Functional analysis of an epitope in the S2 subunit of the murine coronavirus spike protein: involvement in fusion activity

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The monoclonal antibody (MAb) 5B19.2, which has virus-neutralizing and fusion inhibition activities, binds to an epitope (S2A) consisting of nine hydrophobic amino acids in the S2 subunit of the mouse hepatitis virus (MHV) spike (S) protein. This suggests that the S2A epitope may be involved in binding the virus to the MHV receptor and/or in virus–cell fusion. Co-immunoprecipitation analyses demonstrated that while the binding of virus to the receptor was blocked by anti-S1 MAbs, it was not blocked by the S2A antiserum, indicating that S2A was not involved in receptor-binding. The S proteins prepared in this study with mutations in the S2A epitope were either fusogenic or non-fusogenic and their fusogenicity did not correlate with the hydrophobic feature of the S2A epitope. All of these wt and mutated S proteins were similarly transported onto the cell membrane independent of their fusogenicity capability. These results suggest that S2A may mediate the fusion activity of the MHV S protein during virus entry into cells.

The spike (S) protein of mouse hepatitis virus (MHV), a member of the family Coronaviridae, forms a spike projecting from the virion surface. The spike comprises two molecules of the S protein, each of which is a heterodimer consisting of two non-covalently bound subunits, S1 and S2 (Sturman et al., 1985). The S1 subunit makes up the globular part of the spike and the S2 subunit is the stalk portion (De Groot et al., 1987).

An important biological function of the S protein is binding to the virus receptor, which is a member of the carino-embryonic antigen gene family (Dveksler et al., 1991). The receptor-binding domain is located in the N-terminal 330 amino acids of the S1 subunit (S1N330) (Kubo et al., 1994; Suzuki & Taguchi, 1996), rather than on subunit S2 (Taguchi, 1995), as is expected from the topologies of these subunits. Although the receptor-binding site has been mapped to S1N330, it is possible that additional regions in S1 and/or S2 may play an important role in virus–receptor interactions. Another important function is cell–cell and virus–cell fusion (Collins et al., 1982; Taguchi et al., 1992). Various regions in S2 are involved in fusion activity (Bos et al., 1995; Gallagher, 1996; Gallagher et al., 1991; Luo & Weiss, 1998).

Monoclonal antibody (MAb) 5B19.2, which is specific for S2, has been reported to have virus-neutralizing (VN) and fusion-inhibition (FI) activities (Collins et al., 1982). Its binding site, epitope A (S2A), is composed of nine hydrophobic amino acids, Leu–Leu–Gly–Cys–Ile–Gly–Ser–Thr–Cys (Fig. 1). The S2A epitope is located upstream of heptad repeat 1 (Luytjes et al., 1989) and forms a major antigenic determinant in the S2 subunit (Daniel et al., 1993). Antibodies specific to this epitope play an important role in protection in mice (Koolen et al., 1990). Although MAb 5B19.2 was isolated from mice immunized with MHV-4 (JHM strain), it is cross-reactive with other MHV strains (Talbot & Buchmeier, 1985). These findings suggest that S2A plays a critical role in the life-cycle of MHV, presumably in either virus–receptor interactions and/or virus entry into cells. In the present study, we analyse the receptor-binding and fusion activities of the S2A epitope.

The fact that the S2A-specific MAb 5B19.2 displays VN and FI activities suggests that S2A is involved in receptor-binding and/or fusion. In order to confirm that the antibodies recognizing the S2A epitope have VN and FI activities like MAb 5B19.2 and also to test the above possibility, we have made antiserum by immunizing rabbits with a synthetic peptide that encompasses the S2A epitope. The specific VN values (VN titres/ELISA titres) of the S2A antiserum and MAb 5B19.2 to JHMV variant cl-2 (Taguchi et al., 1985) were 103- to 104-fold lower than the VN values of anti-S1 MAbs 3, 6, 13 and 93 (Kubo et al., 1993). In contrast, the specific FI values (FI titres/ELISA titres) of the S2A antiserum were the same or slightly higher than those of anti-S1 MAbs. These results confirm that antibodies against the S2A epitope have VN and FI activities but are not as effective at VN as anti-S1 MAbs that bind to the receptor-binding site of the S protein. These
findings suggest that the S2A antiserum and MAb 5B19.2 neutralize JHMV by a mechanism different from anti-S1 MAbs.

