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SHORT COMMUNICATION

Acquired Fusion Activity of a Murine Coronavirus MHV-2 Variant with Mutations in the Proteolytic Cleavage Site and the Signal Sequence of the S Protein

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The spike (S) protein of a nonfusogenic murine coronavirus, MHV-2, was compared to the S protein of a variant with fusion activity, MHV-2f. Two amino acids differed between the S proteins of these viruses; one was located in the signal sequence and the other was in the putative cleavage site. The amino acid at position 12 in the signal sequence was S in MHV-2 and C in MHV-2f. The amino acid sequence of the cleavage site of MHV-2 was HRARS, while that of MHV-2f was HRARR, showing one amino acid replacement at position 757. In DBT cells infected with MHV-2, the S protein was not cleaved, while the S protein of MHV-2f was cleaved. The S protein of MHV-2f expressed in a transient vaccinia virus expression system was cleaved and was fusogenic in contrast to the nonfusogenic activity of uncleaved MHV-2 S protein. Because the signal sequence is assumed to be removed from the mature S protein soon after synthesis, and because the S protein of MHV-2 was expressed on the cell surface in the same way as the S protein of MHV-2f, the difference in the signal sequence seemed to have had little effect on the transportation and the fusion activity of the S protein. These results showed that MHV-2 does not fuse cells due to the lack of cleavage of its S protein. This conclusion differs from studies on the activity of syncytium formation by the S proteins of fusogenic MHV-JHM and -A59 strains. Possible reasons for these differences in fusion activity are discussed.

Murine coronavirus (MHV) is an enveloped virus with single-stranded, positive-sense genomic RNA of about 31 kb (1–4). The spike (S) protein of MHV is a transmembrane glycoprotein of about 180 kDa and is cleaved by trypsin-like enzymes that are derived from the host cell into two 90-kDa subunits (5). The N-terminal S1 subunit forms the bulbous head and the C-terminal S2 subunit is anchored in the virion envelope (4). The S protein mediates attachment to cells and induces syncytium in fusogenic MHV strains (5, 6). Whether or not S protein cleavage is required for syncytium formation is still controversial. The treatment with exogenous protease enhanced syncytium formation by MHV (5) and the treatment with protease inhibitors causes a delay in the onset of fusion (7). These data suggested that the cleavage of the S protein may be important for fusion activity in much the same way that protein cleavage acts in other fusogenic viruses, such as orthomyxoviruses, paramyxoviruses, and retroviruses; in those viruses, cleavage of the surface glycoprotein exposes the fusion peptide on the N terminus of the membrane-anchored subunit (8). Although these fusion peptides characteristically have a hydrophobic amino acid cluster, such a cluster is not found in the N terminus of the membrane-anchored S2 subunit of coronavirus S protein (8). Studies of mutants MHV-JHMV and -A59 and an MHV-A59 variant, whose S proteins were not cleaved due to amino acid replacements in the cleavage site, suggested that cleavage of the S protein may not be a prerequisite for syncytium formation, although it facilitates that activity (9–12).

Most MHV strains produce cell-to-cell fusion on cultured DBT cells forming syncytia. MHV-2 is the only strain which does not induce syncytia (13, 14). However, we noticed that fusion-type MHV-2 variants were present in our MHV-2 stock virus and we isolated a fusion-type MHV-2 variant by plaque purification. We compared fusion-negative MHV-2 and its fusion-positive variant with respect to cleavage of the S protein and transportation of the S protein to the cell surface. These comparisons suggested that fusion activity of the MHV-2 depends upon cleavage of its S protein.

A fusion-positive variant of MHV-2 was obtained using plaque cloning. About 3–5 × 10^6 PFU of the MHV-2 stock virus were layered on DBT cells growing in a 15-cm dish, and the cells were cultured for 12 to 18 hr. The plaques with syncytium formation were visually distinguished from the cells infected with nonfusogenic MHV-2. Ap-
approximately 1 plaque of \(1-2 \times 10^6\) parental plaques showed a fusion-type plaque. The fusion-type plaques were isolated and plaque-purified three times. The final plaque-purified isolate was designated MHV-2f. By using a panel of monoclonal antibodies against the S protein of JHMV, we confirmed that MHV-2f was closely related to parental MHV-2 and was not a fusion-type contaminant from another MHV strain (data not shown).

To compare cytopathic effects and virus growth, DBT cells were inoculated with MHV-2 or -2f at a multiplicity of 1 to 3. Virus titers in the culture fluids were determined by plaque assay as previously reported (15). No substantial difference in the growth of these viruses was observed. The progeny viruses were detected from 6 hr postinfection (p.i.) after which their titers increased gradually, reaching a plateau at 12 hr p.i. to \(0.8-3 \times 10^7\) PFU/ml (data not shown). Infected cells were also observed for cytopathic effect and expression of viral antigen. At 7 hr p.i., syncytium formation was observed, and viral antigen was detected in the cytoplasm of MHV-2f-infected cells (Fig. 1A). In contrast, parental MHV-2 had not induced syncytia by 24 hr, although viral antigen was detected in the cytoplasm of infected cells (Fig. 1B). Viral antigen was not detected in uninfected DBT cells (Fig. 1C).

