

## Mutational Analysis of the Murine Coronavirus Spike Protein: Effect on Cell-to-Cell Fusion

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The spike (S) protein of murine coronavirus strain A59 (MHV-A59) is a type I membrane protein that induces membrane fusion. In this study we have analyzed the role of two domains in the S protein on fusion. The 180-kDa mature S protein is partially cleaved into two 90-kDa subunits during transport to the plasma membrane. We have identified several amino acids that are important for cleavage of S, and we show that cleavage is not strictly required for fusion. However, the level of cleavage seems to influence the fusion kinetics. After introduction of an arginine at position P<sub>2</sub> to mimic the MHV-JHM cleavage site, full cleavage of the spike protein was obtained. Further, we analyzed the effect of mutations in the transmembrane (TM) domain of the S protein. Maturation and cell surface expression of the mutant proteins were not affected, and all proteins became acylated. The mutant in which the predicted transmembrane domain was shortened did not induce syncytia. From a group of mutants in which several conserved cysteines in the TM domain had been replaced by serines, one was unable to induce syncytia, another showed delayed syncytia formation, and the third mutant induced syncytia as did the wild-type protein. The potential role of the transmembrane domain in fusion is discussed. © 1995 Academic Press, Inc.

### INTRODUCTION

Membrane fusion is a key event in the replication cycle of enveloped viruses. During penetration of the host cell, the viral membrane fuses with either the plasma membrane or endosomal membrane resulting in the release of the viral genome into the cytosol of the infected cell. Whether fusion occurs at the endosomal membranes or at the plasma membrane depends on the pH at which the viral fusion protein is in its fusogenic conformation. Fusion at the plasma membrane occurs when this conformation is reached at neutral pH. Fusion can also be induced when the fusion protein is expressed at the cell surface of infected cells, leading to cell-to-cell fusion. In this case large syncytia are formed which can add significantly to the intracellular spread of these viruses (reviewed by White, 1990).

Membrane fusion in coronavirus-infected cells is mediated by the spike (S) protein and takes place at neutral pH (Sturman and Holmes, 1985). Cell surface expression of S in infected or recombinant S-expressing cells results in the formation of extensive syncytia (Vennema *et al.*, 1990). The S protein is a glycosylated (Niemann and Klenk, 1981) and palmitine-acylated (van Berlo *et al.*, 1987; Niemann and Klenk, 1981; Ricard and Sturman, 1985; Schmidt, 1982) type I membrane protein. During transport of the trimerized (Cavanagh *et al.*, 1983; Delmas and Laude, 1990) precursor protein through the Golgi apparatus, the high mannose side chains are trimmed

and modified, giving rise to an almost endo-H-resistant 180- to 200-kDa protein. A distinctive feature among viral fusion proteins is that, irrespective of the pH optimum, some must undergo cleavage activation (e.g., hemagglutinin of influenza virus, gp160 of HIV), whereas others are fusogenic in an uncleaved form (e.g., G protein of rhabdoviruses) (reviewed by White, 1990).

In several coronaviruses such as infectious bronchitis virus (IBV), murine coronavirus (MHV), and bovine coronavirus (BCV) the S proteins are cleaved (reviewed by Spaan *et al.*, 1988). Proteolytic cleavage of S (resulting in S1 and S2) by host-cell proteases occurs as a late step in transport. The 180-kDa S protein of MHV strain A59 is only partially cleaved, whereas cleavage of strain JHM is almost complete. In 17C11 cells, however, almost no cleavage of MHV-A59 S protein was observed (Frana *et al.*, 1985). Trypsin treatment of MHV-A59 virus purified from 17C11 cells resulted in cleavage of S between amino acid residues 717 and 718. The sequence at the cleavage site is RRAHR ↓ SVS (Luytjes *et al.*, 1987).

In the presence of leupeptin, an inhibitor of serine and thiol proteases, the formation and size of syncytia was delayed and reduced, respectively (Frana *et al.*, 1985). Cell fusion activity of MHV-A59 virions purified from 17C11 cells required the addition of trypsin (Sturman *et al.*, 1985). These data strongly suggested that only A59 virions with cleaved S are fusogenic and are supported by the observations of Gombold *et al.* (1993) who have isolated cleavage-negative MHV-A59 variants, which are fusion-negative. However, it has been shown recently that recombinant S proteins of MHV-JHM in which the cleavage site was mutated were still able to induce cell-

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to-cell fusion (Stauber *et al.*, 1993; Taguchi, 1993). The heptad repeat region just upstream of the transmembrane anchor (De Groot *et al.*, 1987) has also been connected with fusion (Gallagher *et al.*, 1991; Grosse and Siddell, 1994).

The membrane-anchoring sequence is another part of the fusion protein that has been associated with fusion activity in several viruses (Ragheb and Anderson, 1994; Rasile *et al.*, 1993; Mulligan *et al.*, 1992). The coronavirus S protein is anchored in the lipid bilayer by a large (34–45 aa) transmembrane (TM) domain, which is defined by the bordering charged amino acid residues (reviewed by Boyd and Beckwith, 1990). At the start of the TM domain in all coronavirus S protein sequences studied to date a highly conserved stretch of seven large hydrophobic amino acids is found. The first part of the TM anchor is a hydrophobic region of about 20–22 amino acids, followed by a cysteine-rich hydrophilic region of 11–23 amino acids.

We have studied both the cleavage domain and the TM anchor of coronavirus MHV-A59. Here, we demonstrate by site-directed mutagenesis that the endogenous protease, yet to be identified, cleaves the MHV-A59 S protein at the RAHR↓ motif. Several amino acid residues important for cleavage were identified. We also demonstrate that cleavage of S is not a prerequisite for cell fusion. Furthermore, we show that the transmembrane anchor of the MHV-A59 S protein is required for fusion.

## MATERIALS AND METHODS

### Cells and viruses

Mouse L cells, 17C11, and rabbit RK13 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum. Vaccinia virus vTF7.3 stocks (kindly provided by Dr. B. Moss) were grown on RK13 cells. MHV-A59 stocks were grown on 17C11 cells. MHV-JHM was kindly provided by Dr. S. G. Siddell.

