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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 $3–5 \times 10^4$ 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-

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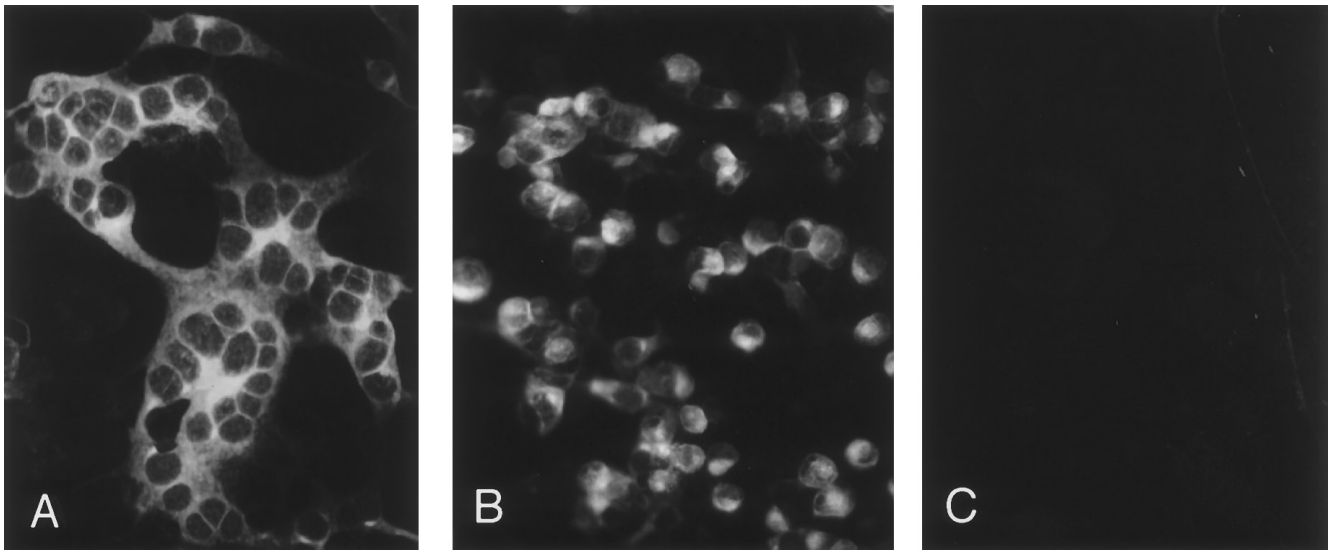

FIG. 1. Immunofluorescent staining of DBT cells infected with MHV-2 or -2f. Viral antigens were detected with rat anti-MHV-2 antiserum and FITC-conjugated anti-rat IgG (MBL, Nagoya) after fixation with chilled acetone. (A) 7 hr p.i. with MHV-2f. (B) 24 hr p.i. with MHV-2. (C) Uninfected DBT cells.

proximately 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 in 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 μg of RNA was reverse transcribed into cDNA using oligo(dT) as a primer, as described previously (*16*). The cDNA transcripts (5 μl) were mixed with 2.5 U Takara Ex *Taq* (Takara Biochemicals), 0.25 μM each dATP, dCTP, dTTP, and dGTP (dNTPs), and 0.2 μM each sense primer, 5′-CGCAAGCTTCTAAACATGCTATTCGTGT-3′ [the MHV-2 S gene around the initiation codon (*17*) and its attached *Hin*dIII site], and complementary primer, 5′-CGGGATCCAGGAGAGGCTGTGATAGTCA-3′ [the S gene around the stop codon (*18*) and its attached *Bam*HI site], in a total volume of 50 μl 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

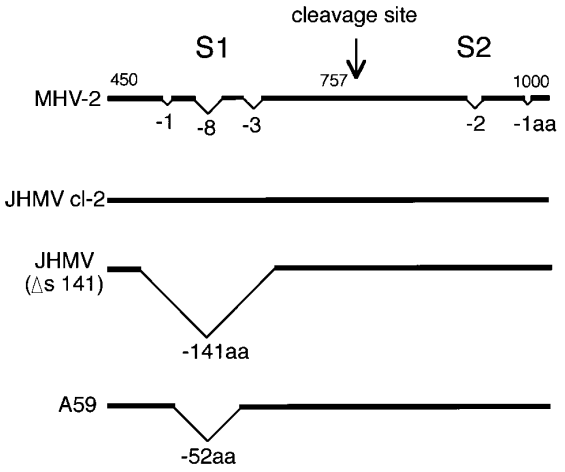

FIG. 2. Diagrammatic representation of the deletion site in the MHV-2 S gene. The sequences of JHMV cl-2, JHMV (△s141), and A59 were cited from published data (*18, 23, 22*). The deletion is indicated by thin lines.

