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## Coronavirus-Induced Membrane Fusion Requires the Cysteine-Rich Domain in the Spike Protein

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The spike glycoprotein of mouse hepatitis virus strain A59 mediates the early events leading to infection of cells, including fusion of the viral and cellular membranes. The spike is a type I membrane glycoprotein that possesses a conserved transmembrane anchor and an unusual cysteine-rich (*cys*) domain that bridges the putative junction of the anchor and the cytoplasmic tail. In this study, we examined the role of these carboxyl-terminal domains in spike-mediated membrane fusion. We show that the cytoplasmic tail is not required for fusion but has the capacity to enhance membrane fusion activity. Chimeric spike protein mutants containing substitutions of the entire transmembrane anchor and *cys* domain with the herpes simplex virus type 1 glycoprotein D (gD-1) anchor demonstrated that fusion activity requires the presence of the A59 membrane-spanning domain and the portion of the *cys* domain that lies upstream of the cytoplasmic tail. The *cys* domain is a required element since its deletion from the wild-type spike protein abrogates fusion activity. However, addition of the *cys* domain to fusion-defective chimeric proteins was unable to restore fusion activity. Thus, the *cys* domain is necessary but is not sufficient to complement the gD-1 anchor and allow for membrane fusion. Site-specific mutations of conserved cysteine residues in the *cys* domain markedly reduce membrane fusion, which further supports the conclusion that this region is crucial for spike function. The results indicate that the carboxyl-terminus of the spike transmembrane anchor contains at least two distinct domains, both of which are necessary for full membrane fusion. © 2000 Academic Press

### INTRODUCTION

Mouse hepatitis virus (MHV) is a neurotropic murine coronavirus that has been used in numerous studies of viral neurovirulence and the mechanism of virus-mediated demyelination in the central nervous system (Fleming *et al.*, 1993; Gilmore *et al.*, 1994; Gombold *et al.*, 1995; Gombold and Weiss, 1992; Hingley *et al.*, 1994; Houtman and Fleming, 1996; Lavi *et al.*, 1984, 1988; Wang *et al.*, 1990; Watanabe *et al.*, 1983; Weiner, 1973). Like all coronaviruses, MHV strain A59 possesses a large (31 kb) single-stranded, positive-sense RNA genome (Lee *et al.*, 1991; Pachuk *et al.*, 1989) that is associated with the viral nucleocapsid protein (Sturman *et al.*, 1980) inside a host cell-derived membrane. The envelope contains four to five additional structural proteins depending on the particular strain of virus. Prominent among these is the spike glycoprotein, which is a 180-kDa transmembrane glycoprotein that functions in receptor binding and membrane fusion (Sturman *et al.*, 1985). It is translated as a 145-kDa polypeptide and glycosylated in the endoplasmic reticulum. For A59 and some other strains of MHV, the spike is posttranslationally cleaved into two 90-kDa subunits (Luytjes *et al.*, 1987; Sturman *et al.*, 1985) termed S1 (N-terminal) and S2 (C-terminal), presumably in the *trans*

Golgi, by what is believed to be a host cell protease. This cleavage event appears to enhance the fusogenic activity of the spike (Gombold *et al.*, 1993). Furthermore, virus infectivity does not require cleavage of the A59 spike protein (Bos *et al.*, 1997) and thus differs from other RNA viruses such as paramyxoviruses, influenza virus, and retroviruses (Glickman *et al.*, 1998; Kawaoka and Webster, 1988; Perez and Hunter, 1987). Expression of the spike on the surface of the infected cell results in extensive cell–cell fusion. It is clear that fusion is mediated solely by the spike since expression of the glycoprotein in the absence of other viral proteins is sufficient to induce cell fusion (Stauber *et al.*, 1993; Taguchi *et al.*, 1992; Vennema *et al.*, 1990).

