GGTCTGGGCACTTACTCTGT-3′	149–168	Y-52→S
S1N 62aaS-P	5′-CTTAAATGCCTCGTTATTGC-3′	184–203	T-62→S
S1N 212aaS-P	5′-GCAGGGTGGTTCTTTTTATG-3′	634–652	T-212→S
S1N 214aaS-p	5′-GGTGGTACTTTTTCTGCGTA-3′	638–657	Y-214→S
S1N 216aaS-P	5′-ACTTTTTATGCGTCCTATGC-3′	644–663	Y-216→S

[a] TC, termination codon.

ered more than 50% of the cells were shown to have high virus titers (10^4 to 10^6 PFU/0.1 ml) (Table 1), which indicated virus growth in BHK cells with the MHV receptor, while all of the MHV strains examined showed no substantial viral growth in BHK cells without receptor. The virus titers in culture fluids of BHK cells with or without MHV receptor infected with MHV-2 and isolated at 24 h postinoculation indicated the growth of MHV-2 in cells with receptor but not in those without receptor. These results indicated that all of the seven MHV strains examined were able to utilize Bgp C as a functional receptor.

Since it has been found that seven MHV strains utilize the Bgp C as a functional receptor, we confirmed that virus particles of these MHV strains bound to the receptor prepared on membrane paper. The receptor protein expressed in RK 13 cells by recombinant vaccinia virus harboring the MHV receptor gene (11) was prepared on membrane paper by Western blotting (immunoblotting) as previously reported (9, 19). The paper-bound receptor protein was incubated in Dulbecco's modified minimal essential medium (Nissui, Tokyo, Japan) with various MHV strains at titers of generally 10^5 to 10^6 PFU/0.1 ml, and receptor-bound virus particles were monitored with monoclonal antibodies (MAbs) reactive to S proteins of these seven strains (10). All of the MHV strains tested were demonstrated to bind the receptor protein, although there were some differences in intensities of the bands (data not shown), indicating that all strains that utilized Bgp C as a functional receptor could bind to receptor protein prepared on membrane paper.

We then examined whether S1N(330) of each MHV strain was responsible for this binding. We have isolated the genes encoding S1N(330) of each strain except MHV-NuU by reverse transcription-PCR (28) with PL-3 as a forward primer and S1N 990 (for MHV-1, -2, -3, and -A59) or S1N 990(MHV-S) (for MHV-S) as a reverse primer (Table 2), expressed these proteins in a transient vaccinia virus expression system using vTF7.3 (provided by B. Moss) (6, 7) in RK 13 cells, and examined whether these S1N(330) proteins bind to receptor prepared on membrane paper as previously reported (11, 20). All S1N(330) proteins were found to bind to the receptor protein, although differences in intensity were observed (data not shown). These data indicated that S1N(330) of each of the various MHV strains is responsible for binding to receptor prepared on membrane paper and suggested that there must be conserved region(s) within the N-terminal 330 aa of each strain that interacts with receptor.

We have determined the nucleotide sequences of the S1N(330) genes and compared the deduced S1N(330) amino acid sequences of the MHV strains for which sequences have not been published as well as those of MHV-A59 (12) and