### Site-directed mutagenesis

The complete MHV-A59 spike gene (MHV-S) has been cloned, sequenced, and reconstructed previously (Luytjes *et al.*, 1987; Vennema *et al.*, 1990). The full-length S gene was cloned as a *Bam*HI fragment between a T7 promoter and terminator in the *Bam*HI site of the expression vector pTUG31 (Vennema *et al.*, 1991). From this clone (pTugMS) a 662-bp *Kpn*I–*Sph*I fragment was isolated and ligated in *Kpn*I- and *Sph*I-digested M13-mp18 DNA. The resulting M13 template was used for site-directed mutagenesis described by Kunkel (Kunkel *et al.*, 1987). Mutant m1 was designed after mutant 4 of paramyxovirus SV-5 (Paterson *et al.*, 1989). Amino acid substitutions in mutants m3, m4, m5, and m6 were designed to mimic the influenza HA cleavage mutants described by Kawaoka and Webster (1988). Mutants m2

and m7 were constructed using Kunkel mutagenesis on the *Bam*HI–*Sph*I fragment of pTugMS cloned in the pBluescript KS<sup>−</sup> vector. Mutants m40, m41, m42, and m43 were constructed by Kunkel mutagenesis on the *Kpn*I–*Sal*I fragment of pTugMS in pBluescript KS<sup>−</sup>. The mutated fragments were sequenced and subsequently used to replace the corresponding nonmutated fragment in pTugMS.

### Virus infection

Confluent monolayers of L cells were infected with MHV-A59 or MHV-JHM in PBS–DEAE, supplemented with 3% FCS, at a multiplicity of infection (m.o.i.) of 10. After absorption for 1 hr at 37°, virus was removed and cells were cultured in DMEM supplemented with 3% FCS.

### Protein expression

Mouse L cells ( $1 \times 10^6$ ) were seeded in 35-mm dishes. Sixteen hours later the cells were infected with the T7 RNA polymerase-expressing vaccinia recombinant (vTF7.3) at a m.o.i. of 5. At 1.5 hr postinfection the cells were transfected with 1  $\mu$ g DNA per 35-mm plate using the lipofectine procedure (Gibco/BRL) as described by the manufacturer.

Before labeling, the cells were incubated for 30 min in DMEM deficient in methionine. Subsequently, the cells were metabolically labeled with 100  $\mu$ Ci <sup>35</sup>S-labeled amino acids (Expre<sup>35</sup>SS label, NEN) in medium lacking methionine, or with 100  $\mu$ Ci [<sup>3</sup>H]palmitic acid (NEN) in complete medium. When a pulse-chase experiment was performed, the radioactive medium was replaced with DMEM containing four times the normal concentration of methionine. Cells were lysed in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8.0) and 2 mM PMSF. The lysate was centrifuged at 4° for 10 min at 13,000 rpm to remove the nuclei and cell debris. Immunoprecipitations were performed on the supernatant using a mixture of S-specific monoclonal antibodies J7.6 and J1.4 (Fleming *et al.*, 1983) and wa3.1 (Gilmore *et al.*, 1987). Antibody A1 (kindly provided by Dr. H. Wege) was used to immunoprecipitate the MHV-JHM S protein. After an overnight incubation at 4°, 50  $\mu$ l Pan-sorbin cells (Calbiochem, La Jolla, CA), and KCl to a final concentration of 0.5 M were added, followed by an incubation for 1 hr at 4°. After washing the samples three times in RIPA, they were boiled in Laemmli sample buffer for 2 min (Laemmli, 1970). Protein samples were endo-H-treated as described by Vennema *et al.* (1990). The immune precipitates were analyzed by SDS–PAGE on 10% gels.

### Indirect immunofluorescence

Cells were cultured on glass coverslips in 35-mm wells and infected and transfected as described above. Coverslips were removed at set intervals and fixed in 4% paraformaldehyde to monitor cell surface expression of

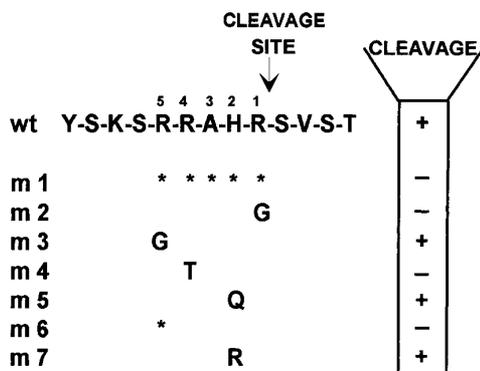


FIG. 1. Mutations introduced upstream of the trypsin cleavage site of MHV-A59 S protein. For the mutants only mutated residues are depicted. Asterisks represent deleted amino acids. Numbers above the amino acids indicate the position of that particular amino acid relative to the trypsin cleavage site. The cleavage phenotypes of the mutants is shown in the table at the right.

S proteins. Cells were washed several times in PBS supplemented with 10 mM glycine, reacted with a 1:5 diluted monoclonal antibody mix of J7.6, J1.4, and wa3.1 for 30 min, and developed with 1:50 diluted goat anti-mouse FITC-conjugated mAb (Dako).

## RESULTS

### Mutagenesis of the cleavage site of the MHV-A59 spike protein

Protein sequencing of the trypsin-generated N-terminus of the MHV-A59 S2 subunit has revealed the position of the cleavage site (Luytjes *et al.*, 1987). To determine whether the trypsin cleavage site is also recognized by the endogenous protease that cleaves S during transport to the cell surface, we have constructed several mutant (m) S genes. In m1 the sequence encoding the peptide R<sub>5</sub>-R<sub>4</sub>-A<sub>3</sub>-H<sub>2</sub>-R<sub>1</sub>, which is located at the C-terminus of trypsin-cleaved S1 (Fig. 1), has been deleted, whereas in the second mutant (m2) the arginine residue at the P<sub>1</sub> position has been replaced by a glycine residue. To study the processing of both mutants, L cells were infected with the vaccinia T7-RNA-polymerase recombinant (vTF7.3) and transfected with the wild-type (wt), m1, and m2 S-expressing plasmids. After metabolic labeling for 1 hr followed by a chase of 2 hr, the cells were lysed and S protein was immunoprecipitated using a mixture of three different S-specific monoclonal antibodies and analyzed by SDS-PAGE (Fig. 2).

In both MHV-infected cells (see below) and in cells transfected with the wt S-encoding plasmid, S was mostly present as a 150-kDa core protein. In addition the mature 180- and 90-kDa species could be detected (Fig. 2, lane 4). During the chase period the intensity of the gp 180 band increased. In m1- and m2-transfected cells only the 150-kDa precursor and the mature 180-kDa S proteins could be detected. Even after the 2-hr chase period cleavage products could not be observed (Fig. 2,

lanes 6 to 7). The lack of cleavage resulted in a higher level of 180-kDa protein compared to wt S-transfected cells. Surface immunofluorescence was detected with wt, m1, and m2 S protein (data not shown). These results strongly suggest that an endogenous protease cleaves the MHV-A59 S protein at the same position as trypsin does. The absence of cleavage of mutant m2 also implies an important role for the arginine residue at position P<sub>1</sub>.