The spike is an unusual viral fusion protein in that proteolytic cleavage does not generate a hydrophobic amino-terminus on S2 that, based on the function of other viral fusion proteins, would be expected to participate in membrane fusion. In addition, the A59 spike has a high (4%) cysteine content (53 residues). Nine of the cysteine residues are found in a stretch of 18 amino acids that overlaps the junction of the membrane-spanning domain and the cytoplasmic tail, and 4 of these 9 residues are conserved among most coronaviruses (Fig. 1). This cysteine-rich (*cys*) domain is of unknown function, but the unusual sequence composition and conservation of the *cys* domain suggest that it is important in the biology of the spike. Indeed, replacement of the

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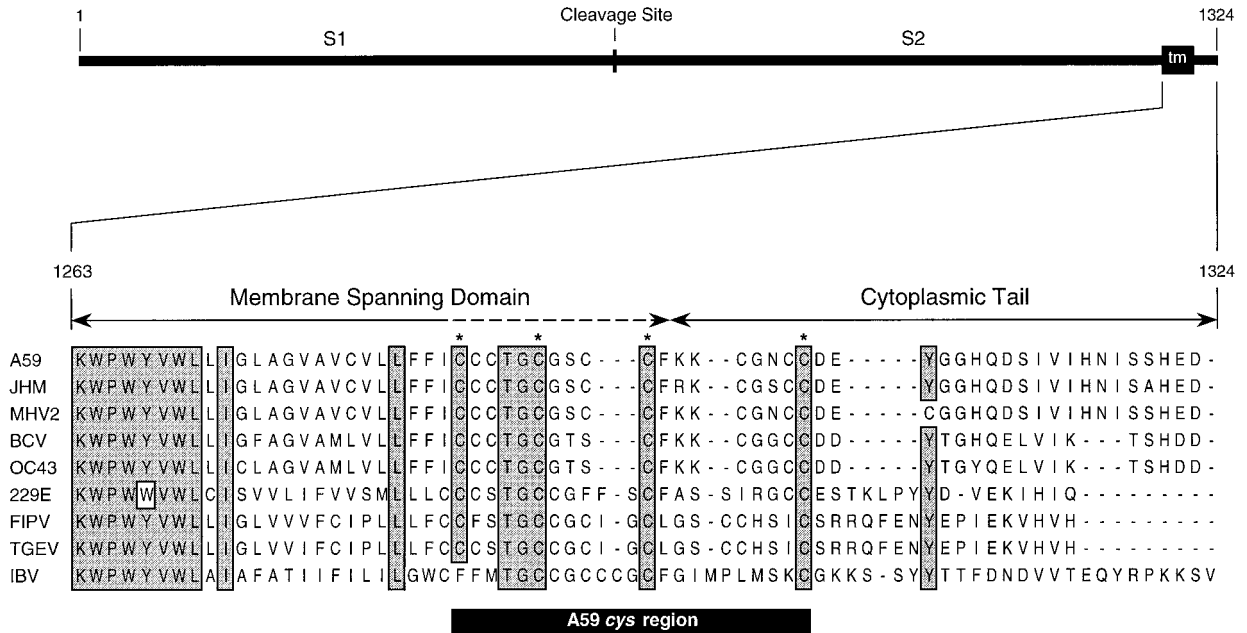


FIG. 1. CLUSTAL alignment of the transmembrane anchor and cytoplasmic tail of the spike glycoprotein from nine different coronaviruses (Abraham *et al.*, 1990; Binns *et al.*, 1985; Delmas *et al.*, 1992; Kunkel and Herrler, 1993; Luytjes *et al.*, 1987; Mounir and Talbot, 1993; Parker *et al.*, 1989; Raabe *et al.*, 1990; Rasschaert and Laude, 1987). Shown on the top is a diagram of the A59 spike protein from amino acid 1 to amino acid 1324. The position of the proteolytic cleavage site that divides the S1 and S2 subunits is also shown. The carboxyl-terminal region of the A59 spike (amino acids 1263 to 1324) that contains the transmembrane anchor (tm) is enlarged below and is aligned with the spike sequence from viruses belonging to antigenic group II [mouse hepatitis virus strains A59, JHM, and MHV2, bovine coronavirus (BCV), and human coronavirus OC43 (OC43)], antigenic group I (human coronavirus 229E (229E), feline infectious peritonitis virus (FIPV), and transmissible gastroenteritis virus (TGEV)) and antigenic group III [infectious bronchitis virus (IBV)]. The solid black bar below the sequences indicates the boundary of the cysteine-rich domain of the A59 spike protein, and the dashed region above the sequences indicates the region of the cysteine-rich domain that is potentially membrane-spanning. The shaded residues are those that are conserved in at least eight of the nine sequences shown, and the asterisks above the A59 sequence indicate four nearly perfectly conserved cysteine residues found within the cysteine-rich domain.