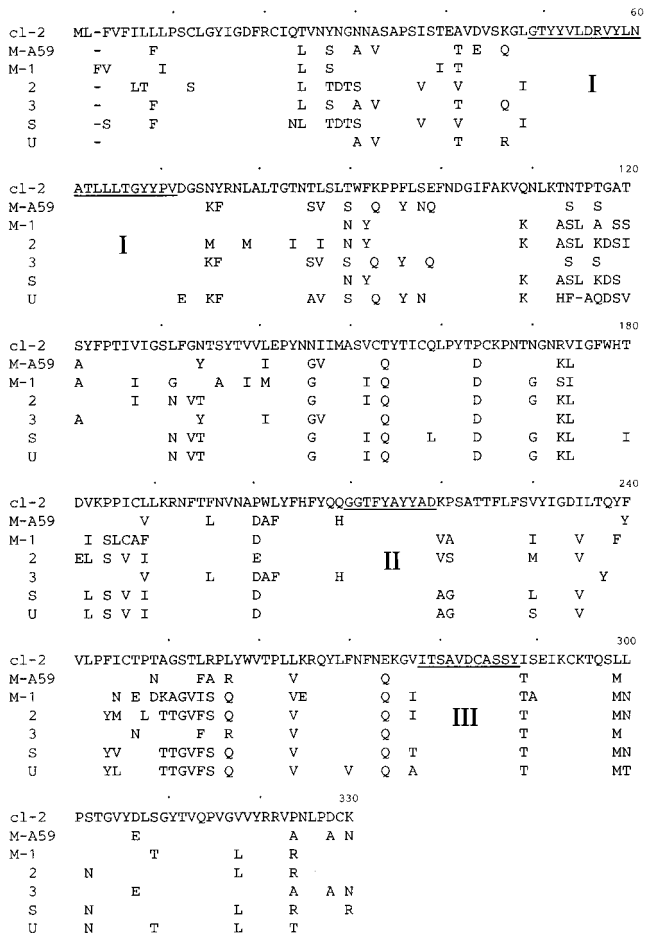

FIG. 1. Comparison of the N-terminal 330 aa of the S1N(330) proteins of seven MHV strains. The amino acid sequences were deduced from nucleotide sequences of S1N(330) genes isolated from these strains by reverse transcription-PCR.

