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ISOLATION AND CHARACTERIZATION OF MURINE CORONAVIRUS MUTANTS RESISTANT TO NEUTRALIZATION BY SOLUBLE RECEPTORS

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1. ABSTRACT

Murine coronavirus mutants resistant to neutralization with soluble receptors were isolated to study the receptor-binding site on the S proteins since such mutants were expected to have mutations in an important site for receptor-binding. We have isolated five soluble receptor-resistant (srr) mutants which had mutations of a single amino acid at 3 different positions in S protein. Srr mutant 11 with an amino acid change at position 65 (Leu to His) in the S1 subunit showed an extremely reduced binding by virus overlay protein blot assay. However srr mutants with a mutation at 1114 (Leu to Phe) (srr mutants 3, 4 and 7) or 1163 (Cys to Phe) (srr mutant 18) in the S2 subunit had receptor-binding activity similar to that of wild type cl-2. These results suggest that an amino acid at position 62 located in a conserved region among MHV strains is in particular important for receptor binding. We also discuss why srr mutants with a mutation in S2 showed high resistance to neutralization by soluble receptor, irrespective of their binding to MHV receptors.

2. INTRODUCTION

The spike projecting from the virion surface of coronavirus is composed of two or three molecules of the spike (S) protein, each of which is a heterodimer consisting of two non-covalently bound subunits, S1 and S2 (Sturman and Holmes, 1984). These S1 and S2 subunits are derived from cleavage of the N-terminal and C-terminal halves of the precursor S protein by a host-cell derived protease (Sturman and Holmes, 1984). The S1 subunit

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is believed to form the globular part of the spike and the S2 its stalk portion (De Groot et al., 1988). The S protein has many important biological functions. One of them is the fusion of cultured cells (Taguchi et al., 1992; Collins et al., 1982). Uncleaved S protein has a fusion activity (Stauber et al., 1993; Taguchi, 1993), suggesting that the mechanism of MHV fusion differs from that of other fusogenic RNA viruses (White, 1990). The S protein is the major target of the neutralizing antibodies induced in mice. It also elicits cytotoxic T cells (Flory et al., 1993). Furthermore, the S protein is suggested to be a major determinant of viral virulence in animals (Matsubara et al., 1991; Dalziel et al., 1986; Fleming et al., 1986).

The binding to the receptor proteins (Williams et al., 1991) is another important biological function of the MHV S protein. The receptor-binding domain on the S protein of MHV is located in the N-terminal region of the S1 composed of 330 amino acids [S1N(330)] (Suzuki and Taguchi, 1996; Kubo et al., 1994) but not on the S2 (Taguchi 1995). Recently, we have identified amino acid residues responsible for receptor-binding using various mutant S1N(330) generated by site-directed mutagenesis (Suzuki and Taguchi, 1996). To further identify the receptor-binding site in the S protein, we have utilized a different approach, the isolation and characterization of mutant viruses that escaped from neutralization by soluble receptor protein. Since such resistant viruses are expected to carry the mutation defective in the receptor binding (Colston and Racaniello, 1994), the mutations found in the S protein of such mutants appeared to be most useful tools to identify the amino acids responsible for receptor binding.

3. MATERIALS AND METHODS

3.1. Cells and Viruses

Cell lines (DBT and RK 13) were grown as previously reported (Kubo et al., 1994). The MHV strain JHMV cl-2 (Taguchi et al., 1985) and the recombinant vaccinia virus vTF7.3 were prepared and assayed as described elsewhere (Fuerst et al., 1987).

3.2. Preparation of Soluble Receptor Protein

The MHV receptor gene of MHVR1 (Kubo et al., 1994) or Bgp C was used for expression. The soluble form of the MHV receptor protein (soMHVR1) with the transmembrane and intracytoplasmic domains deleted was tagged with influenza HA epitope (Ohtsuka et al., 1996) and was expressed by recombinant vaccinia virus vTF7.3 (Fuerst et al., 1987). The soMHVR1 expressed and secreted into the culture was concentrated by ultrafiltration (Millipore) after removing vaccinia virus by centrifuging at 20,000 rpm for 2 h.