We also tested whether the transition of the 150-kDa precursor protein to the gp180/90 mature form could be improved by incubating the vaccinia virus-infected and DNA-transfected cells at 32°, as has been suggested by Marquardt and Helenius (1992). No significant difference in the maturation of gp150 at the two temperatures was observed and therefore all subsequent experiments were carried out at 37°.

Next we examined in more detail the sequence requirements for cleavage of the S protein. Four additional mutants were generated, three containing single amino acid changes at position P<sub>5</sub>, P<sub>4</sub>, or P<sub>2</sub> of the cleavage site and one having a deletion of the arginine residue at position P<sub>5</sub> (Fig. 1). The effect of these mutations on the proteolytic cleavage of S was tested. Immunoprecipitated proteins were endo-H-treated as described under Materials and Methods. In all cases the most abundant S protein was the endo-H-sensitive 150-kDa core protein. Mutant m5, in which the histidine residue is replaced by a glutamine, was cleaved at a level comparable to the wt S protein (Fig. 3, lanes 7 and 8). Substitution of the arginine residue at position P<sub>5</sub> by glycine (m3) had only a limited effect on cleavability (Fig. 3, lanes 3 and 4). When the arginine residue at position P<sub>4</sub> was replaced by a threonine residue, no cleaved S could be detected. Neither did we observe any cleavage in which the arginine residue at position P<sub>5</sub> was deleted (Fig. 3, lanes 5, 6, 9, 10). The presence of the endo-H-resistant 180-kDa S protein in both m4- and m6-transfected cells clearly demonstrates that the cleavage-negative phenotype of

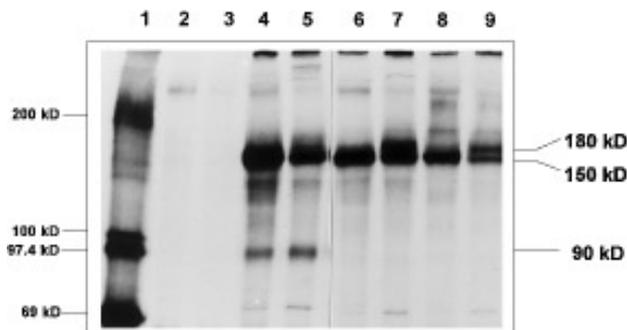
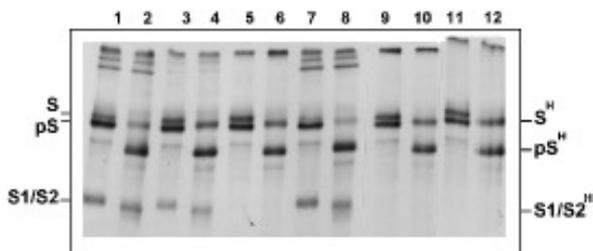


FIG. 2. Cleavage pattern of wt and mutant S proteins. L cells, transiently expressing wt or mutant spike proteins, were pulse labeled with 100  $\mu$ Ci Express<sup>35</sup>S label from 5 to 6 hr posttransfection (lanes 2, 4, 6, 8) and subsequently chased for 2 hr (lanes 3, 5, 7, 9). Spike proteins were immunoprecipitated using a mix of anti-S monoclonal antibodies and analyzed as described under Materials and Methods. Marker (lane 1), mock-transfected cells (lanes 2, 3), wt S (lanes 4, 5), m1 (lanes 6, 7), and m2 (lanes 8, 9).



**FIG. 3.** Acquisition of endo-H resistance of wt and mutant S proteins. L cells expressing S were labeled with 100  $\mu$ Ci Express<sup>35</sup>SS label from 7 to 11 hr posttransfection. Immunoprecipitated S proteins were incubated at 37° for 16 hr without (lanes 1, 3, 5, 7, 9, 11) or with (lanes 2, 4, 6, 8, 10, 12) endo H and analyzed as described. Cells were transfected with wt S (lanes 1 and 2), m3 (lanes 3 and 4), m4 (lanes 5 and 6), m5 (lanes 7 and 8), m6 (lanes 9 and 10), or m1 (lanes 11 and 12). S, pS, and S1/S2 at the left indicate the positions of the 180-kDa, the 150-kDa precursor protein, and the cleaved S1 and S2 subunits, respectively, before endo-H treatment. After endo-H treatment, the proteins shift to the positions indicated at the right; S<sup>H</sup>, pS<sup>H</sup>, and S1/S2<sup>H</sup>.

both mutant S proteins is not due to the lack of transport of the endo-H-sensitive gp150 protein.

#### Introduction of a dibasic cleavage site results in complete processing of the A59 S protein

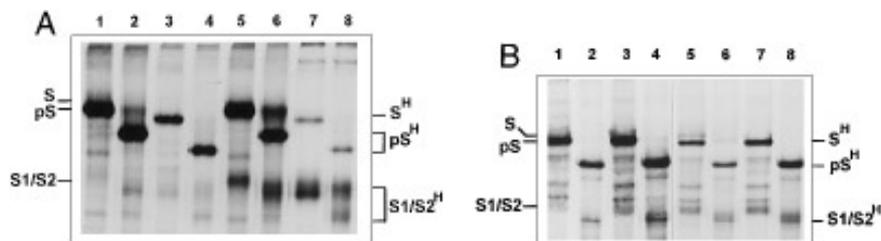
As described above, the mature 180-kDa wt S protein of MHV-A59 is only partially cleaved. In strain MHV-JHM the motif RRARR is present at the cleavage site, and in JHM only the cleaved form of the mature S protein is observed in the virion and in infected cells. It has been suggested (Daya *et al.*, 1989; Mizzen *et al.*, 1983) that the differences in cleavage efficiencies are host related. Because cleavage of A59 and JHM S proteins has never been compared in the same cell line, we have analyzed the maturation of the MHV-A59 and MHV-JHM S proteins in L cells. In the A59-infected cell lysate, most of S was present as the endo-H-sensitive 150 kD form (Fig. 4A). Upon a 2-hr chase, part of the 150-kDa S protein was converted into the 180-kDa form and some protein was chased to 90 kDa (Fig. 4A, lanes 5 and 6). For MHV-JHM a considerable amount of 150-kDa protein S protein was

converted into the 90-kDa cleavage product during the chase period, but no 180-kDa protein was detected (Fig. 4A, lanes 7 and 8). Even after prolonged exposure the 180-kDa was not observed in JHM-infected cells (data not shown). The different cleavage patterns of MHV-A59 and MHV-JHM S proteins are thus not host cell dependent.