Since S2A antiserum neutralizes JHMV infectivity, albeit weakly, we examined whether this neutralization was due to the inhibition of MHV binding to its receptor CEACAM1a (MHVR1) (Dveksler et al., 1991), although S2 has been shown not to bind to a receptor (Taguchi, 1995). MHVR1 tagged with the Influenza virus HA epitope (Ohtsuka et al., 1996) was bound to protein A–Sepharose beads via anti-HA antibodies. JHMV (5 × 10⁶ p.f.u. in 100 µl) was incubated with 20 VN units of either anti-S1 MAbs or S2A antiserum at room temperature for 2 h. In this experiment, 20 VN units of each antibody neutralized more than 95% of JHMV. The virus stock treated with anti-S1 MAbs was also incubated with anti-mouse IgG Fc antibodies in order to prevent direct binding of anti-JHMV-S1 MAb complexes to the protein A–Sepharose beads via the Fc portion of the anti-S1 MAbs. The treated virus stocks were then allowed to interact with the MHVR1-bound protein A–Sepharose beads. The beads were collected by centrifugation, washed, subjected to SDS–PAGE and blotted onto a transfer membrane. As shown in Fig. 2, S protein was still bound by MHVR1 when viruses were treated with S2A antiserum, MAb 13 or control serum. This shows that these antibodies failed to block the binding of viruses to the receptor. In contrast, only trace amounts of the S protein were detected following the treatment of the JHMV stock with MAbs 3, 6 or 93, indicating that these three MAbs blocked the binding of virus to the receptor. These results indicate that the S2A antiserum fails to block the binding of virus to the receptor and argue strongly against the involvement of the S2A epitope in receptor-binding activity. Neutralization of S1-specific MAb 13 was shown to be independent of the interference of virus binding to the receptor. Its VN activity is, however, presumably not due to the inhibition of virus–cell fusion, since its FI activity was slightly lower relative to that of other MAbs. MAb 13 could inhibit virus internalization, as reported for several MAbs against human immunodeficiency virus and influenza virus; these MAbs neutralize these viruses without interfering with the receptor binding of the viruses (Armstrong et al., 1996; Outlaw & Dimmock, 1993; Skinner et al., 1988).

Since the S2A antiserum did not interfere with receptor-binding by JHMV, its VN activity could result from the inhibition of virus–cell fusion. To evaluate the role of the S2A epitope in the process of virus–cell fusion, we have examined whether S2A plays a role in syncytia formation, which may occur by the same mechanism as virus–cell fusion. We have prepared a series of mutated S proteins that have amino acid substitutions in the S2A epitope. These mutated S genes were prepared by PCR using various mutating primers as previously described (Taguchi, 1993). Mut-1 and mut-4 have mutations at amino acid positions 1, 4 and 7, while mut-2 and mut-5 have mutations at amino acid positions 2, 5 and 8. Mut-3 and mut-6

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**Fig. 1.** The S2A epitope in the JHMV S protein. (a) Schematic diagram of the JHMV S protein and S2A. The following areas of the S protein are shown: the S2A epitope (S2A), signal sequence (SS), receptor-binding domain (SIN330), hypervariable region (HVR), cleavage site (CS), heptad repeat 1 (HR1), heptad repeat 2 (HR2), a candidate fusion peptide (PEP1) and the transmembrane domain (TM). The arrowheads indicate the epitopes that are recognized by MAb 30B and 10G. The boxed region depicts a hydrophobicity plot between residues 870 and 970. The hydrophobicity of the region around the S2A epitope, which is indicated by the shadowed box, and the amino acid sequence of the wt S2A epitope is also shown. (b) Amino acid sequences of mutant S2A epitopes. Amino acid residues that are mutated in each S2A mutant are shown. The amino acid residues that are identical to those found in wt S2A are indicated by a dash.

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**Fig. 2.** Inhibition of JHMV binding to MHVR1 by anti-S antibodies as examined by co-immunoprecipitation. JHMV was incubated with 20 VN units of S2A antiserum (S2A), S1-specific MAbs (3, 6, 13 and 93) or normal rabbit serum (cr) at room temperature for 2 h. The treated virus stock was then mixed with either MHVR1-coupled Sepharose 4B beads, (MHVR1 +), or Sepharose 4B beads alone (MHVR1 –). MHVR1-bound JHMV was precipitated and transferred onto a membrane for Western blot analysis. The S proteins were detected by enhanced chemiluminescence using S1-specific MAb 30B and horseradish peroxidase-conjugated antimouse IgG. Lane S1 shows the S1 subunit band prepared from JHMV virions.
are mutated at amino acid positions 3, 6 and 9 (Fig. 1). In mut-1 to mut-3, hydrophobic residues were substituted with different hydrophobic residues. Mut-4 to mut-6 had changes from hydrophobic to hydrophilic residues. These mutated S proteins were expressed in DBT cells using vTF7.3 as previously described (Fuerst et al., 1986; Saeki et al., 1997) and syncytia formation was examined by staining with Giemsa (Kubo et al., 1993). Mut-1 and mut-4 failed to induce syncytia formation in DBT cells throughout the observation period and the syncytia caused by mut-2 were smaller than those produced by the wt S protein; the other mutants exhibited fusion activity similar to the wt S protein (Fig. 3a). In order to determine which of the three residues altered in mut-4 was critical for fusion activity, we made mut-41, mut-44 and mut-47, which contain mutations at positions 1, 4 and 7, respectively. Syncytia formation by mut-41 was slightly but significantly reduced, while the other two mutants induced syncytia in a similar fashion as the wt S protein (Fig. 3a). Fusogenicity was also quantitatively measured by using the pG1NT7 β-galactosidase plasmid, which encodes the β-galactosidase gene under the control of the T7 promoter, as previously reported (Nussbaum et al., 1994). This test showed a similar result to that obtained with Giemsa staining (data not shown). These results suggest that Leu4, Cys4, and Ser7 influence the fusogenicity of the S protein, with Leu4 being the most important. Other residues were also important for fusion activity, as shown by the reduced fusogenicity of mut-2. Furthermore, these results suggest that the hydrophobicity of S2A is not critical for its fusion activity.