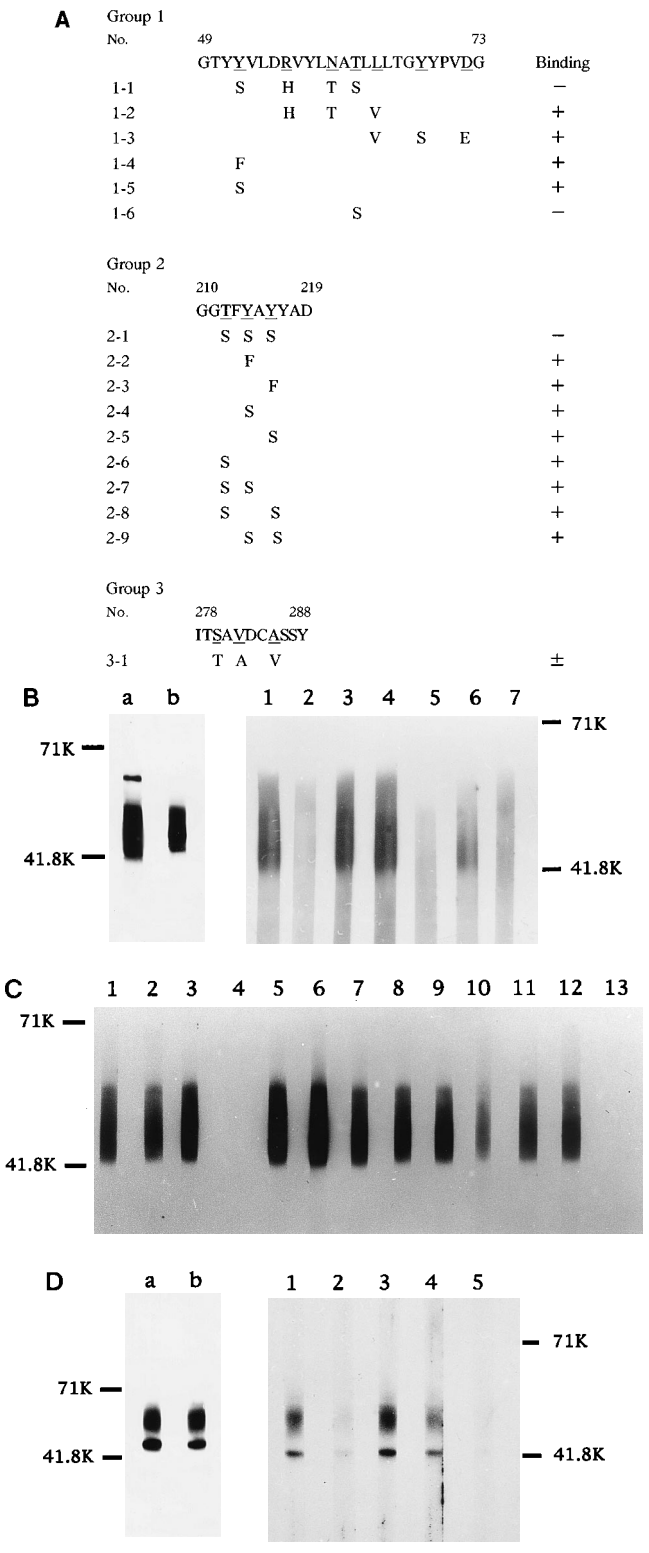

FIG. 2. (A) Amino acid substitutions introduced into each S1N(330) mutant and the receptor-binding ability of each mutant. These mutants were generated by site-directed mutagenesis using the primers shown in Table 2. (B and C) Binding of each mutant S1N(330) protein to receptor protein on membrane paper. (B) Receptor protein prepared on membrane paper was reacted with S1N(330) (lane 1), 1-1 (lane 2), 1-2 (lane 3), 1-3 (lane 4), 2-1 (lane 5), 3-1 (lane 6), or culture fluid of RK 13 cells infected with vTF7.3 (lane 7), and binding of the protein was monitored with an anti-S1 MAb and anti-mouse IgG labeled with peroxidase by ECL. (C) Receptor binding was examined as described for panel B with S1N(330) (lane 1), 1-4 (lane 2), 1-5 (lane 3), 1-6 (lane 4), 2-2 (lane 5), 2-3 (lane 6), 2-4 (lane 7), 2-5 (lane 8), 2-6 (lane 9), 2-7 (lane 10), 2-8 (lane 11), 2-9 (lane 12), or culture fluid of RK 13 cells infected with vTF7.3 (lane 13). (D) Inhibition of virus-receptor binding with the S1N(330) mutants. Receptor protein on membrane paper was reacted with S1N(330) (lane 2), 1-6 (lane 3), 2-1 (lane 4), sp-4 virus (lane 5), or culture fluid of RK 13 cells infected with vTF7.3 (lane 1) and then further reacted with cl-2. Binding of cl-2 to the receptor was monitored with anti-S MAbs reactive to only cl-2 S protein, not to S1N(330), and anti-mouse IgG labeled with peroxidase by ECL. In panels B and D, receptor protein was visualized by anti-human carcinoembryonic antigen rabbit serum that was shown to react with mmCGM1 and anti-rabbit IgG labeled with peroxidase by ECL (lane a). Receptor protein was also reacted with cl-2 virus, and binding was detected with an anti-S1 MAb and anti-mouse IgG labeled with peroxidase by ECL (lane b).

JHMV-cl-2 (22). The S1N(330) gene of MHV-NuU was obtained by reverse transcription-PCR with PL-3 and S1N 1056. As shown in Fig. 1, considerable differences in amino acid sequences were observed among these seven MHV strains throughout the S1N(330) sequences. The amino acid homologies were mostly 75 to 85%. MHV-1 was shown to have an additional amino acid position 3 from the N terminus, and MHV-NuU had one amino acid deletion, at position 115. Despite the high sequence divergence, in three regions there were sequences of more than 10 consecutively identical aa (sites I to III). Site I consisted of a large region with 23 aa from positions 49 to 71. The N-terminal half of site I was shown to be hydrophobic, while the C-terminal half was hydrophilic. Site II was composed of 10 aa from positions 210 to 219 and was hydrophilic. Site III consisted of 11 aa at positions 278 to 288 and was slightly hydrophobic. There was a region with identical amino acids at the N terminus of S1N(330) from positions 13 to 22. This region corresponded to the signal peptide (22) and therefore was expected not to exist in mature S1 protein.

To examine whether the regions conserved in the S1N(330) proteins of seven different MHV strains that utilize Bgp C as a receptor are involved in receptor-binding activity, we made a variety of mutant S1N(330) proteins that have amino acid mutations in site I, II, or III (Fig. 2A) by using a site-directed mutagenesis kit (Clontech, Palo Alto, Calif.), which is based on the method described by Deng and Nickoloff (2), with a selection primer [MCS-Hd-Pst(−)] and various mutagenic primers described in Table 2. pT7 Blue vectors (Novagen, Madison, Wis.) containing mutated S1N(330) genes downstream of the T7 promoter were transfected into RK 13 cells, and the proteins were expressed by the infection of vTF7.3 (20). Aliquots of the culture fluid isolated from RK 13 cells expressing various mutant S1N(330) proteins were analyzed by Western blotting to determine the amounts of the proteins produced in the culture fluids. We then adjusted the amounts of the S1N(330) proteins according to band intensity, incubated the samples with paper-bound receptor protein, and detected receptor-bound S1N(330) proteins with the S1-specific MAbs and anti-mouse immunoglobulin G (IgG) labeled with peroxidase by enhanced chemiluminescence (ECL; Amersham) as described previously (11, 20). We first analyzed mutant proteins with three different mutations in site I (1-1, 1-2, and 1-3), one mutant with mutations in site II (2-1), and one with mutations in site III (3-1). Figure 2B shows that mutants 1-2 and 1-3 bound to the receptor with the same intensity as wild-type S1N(330), while 1-1 and 2-1 were not able to bind. The binding of 3-1 was slightly lower than that of wild-type S1N(330). We further analyzed which amino acids in sites I and II are involved in receptor binding (Fig. 2C). In site I, the change of Tyr to Ser at position 52 or/and the change of Thr to Ser at position 62 must be responsible for loss of binding, since changes in 1-1

