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The S2 Subunit of the Murine Coronavirus Spike Protein Is Not Involved in Receptor Binding

FUMIHIRO TAGUCHI*

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

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The receptor-binding capacity of the S2 subunit of the murine coronavirus S protein was examined by testing the inhibition of virus-receptor binding. Sp-4 virus and S1N(330), which consists of the N-terminal 330 amino acids of the S1 protein, both of which exhibited receptor-binding capacity, were able to prevent the binding of cl-2 virus to the receptor, while the mutant protein S1N(330)-159, which failed to bind to the receptor protein, and S2TM$^-$, which lacks the transmembrane and cytoplasmic domains normally existing in the S2, were unable to prevent the binding of cl-2. By using cultured DBT cells, it was revealed that the infection of cells by cl-2 virus was significantly inhibited by S1N(330) but not by S2TM$^-$. These results indicate that the S2 protein is not involved in the receptor binding of murine coronaviruses.

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 (28, 37, 39). MHV has a genome RNA of about 31 kb which encodes four or five structural proteins as well as several nonstructural proteins (28, 29).

The spike protruding from the MHV virion is composed of two or three molecules of the spike (S) protein, each of which is a heterodimer consisting of two noncovalently bound S-protein subunits, S1 and S2 (29), derived from the N-terminal and C-terminal halves of the S protein (31). S1 is thought to form the globular head of the spike, and S2 is thought to form its stalk portion (5). The S protein of MHV induces fusion formation in cultured cells (1, 34, 38), and even uncleaved S protein has fusion activity (30, 32). The S protein is the major target of the neutralizing antibodies induced in mice (1) and also elicits cytotoxic T cells (21). Furthermore, the S protein is suggested to be a major determinant of viral virulence in animals (3, 9, 10, 23, 35).

Another important biological activity of the S protein is the binding to the MHV-specific receptors (16, 42) that belong to the carcinoembryonic antigen family (7, 8, 42, 44). The topologies of the S-protein subunits suggest that the receptor-binding site is more likely to exist on S1 than on S2. Recently, work in this laboratory revealed that the N-terminal region of S1 of MHV, composed of 330 amino acids (aa) [S1N(330)], has the receptor-binding activity (20). In porcine coronavirus, a domain located at the C terminus of the S1 subunit was reported to have the receptor-binding activity (13). Both of these viruses have various epitopes to elicit the neutralizing antibody in the domain with receptor-binding activity (13, 20). These facts fit the idea that the neutralization by antibodies is due to the inhibition of virus binding to the receptor. It has also been reported that the monoclonal antibodies (MAbs) specific for the S2 subunit of MHV showed the neutralizing activity (1, 4, 10, 22, 40), which may suggest that S2 is also involved in the receptor binding. In a previous study, the capacity of S2 to bind to the receptor failed to be tested because of the lack of an appropriate MAb to detect the receptor-bound S2. In the present study, the question of whether S2 is involved in receptor binding was tested by the inhibition of virus-receptor binding.

First, I established the system for examining the receptor-binding capacities of S proteins by an inhibition test. Both the cl-2 (36) and sp-4 (33) variants of neurotropic JHM virus were able to bind to the receptor prepared on the membrane paper by Western blotting (immunoblotting). The sp-4 S protein has a deletion of 141 aa in the middle, compared with the S1 protein of cl-2 (34). The MAbs against cl-2 S1 protein, MAbs 8, 12, and 56 (19), failed to react with the sp-4 S1 protein with the deletion. Thus, I tested whether the sp-4 viral particles prevented the binding of cl-2 virus using the mixture of these MAbs. The MHV receptor protein identical to MHVR(2d) (7), or Bgp C (24), was prepared on the paper by Western blotting as previously reported (20). The paper with the receptor protein was first incubated at room temperature (20 to 22°C) for 1 h with culture fluid (Dulbecco's minimal essential medium containing 6% tryptose phosphate broth [DMEM-TPB]) of DBT cells infected with sp-4 which had been concentrated by ultrafiltration with ultra-free PF (Millipore). Then the paper was incubated with DMEM-TPB containing 1×10^5 to 5×10^5 PFU of cl-2 per 0.1 ml at room temperature for 1 h. The paper was washed with phosphate-buffered saline, pH 7.2, containing 0.01% Tween 20, and the binding of cl-2 viruses was monitored with the mixture of MAbs described above and anti-mouse immunoglobulin G conjugated with peroxidase. As shown in Fig. 1C (lane 4), sp-4 concentrated 10 times from cultured fluid prevented the binding of cl-2. This indicated that the receptor-binding capacities of various proteins could be tested by the inhibition test.