cytoplasmic portion of the cys domain and the cytoplasmic tail with the VSV G protein cytoplasmic tail resulted in the loss of cell-cell fusion (Bos *et al.*, 1995).

The strong amino acid identity throughout the coronavirus transmembrane anchor, together with the unusual cys domain, led us to examine the potential role of these domains in spike protein function. We report here that unlike other characterized viral fusion proteins, the A59 spike protein has an absolute requirement for the native MHV transmembrane anchor. Substitution of the anchor with an analogous but unrelated transmembrane anchor abrogates fusion activity. Part of this requirement reflects a need for the cys domain, which we show is necessary for fusion activity, but is not the sole functional domain of the transmembrane anchor required for fusion.

## RESULTS

The spike glycoprotein of the mouse hepatitis virus A59 binds the MHV receptor (recently renamed CEACAM 1a), the host cell receptor for the virus, and promotes fusion of the viral envelope and the host cell membrane. Fusion occurs with the plasma membrane in a pH-independent fashion (Frana *et al.*, 1985; Gallagher *et al.*, 1991; Nash and Buchmeier, 1997; Sturman *et al.*, 1985; Ven-

nema *et al.*, 1990). Coronavirus spike proteins are unusual in at least two aspects. First, they contain a cys domain approximately 20 amino acids in length and containing 40 to 50% cysteine. The cys domain bridges the putative border between the hydrophobic membrane-spanning domain and the cytoplasmic tail (Fig. 1). In addition, four of the cysteines present in the A59 cys domain are highly conserved, suggesting that these amino acids contribute to spike function. Second, the transmembrane anchor is one of the most highly conserved regions of the protein. To address the role of these domains in spike function, we have examined the fusion activity of spike proteins with defined mutations in these regions.

To express the spike, we cloned the A59 spike coding sequence downstream of the T7 RNA polymerase promoter and encephalomyocarditis virus (EMC) internal ribosome entry site (IRES) sequence. The T7 transcriptional terminator closely follows the spike coding sequence. Expression of the spike protein was achieved by infection of murine DBT cells with vTF7.3, a recombinant vaccinia virus that produces T7 RNA polymerase (Fuerst *et al.*, 1986, 1987), followed by transfection with plasmid DNA encoding the spike gene. Using this expression

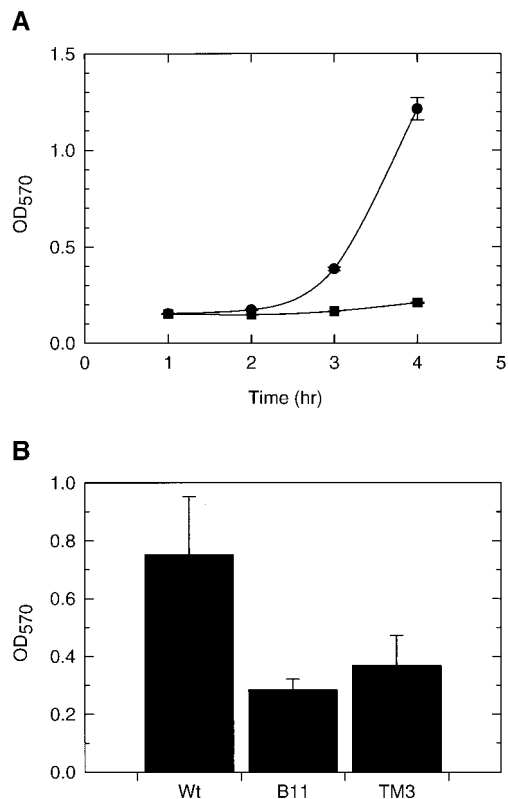
system, we can visually observe fusion of cells as early as 3 to 4 h posttransfection, and by 10 h posttransfection fusion is nearly confluent (data not shown).