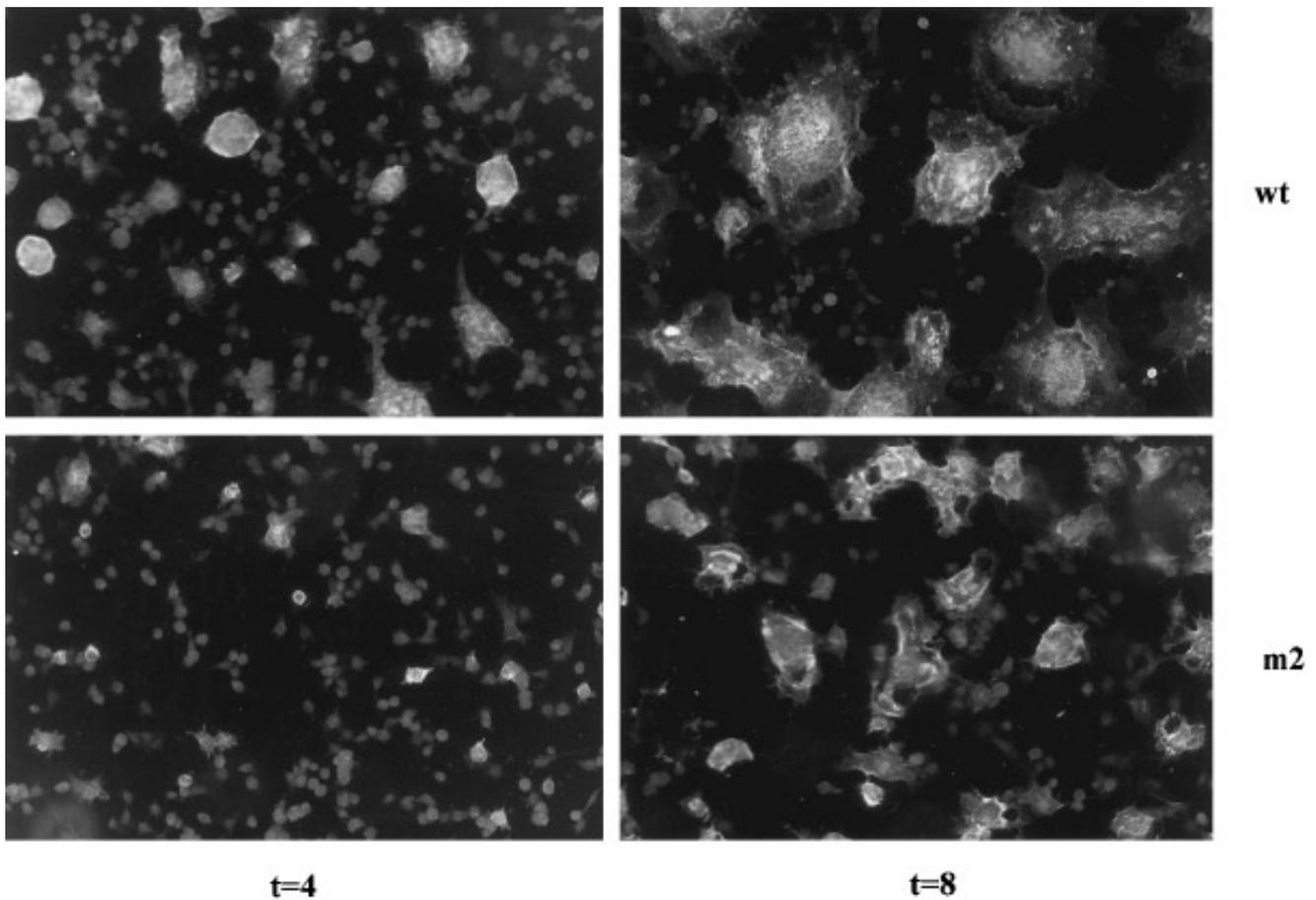
To test whether the difference in cleavability between the S proteins of JHM and A59 was dependent on the amino acid at position P<sub>2</sub> of the cleavage site, an A59 mutant S protein was constructed in which the histidine at position P<sub>2</sub> was replaced by an arginine residue. The sequence of the cleavage domain of this mutant (m7; Fig. 1) is thus identical to the JHM cleavage site. After a 2-hr chase the endo-H-resistant 180-kDa product was observed in the lysate obtained from wt S-transfected cells only. The 180-kDa band could not be detected in the m7 lysates (Fig. 4B, lane 8). The 90-kDa forms of both the wt and m7 S protein were clearly present. These results indicate that the S protein of A59 acquires high cleavability when an arginine residue is introduced at position P<sub>2</sub> instead of a histidine residue. The amino acid at position P<sub>2</sub> is therefore important in determining the efficiency of cleavage of MHV S.

#### Induction of cell-to-cell fusion by cleavage site mutants

The ability of mutant S proteins to induce cell-to-cell fusion was examined in vTF7-infected mouse L cells. Cells expressing the wt and the mutant S proteins were fixed at 4 and 8 hr posttransfection, and S protein expression was detected by indirect immunofluorescence. The wt S protein induced syncytia that were readily visible after 4 hr and increased in time. Cleavage-negative S mutants were expressed at the surface, but were delayed in syncytium induction. Results for wt S and m2 are shown in Fig. 5. The results for the other cleavage-negative mutants were similar to m2, whereas m3 and m5 were almost indistinguishable from wt (data not shown). Thus, cleavage of the S protein is not a prerequisite



**FIG. 4.** Effect on cleavage efficiency of introduction of the JHM cleavage site into the MHV-A59 spike protein. (A) Comparison of cleavage of the MHV-A59 and MHV-JHM S proteins. Immunoprecipitation of S from L cells that were infected with either MHV-A59 (lanes 1, 2, 5, 6) or MHV-JHM (lanes 3, 4, 7, 8) at a m.o.i. of 10. Cells were labeled with 100  $\mu$ Ci Express<sup>35</sup>SS label from 6 to 7 hr posttransfection (lanes 1, 2, 3, 4) and chased for 2 hr (lanes 5, 6, 7, 8). Proteins were immunoprecipitated using a mAb anti-S mix (A59) or anti-S monoclonal antibody A1 (JHM). Part of the lysate was subsequently treated with endo H (lanes 2, 4, 6, 8). (B) Comparison of the cleavage efficiency between wt S and m7. L cells expressing wt S (lanes 1, 2, 5, 6) or m7 (lanes 3, 4, 7, 8) were pulse labeled for 1 hr ( $t = 5$  to 6, lanes 1 to 4) and chased for 2 hr (lanes 5 to 8). Immunoprecipitations were performed using an anti-S mAb mix. Proteins were subsequently incubated at 37° for 16 hr without (lanes 1, 3, 5, 7) or with (lanes 2, 4, 6, 8) endo H. Positions of the different S forms are indicated as described in the legends of Fig. 3.