In order to assess the receptor-binding capacity of S2, an S2 protein without the transmembrane domain and the cytoplasmic tail was prepared, since this protein was expected to be secreted in the culture fluid of cells. The gene encoding the S2 protein with a signal peptide at the N terminus and without the transmembrane domain (S2TM$^-$) was made by PCR from the ssS2 gene, which has signal sequence and transmembrane domain (18). With this gene as a template, two different oligonucleotide primers were constructed: the forward primer, PL-3 (a positive-sense 30-mer oligonucleotide corresponding to the leader sequence of JHMV) (20), and the reverse primer, S-stp-

* Mailing address: National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan. Fax: 423-46-1754. Electronic mail address: taguchi@ncnpja.ncnp.go.jp.

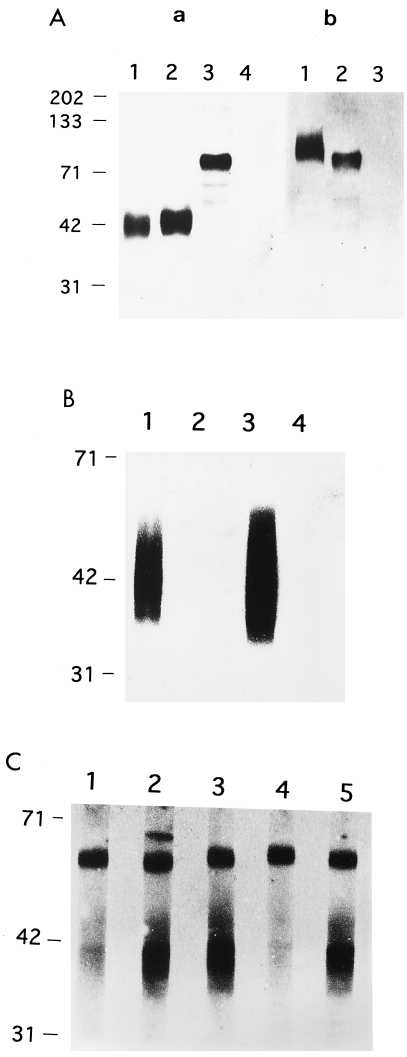

FIG. 1. (A) Western blot analysis of the S1 (a) and S2 (b) proteins transiently expressed by recombinant vaccinia virus vTF7.3. Culture fluids isolated from RK 13 cells producing S1N(330) (lane 1 [a]), S1N(330)-159 (lane 2 [a]), or S2TM$^-$ (lane 1 [b]) as well as from DBT cells infected with sp-4 (lanes 3 [a] and 2 [b]) and RK 13 cells infected with vTF7.3 only (lanes 4 [a] and 3 [b]) were concentrated by ultrafiltration and analyzed by Western blotting with MAbs 11F (a) and 10G (b). (B) Receptor-binding test of the S1 proteins. MHV receptor protein was prepared on membrane paper and was reacted with culture fluids isolated from RK 13 cells producing S1N(330) (lane 1) and S1N(330)-159 (lane 2) and cells infected with vTF7.3 only (lane 4). Culture fluid from DBT cells infected with sp-4 (lane 3) was also examined. The binding of the S protein was monitored with MAb 7. (C) Inhibition of receptor binding of cl-2 by the S proteins. MHV receptor protein prepared on membrane paper was pretreated with S1N(330) (lane 1), S1N(330)-159 (lane 2), S2TM$^-$ (lane 3), or sp-4 (lane 4) and then reacted with cl-2 virus. The binding of cl-2 to the receptor was examined with the mixture of MAbs reactive only to cl-2 S protein. Culture fluid from RK 13 cells infected with vTF7.3 only was used as a control (lane 5).

NdeI-N (5′-TTCATATGTGCCAACTTACTTGAGG-3′), corresponding to nucleotides 3909 to 3933 in the cl-2 S gene from the first nucleotide of the initiation codon (36). S-stp-NdeI-N contained the termination codon TAA, which replaced a codon (GAA) coding for the glutamic acid at position 1306 of the S protein (34). This PCR product encoded a protein which had a signal peptide in the N terminus and which lacked 71 aa containing the transmembrane and intracytoplasmic domains of the S2 protein. This gene was inserted into the pT7Blue Vector (pT7; Novagen, Madison, Wis.) downstream of the T7 promoter for expression. As controls for S2TM$^-$ in the receptor-binding inhibition test, I used two different proteins, S1N(330) and S1N(330)-159 (with and without receptor-binding activity, respectively). The gene encoding S1N(330), composed of the N-terminal 330 aa (from 1 to 330) (20) of S1, was constructed by PCR with a pair of primers as described previously (43). The gene encoding S1N(330)-159 was produced by mutating a 4-aa-encoding region of the S1N(330) gene by site-directed mutagenesis (6, 32a). The substitutions were Tyr to Ser, Arg to His, Asn to Thr, and Thr to Ser at positions 52, 56, 60, and 62, respectively, from the N terminus of the S protein (34). The ~1-kb DNA fragments encoding these proteins were then inserted into the pT7 vector. The pT7 vectors containing the genes for S2TM$^-$, S1N(330), and S1N(330)-159 were designated pT7S2TM(−), pT7S1N(330), and pT7S1N(330)-159, respectively.