**$\beta$ -Galactosidase-based fusion assay.** We have adapted the indirect fusion assay used initially in studies of receptor binding and fusion by human immunodeficiency virus (HIV; Nussbaum *et al.*, 1994) to measure the relative fusion activities of spike proteins containing defined mutations. Effector cells are prepared by infecting DBT cells with vTF7.3 and then transfecting these cells with the cloned spike gene. Reporter cells are prepared by infecting DBT cells with wild-type vaccinia virus (strain WR) and then transfecting with a plasmid containing the *lacZ* gene cloned downstream of the T7 promoter and the EMC IRES. Following transfection, the cells are mixed in a 1:1 ratio and incubated for 1 to 4 h to allow for cell–cell fusion. Fusion results in the production of  $\beta$ -galactosidase, which can be measured by monitoring the hydrolysis of chlorophenol red- $\beta$ -D-galactopyranoside (CPRG) in a spectrophotometer at 570 nm.

The kinetics of fusion using the wild-type spike gene were rapid. Figure 2A shows the kinetics over a 4-h period, which is the time course for all assays described below. Significant fusion activity was seen as early as 3 h after the addition of the  $\beta$ -galactosidase reporter cells and continued to increase for several hours. In contrast, cells transfected with pTM3, the expression plasmid lacking the spike gene, do not undergo fusion. The basal optical density (OD) shown for pTM3 in Fig. 2A is due to the absorption characteristics of the  $\beta$ -galactosidase substrate CPRG.

We verified that this assay can discriminate between wild-type and fusion-defective spike proteins by expressing a cloned copy of the spike gene from B11, a fusion-defective mutant of A59 isolated from a persistent infection of primary glial cells (Gombold *et al.*, 1993). Assay of  $\beta$ -galactosidase activity 4 h after the addition of reporter cells showed no significant difference in the levels of fusion induced by the B11 spike protein and the negative control, pTM3 (Fig. 2B). In contrast, fusion in cells transfected with the wild-type spike protein was significantly greater than B11 or that in cells transfected with pTM3 (Student's *t* test,  $P < 0.05$ ). Again, the optical density shown for lysates from cells transfected with pTM3 (and B11 as well) is due to the absorption characteristics of CPRG and not to  $\beta$ -galactosidase activity. For all subsequent figures, OD values were corrected for this background and normalized to wild-type.

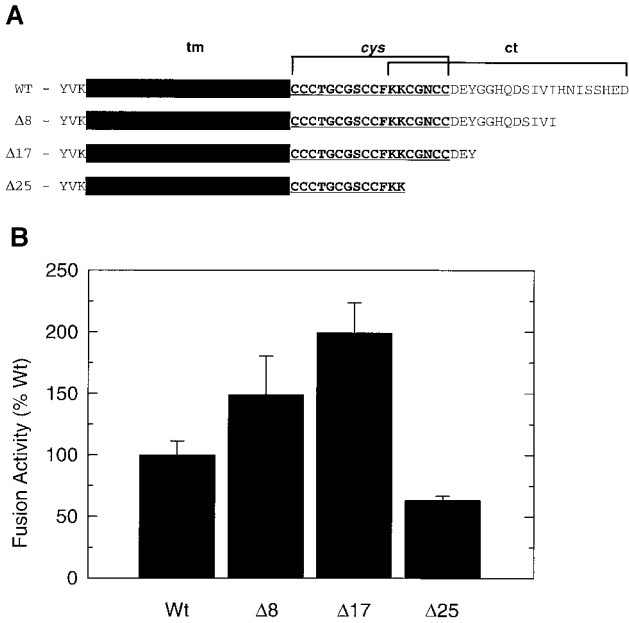
**Role of the cytoplasmic tail in membrane fusion.** This colorimetric fusion assay was used to examine the fusion activity of spike proteins with mutations in the transmembrane anchor and the cytoplasmic tail. Extensive identity in amino acid sequence in these domains is apparent upon comparison of the protein sequences of a number of different coronaviruses (Fig. 1), which led us to hypothesize that the carboxyl-terminus of the spike