**FIG. 5.** Cell surface expression of S proteins on L cells using the vaccinia expression system. After vTF7-3 infection and DNA transfection, cells were fixed using paraformaldehyde at 4 and 8 hr posttransfection. The cells were subsequently incubated with an anti-S mAb mix, followed by FITC-conjugated goat anti-mouse antibodies. Results obtained with wt S and m2 are shown.

for fusion, but the data indicate that it accelerates the appearance of syncytia.

#### Role of the transmembrane region in fusion activity of the spike protein

Comparison of the potential transmembrane regions, defined by the region between two charged residues (reviewed by Boyd and Beckwith, 1990), of all known coronavirus S genes sequenced to date shows that they have several features in common (Fig. 6A). All TM regions contain a stretch of about 20 hydrophobic amino acids followed by 11 to 23 hydrophilic amino acids. Within this hydrophilic stretch, a polar region with many cysteine residues is present. At the N-terminus, seven highly conserved large hydrophobic amino acids are found (Fig. 6B).

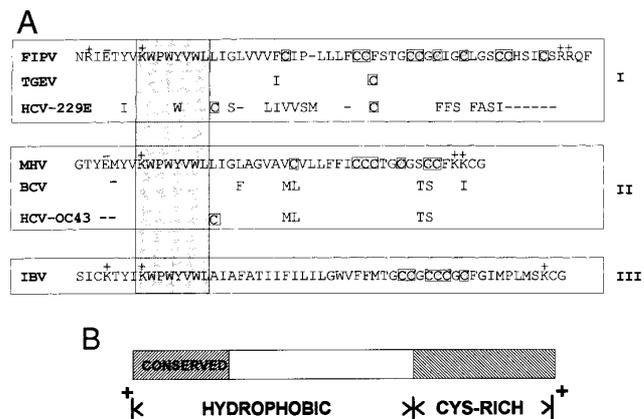
We had already observed that the cytoplasmic tails of MHV-A59 and feline infectious peritonitis coronavirus (FIPV) can be exchanged without loss of fusogenicity of the S protein (H. Vennema, L. Heijnen and W. Spaan, unpublished results). However, an MHV/VSV chimeric protein that consists of amino acid residues 1–1286 (containing the 20 hydrophobic amino acids of the TM region) of the MHV-A59 spike protein followed by the

cytoplasmic tail of the VSV G protein (mutant msg-c; Fig. 7) was not fusogenic. The hybrid protein was transported to the *trans*-Golgi network with similar kinetics to the wt S protein, as measured by the rate of acquisition of resistance to digestion by endo H (data not shown). It was also expressed at the cell surface. Fusogenicity was restored by reintroducing the cysteine-rich polar region of the MHV-A59 spike TM region, resulting in chimera msg-b (Fig. 7).

The differences between the two MHV/VSV chimeras are the absence of the cysteine-rich polar domain and the smaller distance between the two charged residues that border the predicted transmembrane region in the fusion-negative chimera msg-c. This suggests that either one of these differences or both play a role in the fusogenicity of S. To test this we constructed a set of mutants of the MHV-A59 S protein that have alterations in the transmembrane region (Fig. 8A).

#### Mutagenesis of the transmembrane region of the MHV-A59 spike protein

A mutant was constructed in which the isoleucine at position 1286 was replaced by a positively charged lysine



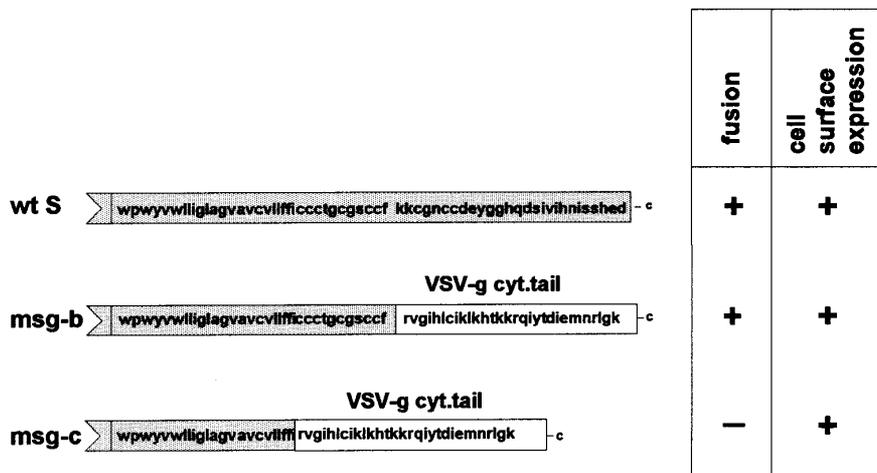
**FIG. 6.** Comparison of the transmembrane regions of coronavirus S proteins. (A) The amino acid sequences of the TM region of S from the three antigenic clusters of coronaviruses are compared (I, II, and III). In each cluster only one full sequence is depicted; of the other sequences, only the differences are shown. Deletions are indicated with a horizontal line. The shaded boxes indicate the conserved WYV domain, and cysteines in the TM region. Charged residues (lysines, arginines, and glutamic acids) are indicated with +. (B) The region between the charged residues that border the predicted transmembrane region is divided into three regions, the domain with the seven large hydrophobic amino acids (left region), the stretch of 23 hydrophobic amino acids (middle region), and the stretch of hydrophilic amino acids (right region).

residue. This would generate an S protein in which the length of the predicted TM region was similar to that of the fusion-negative msg-c VSV chimera (m40; Fig. 8A). To study the putative role of the clustered cysteine residues in cell-to-cell fusion, three additional mutants were constructed. In m41, the first set of three cysteines was replaced by a set of serines (Fig. 8A). The S protein of mutant 42 has both cysteines at positions 1295 and 1296 replaced by serines, whereas in m43 only C1295 was changed to a serine (Fig. 8A). The exchange of phenylalanine at position 1297 for asparagine in m42 and m43 was