The S1N(330), S1N(330)-159, and S2TM$^-$ proteins were expressed in RK 13 cells by the transfection of pT7S1N(330), pT7S1N(330)-159, and pT7S2TM(−) and subsequent infection of vTF7.3, kindly provided by B. Moss (11, 12). All of these proteins lacked the transmembrane domain and hence were expected to be secreted into the culture fluids of cells expressing these proteins. The amounts of these proteins as well as those of S1 and S2 in the concentrated sp-4 culture fluids were analyzed by Western blotting. The S1 and S2 proteins were detected with MAbs 11F and 10G, respectively, kindly provided by S. Siddell (27). As shown in Fig. 1A, S1N(330), S1N(330)-159, and S2TM$^-$ were all present in amounts equivalent to those of S1 or S2 of sp-4. The molecular masses of S1N(330) and S1N(330)-159 were more or less 42 kDa (Fig. 1A), which is similar to our previous observation (20). S2TM$^-$, which was about 95 kDa, was, however, slightly larger than the authentic S2 protein on the virions, which was 86 kDa, as shown in Fig. 1A. The structure of the S2TM$^-$ gene suggested that the protein was smaller than the native S2 protein, since it lacked 71 C-terminal aa. It was shown in our previous work that ssS2 expressed in a vaccinia virus expression system was larger than the authentic S2 protein produced in cells infected with cl-2 virus (18).

I then examined whether these S1 proteins as well as sp-4 virus bound to the receptor prepared on the membrane paper. The paper with receptor was incubated with S1N(330), S1N (330)-159, or sp-4, and the binding of these proteins was monitored with MAb 7. As shown in Fig. 1B, a heavy band was observed at the position corresponding to the receptor protein of ca. 42 kDa when S1N(330) and sp-4 were incubated with the receptor-bound paper, while no band was seen with S1N(330)-159. S1N(330)-159 concentrated 10 times was also unable to bind to the receptor (data not shown). The S2TM$^-$ was not determined to bind to the receptor because of the lack of MAb that reacts with the receptor-bound S2 protein as previously reported (20).

In the receptor-binding inhibition test, the paper with receptor protein was first incubated with sp-4, S1N(330), S1N(330)-159, and S2TM$^-$ at room temperature for 1 h. Then, the receptor on the membrane was reacted with cl-2, and the binding of cl-2 to the receptor was monitored with cl-2-specific MAbs. As shown in Fig. 1C, the receptor binding of cl-2 was apparently inhibited when the receptor was pretreated with sp-4 and S1N(330), both of which showed receptor-binding activity, as shown in Fig. 1B. However, such inhibitory effects were not detected when the receptor was pretreated with S1N(330)-159, which lacks receptor-binding activity, or with S2TM$^-$. These results suggested that the S2 subunit of the S protein failed to react with the receptor protein. An apparent band of ca. 65 kDa was observed in all samples in Fig. 1C. This