of Arg to His at position 56 and Asn to Thr at position 60 were shown to have no effect with respect to the receptor-binding ability of mutant 1-3, which has identical mutations at positions 56 and 60. The binding test using S1N(330) with a change of Tyr to Ser at position of 52 (1-5) or that with a change of Thr to Ser at position 62 (1-6) revealed that S1N(330) with a mutation at position 62 lacked receptor-binding ability. The change of Tyr at position 52 to Phe (1-4), categorized in a different amino acid group, had no effect. Mutant S1N(330), in which Thr, Tyr, and Tyr at positions 212, 214, and 216 were singly (2-4, 2-5, and 2-6) or doubly (2-7, 2-8, and 2-9) mutated to Ser, bound to the receptor almost to the same extent as wild-type S1N(330); the exception was 2-7, with mutations at positions 212 and 214, which was slightly lower in receptor-binding ability. These results indicated that these three amino acids in site II, in particular those at positions 212 and 214, were important for receptor-binding ability.

We confirmed this observation by performing an inhibition test of receptor-virus binding by a method described previously (20). The receptor protein prepared on membrane paper was first incubated with equal amounts of S1N(330) with receptor-binding ability and of S1N(330) 1-1 and 2-1, which failed to bind to the receptor. Then, JHMV cl-2 was further reacted with receptor pretreated with S1N(330) proteins, and the binding in cl-2 was monitored by using an MAb specific for cl-2 S protein that could not react with the S1N(330) proteins with sp-4 S protein. Sp-4 virus (21), which has been shown to bind to receptor (20), was used as a control. As shown in Fig. 2D, S1N(330) as well as sp-4 virus prevented the binding of cl-2 virus to the receptor, while S1N(330) 1-1 and 2-1 failed to prevent it. This result confirmed that S1N(330) had receptor-binding ability and that the other two mutant S1N(330) proteins lacked such ability.

In this study, we have tried to identify amino acids in S1N(330) that are important for receptor-binding activity. The binding of S1N(330) to an MHV-specific receptor was found to be a common feature among a variety of MHV strains. Such a feature should be conserved at the amino acid sequence level within 330 aa of all of those MHV strains. Our approach to identify the conserved regions in S1N(330) revealed three regions composed of more than 10 consecutively identical aa. Site-directed mutagenesis analyses of these regions demonstrated that the Thr at position 62 as well as amino acids at positions 212, 214, and 216 played an important role in receptor binding. However, the mutations in region III also slightly reduced receptor-binding activity, which shows that this region is also involved in receptor binding. These results may indicate that the receptor-binding site is composed of multiple, dissociated regions in the primary structure of S1N(330) and that there is a secondary or tertiary structure critical for the activity. This idea is supported by the finding that denatured S protein or S1N(330) was unable to bind to the receptor (11).

The receptor-binding site of rhinoviruses is thought to form a so-called canyon, a small concave region too small for immunoglobulin molecules to gain access (15). This implies that all neutralizing MAbs against rhinoviruses do not recognize the receptor-binding active site but instead probably recognize neighboring sites and that neutralization of rhinoviruses in most cases is due to the steric hindrance of virus-receptor interaction by immunoglobulin molecules. In the case of MHV, all neutralizing MAbs against variant JHMV cl-2 presumably do not recognize the receptor-binding site, since these MAbs are highly specific for JHMV variants and not reactive to other MHV strains, although as shown in this report, all other MHV strains should have a conserved receptor-binding site within 330 aa of the S protein N terminus. This assumption also leads to the idea that the receptor-binding site of MHV comprises the structure inaccessible by immunoglobulin molecules. This idea suggests that the MHV receptor-binding site may form a structure similar to the rhinovirus small canyon. To ascertain this possibility, detailed analysis of the amino acids involved in receptor-binding activity is needed.

Nucleotide sequence accession numbers. The sequences reported here are deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers: MHV-1 genomic RNA, D83333; MHV-2 genomic RNA, D83334; MHV-3 genomic RNA, D83335; MHV-U genomic RNA, D83336; and MHV-S, D83337.

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