3.3. Isolation and Sequencing of the Srr Mutant S Genes

Total RNA (0.2 μ g) from DBT cells infected with virus was reverse transcribed and cDNAs were used for PCR using a pair of primers (5'-CGCAAGCTTCTAAA-CATGCTGTTCGTC-3' and 5'-ATCTTGGGACCGATGA-GGGCCAT-3' corresponding to the cl-2 S gene initiation codon and termination codon, respectively. The S cDNAs were directly sequenced on both strands using a series of S gene specific primers (Taguchi et al., 1992) labeled with FITC and Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham) in a DNA sequencer (Pharmacia, Uppsala, Sweden).

3.4. Western Blotting

Western blotting analysis was carried out using the S1-specific monoclonal antibody 30 B (Routledge et al., 1991) as previously reported (Taguchi, 1993).

3.5. Virus Overlay Protein Blot Assay (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 (Ohtsuka et al., 1996; Kubo et al., 1994).

4. RESULTS

4.1. Isolation of Srr Mutants Resistant to Neutralization by soMHVR1

To identify the amino acid residues of the S protein involved in the virus-receptor interaction, we isolated virus mutants resistant to neutralization by soluble receptor protein. In order to isolate the srr mutants, we used the concentrated soMHVR1 expressed by recombinant vaccinia virus vTF7.3. A mixture of 100 μl of cl-2 titered virus (2×10^5 PFU) and 500 μl of soMHVR1 was incubated at room temperature for 60 min and then inoculated onto DBT cells. The plaques produced were isolated 1 to 2 days later and subjected to 3 further plaque-purifications. As shown in Table 1, five virus clones (designated as srr 3, srr 4, srr 7, srr 11, and srr 18) were highly resistant to neutralization by soMHVR1. Wild type virus titer was reduced by 1/50 by incubation with soMHVR1 solution, while the five srr mutants were hardly affected. This result suggested that the five mutant viruses were selected though the resistance to neutralization with soMHVR1. Srr mutants were isolated with a frequency of about 1 per $1x10^5$ PFU of the wild-type cl-2 population.

4.2. Sequence Analysis of S Gene in Srr Mutants

To identify the mutated amino acid residues in the S proteins of srr mutants, we determined the sequence of the entire S protein coding region. Sequence analysis of S cDNA containing 4,131-nucleotide revealed that each srr mutant has a single nucleotide mutation which creates an amino acid change in S1 or S2 subunit (Fig. 1). Srr 11 had an amino acid

Table 1. Neutralization of MHV-JHM cl-2 and srr mutants by soMHVR1

	Titer (pfu/0.1ml)		
Virus	+SoMHVR1	-SoMHVR1	Relative neutralization efficiency
Srr 3	2.5×10^5	2.2×10^5	1.14
Srr4	2.3×10^5	2.4×10^5	0.96
Srr7	1.2×10^5	1.4×10^5	0.86
Srr 11	1.0×10^5	1.0×10^5	1.00
Srr 18	1.1×10^5	1.1×10^5	1.00
JHM cl-2	2.3×10^3	4.8×10^5	0.005

Figure 1. Distribution of amino acid changes on srr mutant S proteins. The S protein (1376 residues) is depicted by a horizontal line. Arrows indicate amino acid alterations in the mutant S proteins. Signal and transmembrane peptides are indicated by dotted and dashed bars respectively.

change in S1 subunit at position 65 (Leu to His) in a highly conserved region (at positions between 49 and 71) among MHV strains (Suzuki and Taguchi, 1996). Other alternations at positions 1114 (Leu to Phe) (srrs 3, 4 and 7) and 1163 (Cys to Phe) (srr 18) were present between two heptad repeat domains in the S2 subunit (De Groot et al., 1988). We chose three srr mutants (srr 7, srr 11, and srr 18) for further characterization because each of them had a single amino acid substitution in different positions of the S protein.

4.3. VOPBA of Srr Mutant S Proteins of soMHVR1

To compare the receptor-binding activities of wild type and srr S proteins, we assessed the ability of mutant S proteins to bind to the soMHVR1 by using VOPBA. Equivalent amounts of Srr mutant and wild-type S proteins were subjected to VOPBA. Srr 11 with a substitution at position 65 (Leu to His) in S1 subunit resulted in more than 98% loss of soMHVR1 binding activity compared to that of wild type (Fig. 2, lanes 1 and 3), while the S protein mutated at position 1114 (Leu to Phe, mutant 7) or 1163 (Cys to Phe, mutant 18) in the S2 subunit showed receptor-binding similar to that of wild type cl-2 (Fig. 2, lanes 1, 2 and 4). We have also assayed the binding activity of the mutant S proteins under several different S protein concentrations. However, the binding patterns for the srr 7 and srr 18 S proteins were similar to that of wild type S protein (data not shown). These results indicate that the substitution at position 65 in the S1 subunit remarkably reduced the capacity to bind to soMHVR1. However, the alteration at position 1114 or 1163 in the S2 subunit did not affect the binding to soMHVR1.