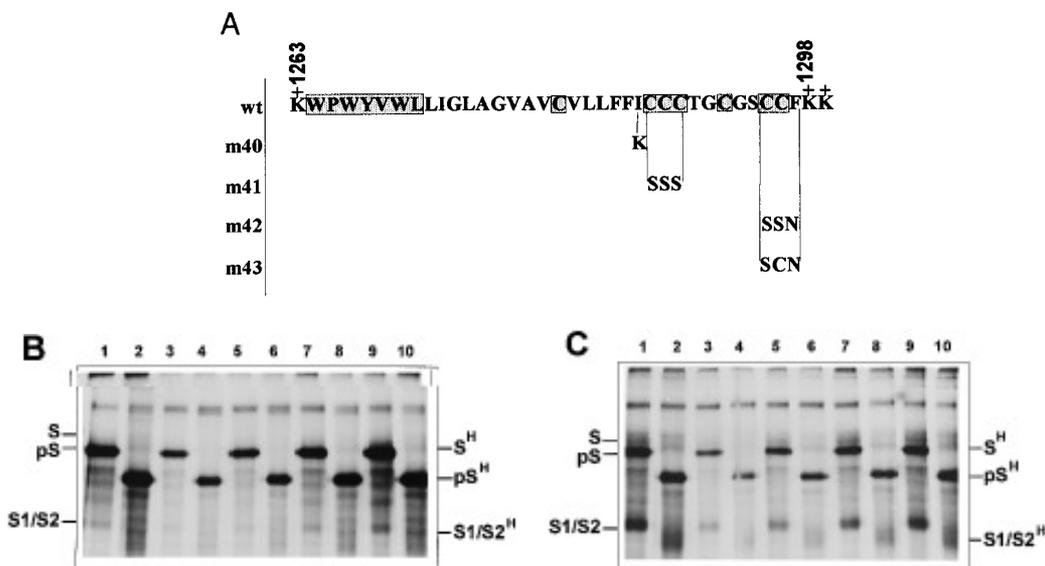
serendipitously obtained during the Kunkel mutagenesis procedure.

First, the maturation of the S proteins encoded by mutants m40, m41, m42 and m43 was analyzed in a pulse-chase experiment. Cell lysates were immunoprecipitated using the mixture of S-specific mAbs mentioned earlier. In the pulse-labeled lysates, the majority of the S protein of both wt and mutants is in the 150-kDa endo-H-sensitive form (Fig. 8B). After the 2-hr chase period, part of the 150-kDa endo-H protein had been converted to the 180- and 90-kDa endo-H-resistant proteins (Fig. 8C, lanes 1 and 2). The level of cleavage of all mutant S proteins was comparable to that of the wild-type S.

Next the fusogenic properties of the TM mutants were tested. Transfected L cells were fixed in paraformaldehyde at 6 and 12 hr posttransfection. At 6 hr posttransfection all mutant proteins were already present at the cell surface (data not shown). Results are shown for the cells that were fixed at 22 hr posttransfection (Fig. 9). In cells transfected with wt S or the TM mutants, the S protein was clearly expressed at the cell surface. The insertion of a charged amino acid between the small hydrophobic amino acids and the cysteine-rich region destroyed fusion. This mutation may have shortened the transmembrane region. Replacement of the three cysteines with serines destroyed fusion indicating that one or more of these cysteines are critical for fusion. Replacement of the two cysteines with serines decreases, but does not destroy fusion, indicating that these cysteines are important but not absolutely required. Replacing the first of this pair of cysteines with serine has no noticeable effect on fusion, indicating that it is not critical. The higher amount of [<sup>35</sup>S]methionine that was incorporated into the S protein expressed in wt and m43-transfected cells compared to the other mutant S-transfected cells is most likely due to an increase in the number of cells expressing S because of extensive syncytia formation before the



**FIG. 7.** Amino acid sequence of the VSV-MHV chimeras. The amino acids in the transmembrane region and cytoplasmic tail are depicted. Amino acids in shaded boxes are derived from MHV-A59 spike. The amino acids in white boxes are derived from the VSV G protein. Cell surface expression and fusogenic properties of the chimeras are shown at the right.



**FIG. 8.** Cleavage and endo-H resistance of the transmembrane mutants. The mutations that were introduced in the transmembrane region are depicted in A. For the mutants only altered amino acids are shown. The position of the amino acids are indicated above the residues (A). Transfected L cells were pulse-labeled for 30 min at  $t = 6$  hr posttransfection (B) and subsequently chased for 2 hr (C). Cell lysates were immunoprecipitated, followed by endo-H treatment (lanes 2, 4, 6, 8, 10) or no endo-H treatment (lanes 1, 3, 5, 7, 9) and analyzed by SDS-PAGE. Cells were transfected with wt S (lanes 1 and 2), m40 (lanes 3 and 4), m41 (lanes 5 and 6), m42 (lanes 7 and 8), and m43 (lanes 9 and 10). Positions of the different S forms are indicated as described in the legend to Fig. 3.

pulse labeling. Only 30 to 50% of the cells are transfected with the DNA and express the spike protein. However, since the wild-type spike and m43 induce cell fusion, more cells (up to 80 to 90%) are involved in protein expression at the time of labeling. Therefore more protein is expressed and labeled with these two constructs.

### Palmylation of the transmembrane mutants

For influenza virus it has been shown that palmylation of the HA protein can affect its fusogenic property. Whether palmylation affects fusion depends on the influenza strain (Naeve and Williams, 1990; Lambrecht and Schmidt, 1986; Veit *et al.*, 1991; Steinhauer *et al.*, 1991). For MHV-A59 it has been shown that the S protein is acylated (Niemann and Klenk, 1981; Ricard and Sturman, 1985; Schmidt, 1982; van Berlo *et al.*, 1987), but the function of this modification has not yet been studied. Potential palmylation sites are known to be the cysteine residues that are clustered around the borderline between the transmembrane region and the cytoplasmic tail (reviewed by Sefton and Buss, 1987). The cysteine residues in the polar region of the coronavirus S TM domains could therefore potentially be palmylated.