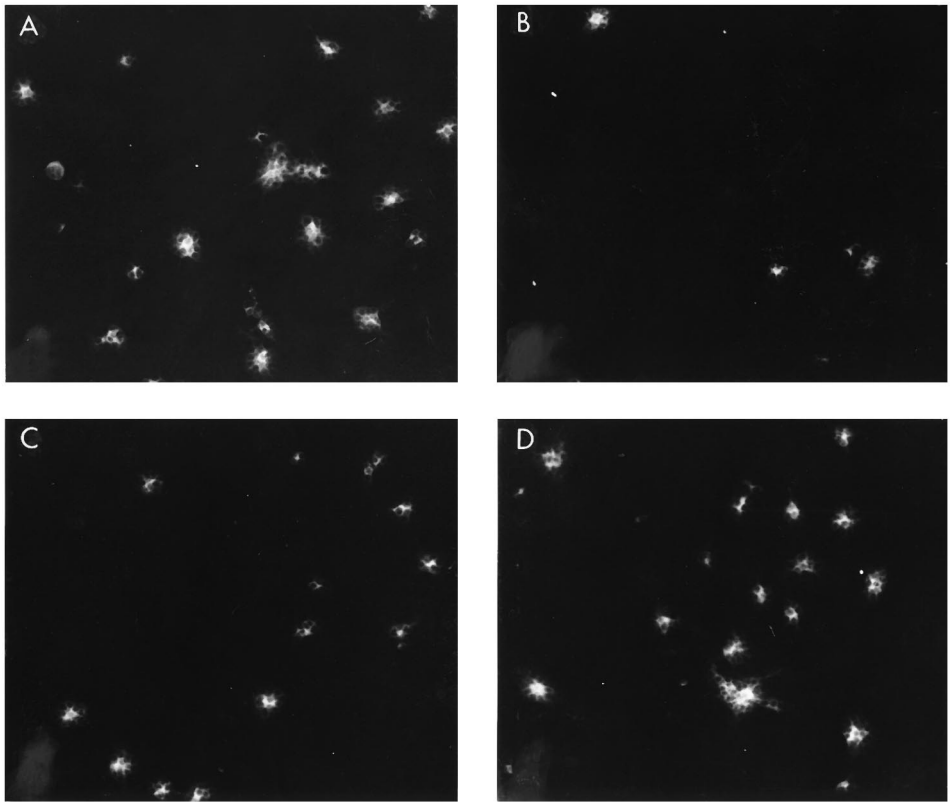

FIG. 2. Immunofluorescence of cl-2-infected DBT cells pretreated with S1N(330) (B), S1N(330)-159 (C), or S2TM$^-$ (D) and of mock-treated cells (A). Pretreated DBT cells were infected with cl-2, and the foci of cells with cl-2 S antigen were examined with MAbs specific for the cl-2 S protein.

band was not detected when receptor-bound S1N(330) and sp-4 virus were monitored with MAb 7, as shown in Fig. 1B. Thus, this band could result from the binding of the mixture of MAbs 8, 12, and 56 to a substance unrelated to the receptor protein.

Next it was examined whether infection of DBT cells with cl-2 was inhibited by pretreatment with S1N(330), S1N(330)-159, or S2TM$^-$ proteins. DBT cells (15) cultured in a 7-mm-diameter well on a glass slide were incubated with S1N(330), S1N(330)-159, or S2TM$^-$ at 4°C for 1 h. Then, the cells were inoculated with cl-2 virus at a multiplicity of infection of 0.02 PFU per cell and incubated at 4°C for 1 h. The number of cells (single or fused) with MHV antigen was monitored by staining with anti-cl-2 S MAb at 7 to 10 h after incubation at 37°C. As shown in Fig. 2, the number of cells with viral antigen was significantly reduced when cells were pretreated with S1N(330) (more than 90% reduction) but was not different in cells pretreated with S2TM$^-$ (less than 10% reduction) compared with the number of antigen-positive cells or foci in untreated cells. The number of antigen-positive cells in cells pretreated with S1N(330)-159 was slightly reduced (30 to 40%) compared with the number in control cells.

Thus, both the receptor-binding inhibition test and the infection inhibition test suggested that the S2TM$^-$ failed to bind to the receptor, in contrast to S1N(330). Although the S2TM$^-$ protein lacked 71 aa located at the C terminus in the authentic S2 protein, this deletion could not explain the lack of receptor-binding capacity of expressed S2TM$^-$, since this region includes the transmembrane and intracytoplasmic domains that are located inside the viral envelope and is not likely to be involved in the receptor binding of the S protein.

The neutralizing activity of the MAbs specific for S2 of MHV and other coronaviruses (4, 13, 17, 25, 40) suggests that the epitopes recognized by such MAbs or their neighboring regions are involved in receptor binding, which is not in agreement with the results of the present study. Taking these conflicting data together with the observations that the S proteins of coronaviruses are extremely conformational (2, 14, 19), it is conceivable that the neutralization (inhibition of receptor binding) by anti-S2 MAbs is mediated not by their interaction with the receptor-binding site but by their interaction with a region which is distant in the primary structure from the binding site, which results in the inhibition of the binding of S1N(330) with the receptor presumably by steric hindrance. Alternatively, it might be possible that the S2, like the membrane-anchoring subunit of other enveloped virus glycoproteins (41), plays an important role in virus penetration after virus binds to the receptor and that the neutralization by S2-specific MAbs results from the inhibition of internalization of receptor-bound viruses. This possibility is currently under investigation.

The receptor-binding site of picornaviruses is believed to be formed by the small concave region, the "canyon," that is not accessible by the immunoglobulin molecule (26). This implies that neutralizing antibodies to picornaviruses cannot bind to the receptor-binding site of the viruses. Also, it is thought that no neutralizing MAbs against JHMV directly interact with the receptor-binding site located in the N-terminal 330 aa, since all neutralizing MAbs we have isolated were found to be specific for the JHM virus strain (19); however, the receptor-binding site must be conserved in variety of MHV strains, since those viruses can utilize Bgp C as a functional receptor (24, 32a). It

is likely that the MAbs that bind to the epitopes located in the vicinity of receptor-binding site neutralize virus infectivity by preventing the binding of viral protein to its receptor. It seems also likely that the receptor-binding active site of MHV has a structure with which the antibodies cannot directly interact, as in the case of the picornavirus receptor-binding site. Further analysis of the receptor-binding site of MHV will answer this question.

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