5. DISCUSSION

We have previously demonstrated by site-directed mutagenesis that the amino acids at positions 62 and a region consisting of amino acids 212 to 216 are important for receptor-binding (Suzuki and Taguchi, 1996). In the present study, we have used another procedure

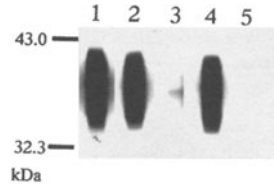

Figure 2. Analysis of receptor-binding activities by VOPBA. Wild type (Lane 1); srr 7 mutant position 1114 (Leu to Phe) (Lane 2); srr 11 mutant position 65 (Leu to His) (Lane 3); srr 18 mutant position 1163 (Cys to Phe) (Lane 4) and control DBT culture medium (Lane 5) were incubated at RT for 1 h with soMHVR1 transferred to the membrane paper by Western blotting. The binding of viruses were evaluated with the S1 specific monoclonal antibody No. 7 and horseradish peroxidase labeled anti-mouse IgG by enhanced chemiluminescence.

to define the region involved in receptor-binding. The srr mutants that are resistant to neutralization by the soluble receptor protein are supposed to contain amino acid changes in the receptor-binding site, as studied in detail for poliovirus (Colston and Racaniello, 1994). Three different srr mutants were isolated, each of which contained only one amino acid change in the S protein. Srr 11 contained the mutation at amino acid position 65 (Leu to His) in S1 subunit. This mutant showed high resistance to neutralization by soluble receptor and its binding activity to the soluble receptor was dramatically decreased. Thus, this particular amino acid at a position 65 appeared to be responsible for the receptor binding activity. This amino acid is located in the vicinity the amino acid 62 which was previously found to be important for receptor binding (Suzuki and Taguchi, 1996). These facts suggest that a region composed of amino acids from 62 to 65 is critical for receptor binding. Our previous study has shown that the mutations at different amino acids between 49 to 70 of the S protein, conserved in all MHV strains examined, did not influence the receptor-binding activity with the exception of the amino acid at position 62 (Suzuki and Taguchi, 1996). These data indicate that the amino acids at positions 62 and 65 are particularly important and that not necessarily all of the amino acids in this region are involved in the receptor-binding activity.

Srr 7 and srr 18 contained an amino acid mutation in the S2 subunit, at positions 1114 (Leu to Phe) and 1163 (Cys to Phe), respectively. We first speculated that the mutations at 1114 and 1163 reduce the receptor-binding activity of the S1, since the mutation in S2 subunit has been reported to influence the conformation of the S1 (Grosse and Siddell, 1994). However, that was not the case. Although these two mutants showed resistance to neutralization with soluble receptor protein, the receptor-binding capacity was not different from that of wild type virus. This means that the mutant S proteins bind to the soluble receptor protein as efficiently as did wild type S protein and that such soluble receptor-bound virus particles are still infectious. At present, no experimental data is available to account for this phenomenon. However, a difference was found between the mutant S proteins and that of wild type in the stability of the S1-S2 association. Wild type S1 and S2 proteins could easily be dissociated after binding to the receptor protein, but the S1-S2 association of srr 7 and srr 18 was more stable than that of wild type (data not shown). Such a stable association between S1 and S2 after binding to the receptor protein may account for the characteristics of srr of these two mutant viruses. Recently, it was reported that the S1 of wild type JHM with a large S protein can easily be dissociated from S2 after binding to receptor, however, the JHMX which contained a large amino acid deletion in the S1 subunit showed a stable association of S1 and S2 (Gallagher, 1997). We have found that JHMX is resistant to neutralization by soMHVR1, that is , JHMX has characteristics similar to our srr mutants (data not shown). These data suggest that the stable association of the S1 and S2 subunits after binding to the receptor protein may be related to the characteristics of the srr mutant.

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