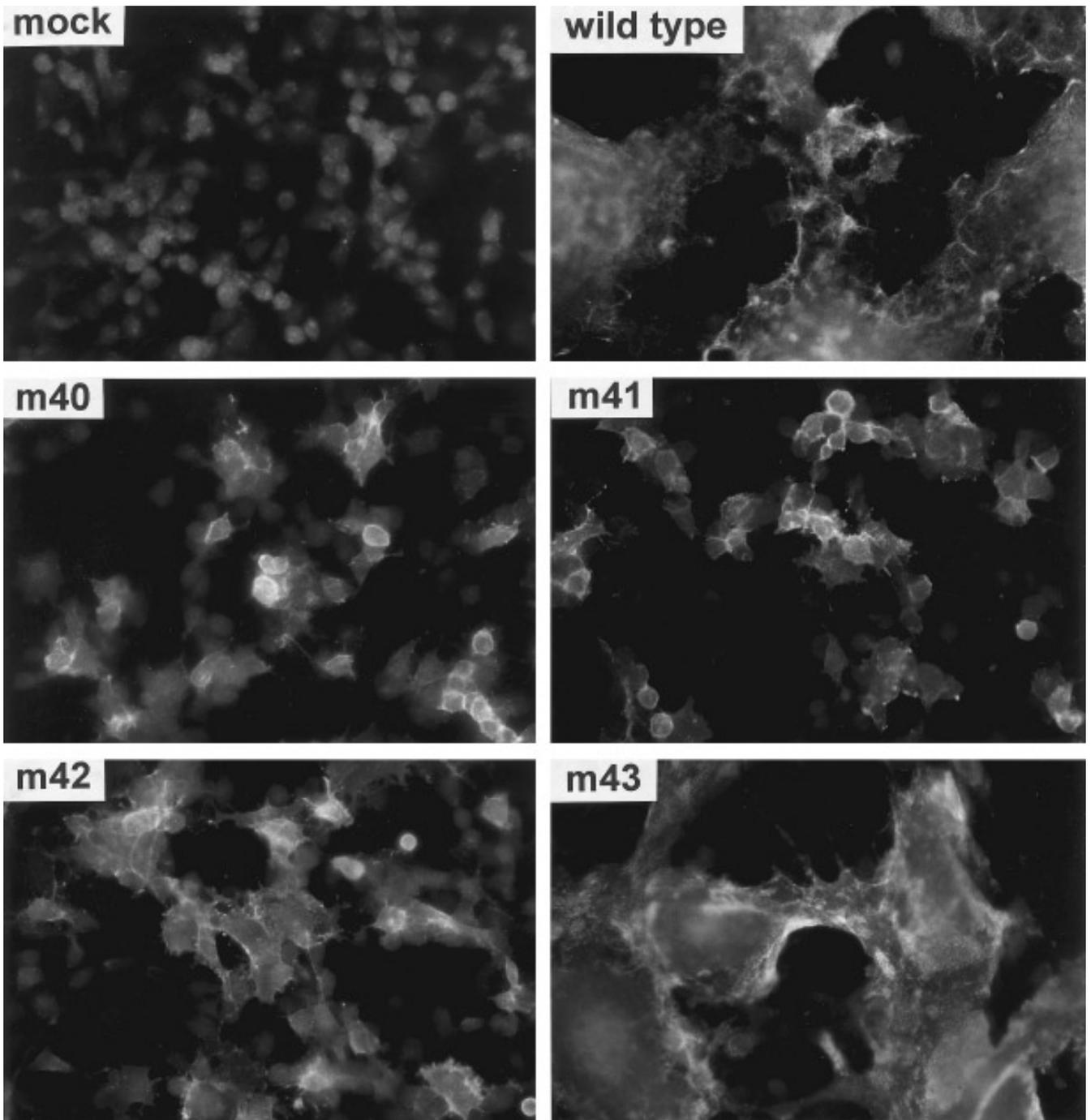
To study palmylation, we labeled the transfected cells with [<sup>3</sup>H]palmitic acid for 3 hr. Parallel transfections were labeled using Expre<sup>35</sup>SS label to study the level of expression of the S proteins. L cells were transfected with wt S, m40, m41, m42, and m43, and the immunoprecipitated S proteins were analyzed by SDS-PAGE (Fig. 10). The results clearly demonstrate that all mutant S proteins became acylated. However, comparison of the ratio of

[<sup>3</sup>H]palmitic acid to [<sup>35</sup>S]methionine incorporated in the S protein of m40 (fusion negative), m41 (fusion negative), and m42 (fusion strongly impaired) suggests that acylation in m41 and m42 was diminished. This indicates that the clusters of three and two cysteine residues just upstream of the charged lysine at position 1298 are possible acylation sites. Comparison of m40, m41, and m42 to wt or m43 was not possible, due to the increase of radioactive label in wt and m43 through extensive syncytia formation.

### DISCUSSION

The spike protein of coronaviruses is involved in attachment to the host cell and fusion with its membrane. In this paper we investigated the involvement of the MHV-A59 spike in cell-to-cell fusion in a system in which the spike gene is expressed using the vaccinia vTF7 expression system. Two domains on the spike protein were studied. The first domain is the cleavage site: we have shown here that cleavage of the MHV-A59 S protein is not strictly required for fusion, although the level of cleavage of S does influence the kinetics of fusion. We have identified several amino acids that are important for cleavage by the (as yet unidentified) endogenous protease. Second, we demonstrated that the transmembrane region of the MHV-A59 S protein plays an important role in fusion.

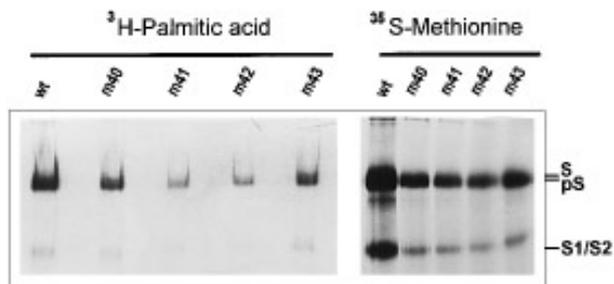
Stauber *et al.* (1993) and Taguchi (1993) have recently published data concerning the effect of a substitution or deletion of five amino acids at the trypsin cleavage site on cleavage of the MHV-JHM S protein. These mutations



**FIG. 9.** Immunofluorescence and fusogenic properties of the transmembrane region mutants. vTF7-3-infected L cells expressing the (mutant) spike proteins were fixed with paraformaldehyde at 22 hr posttransfection. S proteins at the surface were detected using a mix of anti-S monoclonal antibodies, followed by FITC-conjugated goat anti-mouse antibodies.