The S protein is responsible for syncytium formation of MHV infected cells (5, 6, 9–12); therefore, we compared the amino acid sequences of the S proteins of nonfusogenic MHV-2 and fusogenic MHV-2f. The full-length coding region of the S protein was amplified using PCR. At 16 hr p.i., total cellular RNA was extracted from the infected DBT cells and 10 \(\mu\)g of RNA was reverse transcribed into cDNA using oligo(dT) as a primer, as described previously (16). The cDNA transcripts (5 \(\mu\)l) were mixed with 2.5 U Takara Ex Taq (Takara Biochemicals), 0.25 \(\mu\)M each dATP, dCTP, dTTP, and dGTP (dNTPs), and 0.2 \(\mu\)M each sense primer, 5'-CGCAAGCTTCTAAACATGCTATTGTA3' [the MHV-2 S gene around the initiation codon (17) and its attached HindIII site], and complementary primer, 5'-CGGGATCCAGGA-AGGGCTGATAGTCA-3' [the S gene around the stop codon (18) and its attached BamHI site], in a total volume of 50 \(\mu\)l Ex Taq buffer. The mixture was amplified for 30 cycles of denaturation at 94° for 0.5 min, annealing at 60° for 1 min, and elongation at 72° for 3.5 min, and the amplified samples were electrophoresed on a 0.5% agarose gel. Bands of about 4 kb were clearly amplified from MHV-2 and -2f, and were purified from agarose gel by the Prep-A-Gene DNA purification kit (Bio-Rad). Sequence analysis of PCR products was performed by a dideoxy termination labeling method according to the manufacturer's instructions (Applied Biosystems; Model 373A-18 DNA sequencing system). Sequencing oligonucleotide primers were synthesized to fit every 300 to 400 bases of the MHV-2 S gene in both directions of genomic and complementary sense. The deduced amino acid sequence of MHV-2 showed that the MHV-2 S protein was composed of 1361 amino acids. The length of the S gene is assumed to vary among the MHV strains. In MHV-JHMV, several types are reported; JHMV cl-2 is known to have a long S gene (18). The amino acid sequence of MHV-2 was compared with the published amino acid sequence of JHMV cl-2 (schematically shown in Fig. 2). Fifteen amino acids were deleted from the corresponding region of cl-2. Among these deleted amino acids, 12 amino acids were located in a hypervariable region in the S1 subunit where a nucleotide deletion occurs in many MHV strains (19, 20). Three additional amino acids
were deleted from the S2 subunit. The overall S gene-
sequence homology between MHV-2 and cl-2 was
82.23%, excluding the deleted sequence. Comparison of the nucleotide sequences of MHV-2 and -2f revealed only 2 nucleotide replacements (35, C to G and 2271, C to A).
Both of these changes led to the replacement of the predicted amino acids. One was the 12th amino acid from the initiation codon which is located in the signal sequence (Fig. 3). The amino acid at position 12 in MHV-2 was S and in MHV-2f was C. The other replacement was located in the basic amino acid cluster of the cleavage site (Fig. 3). The amino acid at position 757 was S in MHV-2 and R in MHV-2f. To compare these two positions among various MHV strains, DNA fragments including MHV-2 and R in MHV-2f were analyzed by RT-PCR from total cellular RNA of DBT cells infected with MHV-2, -2f, -JHMV, -A59, -1, -3, -S, -NuU, and -D. The cDNA tran-
scripts (5 µl) of various MHV strains were mixed with 0.6 U Taq DNA polymerase (Perkin Elmer Cetus), 0.2 µM each dNTP in a total volume of 50 µl reaction buffer. To amplify the signal sequence, 0.5 µM each sense primer, 5'-TATAAGAGTGATGGGCCTCC-3' (1-20 of leader sequence (26)), and anti-sense primer, 5'-ACAGGGTAATAACCAGTAG-
3' (193-212 of the MHV-2 S gene), were added in reaction mixture. To amplify the putative cleavage site sequence, sense primer, 5'-CCA-
GCTACTATCCCGGT-3' (2065-2081 of the MHV-2 S gene), and anti-sense primer, 5'-GACTCAACACATACCT-3' (2329-2345), were added for MHV-S or sense primer, 5'-GGTTGTGTTGATAGCTGCT-3' (2161-
2180), and anti-sense primer, 5'-TACCTAACACCTGCT-3' (2482-
2498), for other strains. After initial heating at 94°C for 4 min, amplification was performed for 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, and elongation at 72°C for 2 min followed by final extension at 72°C for 5 min. The amplified samples were electrophoresed on a 1% agarose gel and purified. Sequence analysis was performed as mentioned in the text using both directions of primers. The number of bases was determined by the nucleotide sequence of MHV-2. The arrow shows the putative cleavage site.
The replacement on the S protein. The mutation found at position 757 was located at the putative meric S proteins. Fusion chimeras of the S protein could amino acids at positions 12 and 757 from the N terminus and that the S proteins of A59 and JHMV are actively characteristics of the S protein, which is known to mediate fusion due to a lack of cleavage. Perhaps the S proteins of fusogenic MHV strains induce fusion without cleavage of the S protein. In contrast, the MHV-2 S protein required cleavage for activation of its fusion ability. Cleavage of the MHV-2 S protein may alter its conformation thereby activating the otherwise nonfusogenic character of this protein. Such a process would be very similar to that of other fusogenic orthomyxo-, paramyxo-, and retroviruses (8). In these viruses, the newly appearing hydrophobic N terminal region of the membrane-anchored subunit is postulated to work as a fusion peptide. In the N terminus of the MHV-2 S2 subunit, however, a similar fusion peptide with a stretch of apolar amino acids, containing mainly alanine and glycine, was not found. We speculated that the region affecting fusion activity is possibly located inside S2, as is postulated for Semliki Forest, Sindbis, and Rous sarcoma viruses (8), because fusion activity is thought not to reside in the N terminus of the MHV S protein (14). The hypothesis that the MHV-2 S protein is inactive with respect to fusion and that the S proteins of A59 and JHMV are actively fusogenic without cleavage could be tested by using chimeric S proteins. Fusion chimeras of the S protein could also help identify the region responsible for fusion activity on the S protein.
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