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 the signal sequence region and the putative cleavage site were amplified. Sequence analysis of the PCR product showed that MHV-2 had unique amino acids at positions 12 and 757 and that MHV-2f had the same sequences as the other fusogenic MHV strains in both positions (Fig. 3).

To demonstrate the role of the S protein of MHV-2f in syncytium formation, full-length S genes of MHV-2 and -2f were transiently expressed in a vaccinia virus expression system (*21*). DBT cells were infected with a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3, kindly provided by Dr. B. H. Moss), and then 5 μg of pGEM4Z plasmids (Promega) harboring the MHV-2 or -2f S gene in the *Hin*dIII and *Bam*HI sites were transfected with a DOTAP transfection reagent (Boehringer Mannheim). At 16 hr posttransfection, syncytium formation was observed in cells transfected with the MHV-2f S gene. On the other hand, syncytium formation was not detected in cells transfected with the MHV-2 S gene, even though the expression of S protein was detectable by immunofluorescent staining with S protein-specific monoclonal antibody (data not shown). These data showed that the difference in the fusogenicity of MHV-2 and -2f depended on the S protein.

The first amino acid replacement was located in the signal sequence, which is reported to be composed of 20 amino acids from the N terminus of the S protein and to be removed from the S protein soon after synthesis (*22*). A possibility exists that the amino acid difference in the signal sequence can influence the transportation of the S protein to the cell surface. To determine the cell-surface expression of the MHV-2 S protein, live monolayers of MHV-2- and -2f-infected cells were reacted with S protein-specific monoclonal antibody at 4° for 1 hr, followed by FITC-labeled anti-mouse Ig's (TAGO). Granular positive dots were detected on the cell surface at 7 and 24 hr after MHV-2 infection, and by 7 hr after MHV-2f infection a wide granularly stained area appeared in the syncytium, demonstrating that both S proteins were transported to the cell surface (data not shown).

The second amino acid replacement was located in the putative cleavage site. This mutation could influence the cleavage of the S protein, since a host cell-derived proteolytic enzyme is reported to recognize the amino acid sequence RRARR in this position for JHMV and

	region	
	Signal sequence	Cleavage site
	12	757
MHV-2	TLLPSSLGYI	HRARS SVST
MHV-2f	-----C----	----R ----
JHM	L----C----	R---R ----
A59	LF---C----	R--HR ----
MHV-1	L-I--C----	----R -I--
MHV-3	LF---C----	R---R ----
S	LF---C----	----R P---
NuU	L----C----	----R -I--
D	L----C----	----R-I--

FIG. 3. Comparison of predicted amino acid sequences around the signal sequence and the putative cleavage site. Nucleotide sequences 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 transcripts (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′-TATAAGAGTGATTGGCGTCC-3′ [1–20 of leader sequence (*26*)], and anti-sense primer, 5′-ACAGGGTAATAACCAGTAAG-3′ (193–212 of the MHV-2 S gene), were added in reaction mixture. To amplify the putative cleavage site sequence, sense primer, 5′-CCAGCGCTACTATATCG-3′ (2065–2081 of the MHV-2 S gene), and anti-sense primer, 5′-GACTCAACACTATCATT-3′ (2329–2345), were added for MHV-S or sense primer, 5′-GGTTGTGTTGTTAATGCTGA-3′ (2161–2180), and anti-sense primer, 5′-TACTCAACCAACTGCTG-3′ (2482–2498), for other strains. After initial heating at 94° for 4 min, amplification was performed for 30 cycles of denaturation at 94° for 1 min, annealing at 55° for 1.5 min, and elongation at 72° for 2 min followed by final extension at 72° 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 is based on the amino acid sequence of MHV-2. The arrow shows the putative cleavage site.