Since the failure of mut-1 and mut-4 to induce fusion and the reduced fusogenicity of mut-2 could be due to their inappropriate processing, we examined the size and cleavability of the mutant S proteins by Western blot analysis. Both uncleaved and cleaved forms of S proteins were detected, though cleavability differed among the mutants (Fig. 3b). Differences in cleavability did not correlate with differences in fusogenicity (Fig. 3a, b), consistent with the finding that the S protein cleavage was not necessary for its fusogenicity (Taguchi, 1993). We further examined whether the S proteins were transported onto the cell membrane. DBT cells transfected with plasmids containing the various S genes and infected with vTF7.3 were labelled with biotin 6–8 h after transfection. The procedure used only allowed labelling of the proteins exposed on the cell surface (Hernandez & White, 1998). Lysates prepared from biotinylated cells were mixed with avidin coupled to agarose, which selected only the biotinylated proteins. We analysed both the biotinylated and total cell proteins by Western blot. In all samples, an almost identical proportion of cell surface-localized S protein relative to total cell S protein was detected after selection with avidin–agarose, showing that the same proportion of S protein synthesized was transported onto the cell membrane, independent of the fusogenicity of the S protein (Fig. 3c). Large amounts of actin, a protein not transported onto the membrane,
were detected in the total lysates but only trace amounts were detected in the samples selected by avidin–agarose, indicating that the majority of biotinylated S proteins were exposed on the cell surface (Fig. 3c). The transport of fusogenic and non-fusogenic S proteins onto the cell membrane was also confirmed by membrane immunofluorescence (data not shown). These results indicate that the mutated S proteins are appropriately processed and are transported onto the cell membrane, irrespective of their fusogenicity. Taken together, our analysis of mutations in the S2A epitope supports the idea that S2A plays a critical role in fusion formation with the S protein, despite lacking the features of an ordinary fusion peptide.

It was recently reported that a region, PEP1, in the heptad repeat 1 of MHV strain A59 could work as a fusion peptide (Luo & Weiss, 1998). The fusion peptide motif has several common features: it is relatively hydrophobic, it adopts an alpha-helical conformation and it is rich in Ala and Gly (White, 1990). Further, substitution of hydrophobic amino acids in the fusion peptide with hydrophilic residues usually destroys fusion activity (Bosch et al., 1989; Gething et al., 1986; White, 1990). The PEP1 fusion peptide has these features (Luo & Weiss, 1998). However, the hydrophobic feature of S2A is not critical for fusion activity. Moreover, the S2A epitope is unable to form an alpha-helix according to computer analysis. These findings suggest that S2A plays an important role in fusion activity, yet the mechanism by which the S2A epitope operates in fusion events appears to be different from that of the ordinary fusion peptide (White, 1990). Completion of MHV fusion events may require several distinct steps of interaction between the viral envelope protein and the cell membrane, with some functions performed by the PEP1 fusion peptide and others by S2A.

MAb 5B19.2 cross-reacted with other MHV strains suggesting that the S2A epitope exists in these MHV strains (Talbot & Buchmeier, 1985). JHMV and MHV-A59 S proteins have an identical 15 amino acid cluster encompassing the S2A epitope (Luytjes et al., 1987; Parker et al., 1989; Schmidt et al., 1987). Four other MHV strains have a stretch almost identical to this cluster with one to four amino acid substitutions (Kunita et al., 1995; Yamada et al., 1997; Yamada & Yabe, 2000). Moreover, coronaviruses classified in group 2, bovine coronavirus and human coronavirus OC43, do have a similar stretch with four or five substitutions out of 15 amino acids (Boireau et al., 1990; Mounir & Talbot, 1993; Parker et al., 1990). However, coronaviruses classified in groups 1 and 3 lack such a sequence. This suggests that the hydrophobic region including the S2A epitope may be an important site for fusion activity for the coronaviruses classified in group 2.

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References


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