abolished cleavage. We analyzed in more detail the sequence requirements for cleavage of the MHV-A59 S protein. Arginine residues at positions P<sub>1</sub> and P<sub>4</sub> were found to be important for cleavage. The results obtained with mutant m6 (cleavage-negative), which has a deletion at position P<sub>5</sub>, and mutant m3, which has a glycine residue at this position, show that the residue at position P<sub>5</sub> is also involved. The amino acid at position P<sub>5</sub> is not directly involved in a primary sequence requirement for

cleavage, since the R to G mutation (m3) does not affect cleavage. However, deletion of this residue does abolish cleavage, it may therefore be important for a correct conformation or spacing. With respect to the second possibility, Vey *et al.* (1992) found that the cleavage motif of the avian influenzavirus H7 had to be presented in the correct sequence position for cleavage to occur. All mutants had an endo-H profile similar to the wild-type spike protein, thus different levels of cleavage do not affect



**FIG. 10.** Palmitylation of transmembrane mutants. Cells were transfected and labeled at 6 to 9 hr posttransfection with either 100  $\mu$ Ci [ $^3$ H]palmitic acid or with 100  $\mu$ Ci Expre $^{35}$ S label. Immunoprecipitations were performed on the cell lysates and analyzed as described. Cells were transfected with wt S, m40, m41, m42, and m43.

transport of the proteins from the Golgi stacks, and all proteins were expressed at the cell surface. However, we did observe a delay of 2 to 3 hr in the start of syncytia formation induced by the cleavage-negative MHV-A59 S mutants. This is consistent with the findings of Stauber *et al.* (1993) and of Taguchi (1993) obtained for the MHV-JHM S protein. Thus, cleavage of the MHV S protein is not strictly required for fusion, but it does enhance the induction of syncytia. However, we cannot exclude that cleavage does occur at a level below detection, possibly at the KSRR site just upstream of the cleavage site, which is also a potential furin cleavage site. Mutant m5 shows that a polar glutamine residue is allowed at the P<sub>2</sub> position, without loss of cleavage or fusogenicity. This is in contrast to the data obtained by Gombold *et al.* (1993) for the cleavage-negative revertant isolates, where only nonpolar residues were found at this position.

The wt A59 S protein is not cleaved completely, but the JHM spike protein is. Full cleavage of the endo-H-resistant A59 S protein was obtained when the histidine residue at position P<sub>2</sub> was replaced by an arginine (m7), mimicking the MHV-JHM cleavage site. This shows that the endogenous protease cleaves the RARR motif more efficiently than the RAHR motif. One candidate for the endogenous protease could be furin. Furin has been shown to cleave a number of viral and cellular proteins at the RXR/KR or RXXR sequence (reviewed by Barr, 1991; Molloy *et al.* 1992). The biological role of the difference between the extent of cleavage of the A59 and JHM spike proteins remains unclear, but could be related to the different pathological behavior of these viruses (reviewed by Wege *et al.*, 1982).

It has been shown for other viruses that mutations introduced in the transmembrane region or cytoplasmic tail of the surface proteins abolish the fusion activity without affecting transport to the cell surface (Mulligan *et al.*, 1992; Dubay *et al.*, 1992; Owens *et al.*, 1994). Using a number of mutants, we have obtained several pieces of evidence indicating that the transmembrane region of the MHV spike protein is also involved in fusion. The transmembrane mutants can be divided in two categories: (i) mutant m40, in which a charged residue at the

end of the hydrophobic stretch of amino acids leads to a potential stop transfer signal for translocation across the membrane at this position resulting in a shorter predicted TM domain; (ii) the cysteine mutant group (mutants m41, m42, and m43), in which cysteine residues in the TM domain are replaced by serine residues. The MHV/VSV chimeric mutant msg-c has a shortened predicted TM domain but also lacks the two clusters of cysteine residues. Three of the mutants (msg-c, m40, and m41) were incapable of inducing cell-to-cell fusion, whereas the fusion induced by m42 was clearly impaired. However, the fusogenicity of mutant m43 was not altered. The fusion activity of the MHV/VSV chimeric spike mutant msg-b, in which the cytoplasmic tail of the MHV S protein was replaced by the corresponding tail of the VSV G protein, clearly demonstrates that the amino acid sequence of the cytoplasmic tail is not important for cell-to-cell fusion. Maturation of the mutant S proteins was not affected as shown by endo-H profiles, and all proteins were also expressed at the cell surface. Since transport of misfolded proteins or monomers is prevented by a quality control system in the ER (Hammond *et al.*, 1994; reviewed by Doms *et al.*, 1993), we presume that our mutant proteins are generally folded correctly.

We hypothesize that the transmembrane domain of the S protein is involved in conformational changes which occur at mild alkaline pH (Weismiller *et al.*, 1990; Sturman *et al.*, 1990): changes that are induced after binding to the receptor and that are required for subsequent membrane fusion. The large size of the predicted transmembrane region of MHV S may allow for flexibility in the anchoring of the protein. The cysteine-rich region may play a role here, particularly the cysteine residues themselves. In m40, the region might have become too short and therefore the protein too rigid. This could prevent some conformational change at the membrane that is required for fusion. However, we cannot rule out the possibility that the fusion-negative mutant m40 is blocked in its receptor binding capacity. The transmembrane region, or part of it, might be involved directly in the fusion process. The region with the seven large hydrophobic amino acids, e.g., could possibly act as fusion peptide and the transmembrane region may participate in the destabilization of the host membrane. Evidently, several regions in the protein are involved in the complex fusion process.

In none of the cysteine mutants was the incorporation of [ $^3$ H]palmitic acid abolished and therefore the role of acylation in coronavirus-induced syncytium formation remains unclear. Since all three forms of the S protein (150, 180, and 90 kDa) were labeled with palmitic acid, palmitylation of MHV S occurs before the protein reaches the medial-Golgi, similarly to the VSV G and Sindbis E1 proteins (Bonatti *et al.*, 1989). Mutant m41 and m42 seem to have the lowest level of acylation. This suggests that at least one of the cysteines, that are mutated in m41 or m42, becomes acylated. Fatty acids linked to these cysteines could also have an influence on the conforma-

tion of the TM region and thereby on fusion. In any event the results obtained with the cysteine mutants point to an important function of acylation in cell-to-cell fusion which remains to be elucidated.

In this study, we have determined the influence of differential cleavage and the effect of mutations in the TM domain of an expressed spike protein on cell-to-cell fusion. The fusion mechanism at work in coronavirus-induced cell-to-cell fusion is most likely similar to that in virus-to-cell fusion during infection. Therefore the mutant proteins probably behave the same in virus-to-cell fusion as in cell-to-cell fusion. To study this, the mutant proteins will have to be inserted into MHV virions or in pseudovirions. We are currently in the process of setting up a system that can accomplish this.

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