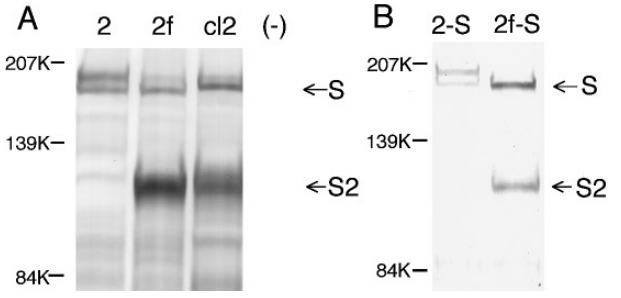

FIG. 4. Western blot analysis of S proteins. (A) DBT cells grown in 12-well plates were infected with MHV-2, -2f, and cl-2. (B) DBT cells were infected with vTF7-3 and transfected with plasmid pGEM4Z harboring the MHV-2 or -2f S genes. After 16-hr incubation, cells were washed with PBS and lysed with 100 μl of PBS containing 1% Nonidet-P40. The lysed samples were centrifuged at 15,000 rpm for 10 min at 4° and the supernatants were collected. The lysates were boiled in a sample buffer and electrophoresed in a 7.5% SDS–polyacrylamide gel. Western blotting analysis of the lysates was carried out as described previously (*27*), using S2 subunit-specific monoclonal antibody and horseradish peroxidase-conjugated anti-mouse Ig's (TAGO). The bands were visualized with PBS containing 0.05% diaminobenzidine and 0.015% hydrogen peroxide.

RRAHR for A59 (*23, 22*). To determine whether the S proteins of MHV-2 and -2f are cleaved, we performed Western blotting analysis using cell lysates infected with MHV-2, -2f, and JHMV cl-2 (Fig. 4A). Cell lysates collected 18 hr p.i. were used in Western blotting, and the S protein was detected with S2-specific monoclonal antibody (kindly provided by Dr. S. Siddell). Two major bands at 150 and 180 kDa were detected in the lysate of MHV-2-infected cells. The 150-kDa protein may represent a nonglycosylated form of the mature 180-kDa protein that is observed in the MHV-A59 S protein (*12*). In contrast, the 150- and 90-kDa bands were detected in the lysates of MHV-2f- and JHMV cl-2-infected cells. Most of the mature 180-kDa S protein was cleaved to S1 and S2 subunits which were detected as the 90-kDa band. In MHV-2-infected cells, a 90-kDa band was not detected, revealing that the MHV-2 S protein was not cleaved. Faint bands were seen just above the location of the S2 band in MHV-2-, -2f-, and cl-2-infected cells; these bands probably do not represent S protein cleavage products, because they are absent from the S proteins expressed in the vaccinia virus system, as shown in Fig. 4B. These results showed that the replacement of amino acid 757 changed the cleavability of the MHV-2 S protein.

We have investigated differences between nonfusogenic MHV-2 and its fusogenic variant MHV-2f, looking specifically at differences in fusion activity and in the characteristics of the S protein, which is known to mediate fusion in MHV. Sequence analysis showed that two amino acids at positions 12 and 757 from the N terminus of the S protein differed between the two viruses. The mutation found at position 757 was located at the putative cleavage site, which is reported to be digested with a trypsin-like host cell-derived enzyme. The replacement of serine with arginine at position 757 appeared to influence the cleavage of the S protein. As expected, the MHV-2f S protein was cleaved, in contrast to the cleavage resistance of nonfusogenic MHV-2 S protein. The other mutation at position 12 was located in the signal sequence that is considered to be removed from the mature S protein soon after synthesis. Transportation of the S protein to the cell surface was detectable in both MHV-2- and -2f-infected cells, indicating that this mutation in the signal sequence had little effect on transportation. These results allowed us to conclude that MHV-2f acquired the ability to form syncytia because of its ability to cleave the S protein. This does not rule out the possibility, however, that the replacement at position 12 might have some effect on fusion activity. Point mutation analysis should show whether the mutation at position 12 affects syncytium formation by MHV-2f.

In addition to the putative trypsin cleavage site of the S protein, three regions of the S protein are involved in fusion activity of MHV. One is the hypervariable region of the S1 subunit (*24*). The transmembrane region (*12*) and a predicted heptad repeat region of the S2 subunit (*25*) are also proposed to influence the fusion activity. These regions were identical in fusogenic MHV-2f and nonfusogenic MHV-2, and therefore are unlikely to have affected the fusogenic activities of MHV-2 and -2f.

In fusogenic strains JHMV and A59, cleavage of the S protein is not absolutely required, because the S proteins of these viruses with mutations in the putative cleavage site can induce syncytium, yet are not cleaved (*9–12*). We describe here that the S protein of MHV-2 failed to induce 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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