**FIG. 2.** (A) Fusion kinetics of wild-type spike protein. Wild-type spike (●) was expressed in DBT as described in the text, and the level of fusion was quantitated at 1-h intervals from 1 to 4 h after the addition of reporter cells. Control cells were transfected with pTM3, the expression plasmid lacking the spike gene (■). Plotted is the mean optical density ( $\pm$  standard deviation) measured as described under Materials and Methods. (B) Relative levels of fusion by the wild-type spike protein (Wt) and a fusion-defective spike mutant (B11) compared to the negative control, pTM3. Data represent the mean optical density ( $\pm$  standard deviation) at 4 h following the addition of reporter cells.

protein was important in the function of the protein. To test this hypothesis initially, we used a polymerase chain reaction (PCR)-based mutagenesis procedure (Chen and Przybyla, 1994; Li and Wilkinson, 1997) to construct three C-terminal truncation mutants that removed increasing amounts of the cytoplasmic tail (Fig. 3A). We found that removing up to 17 amino acids ( $\Delta 17$ ) from the C-terminus had no detrimental effect on cell–cell fusion (Fig. 3B). In fact, removal of either 8 or 17 amino acids increased the spike fusion activity 50 and 100%, respectively. However, when the complete cytoplasmic tail was removed ( $\Delta 25$ ), cell–cell fusion was decreased approximately 40% relative to that of the wild-type spike. These data argue that the cytoplasmic tail has the capacity to modulate spike fusion activity but is not absolutely required for activity.

**Requirement for the A59 transmembrane anchor in membrane fusion.** To further examine the role of the carboxyl-terminal domain of the spike protein in membrane fusion, we made a chimeric spike protein (YS24) containing the A59 spike ectodomain and the transmem-



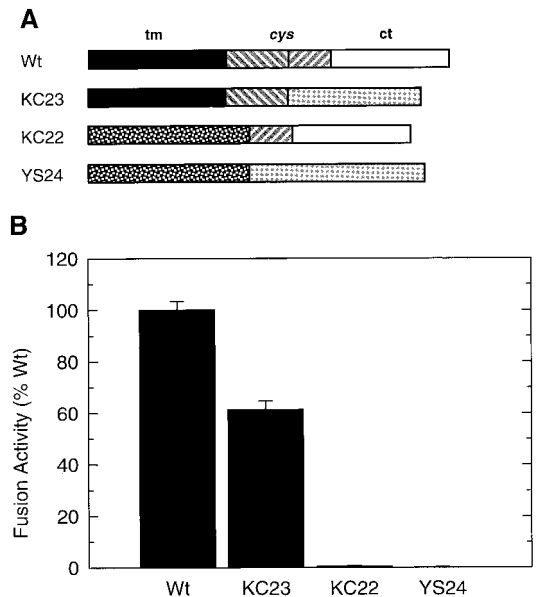
**FIG. 3.** The spike cytoplasmic tail is not required for cell-cell fusion. (A) Diagram of the cytoplasmic tail truncation mutants of the spike protein generated through PCR mutagenesis. Shown are the hydrophobic membrane-spanning domain (tm, ■), the cysteine-rich domain (cys), and the cytoplasmic tail (ct) of the spike protein. The spike ectodomain is omitted from the figure but would lie to the left of the diagram. (B) Fusion activities (mean  $\pm$  standard deviation) of the cytoplasmic tail truncation mutants quantitated using a  $\beta$ -galactosidase-based fusion assay. The data have been normalized to the fusion activity of the wild-type spike protein and show the activity measured 4 h after addition of reporter cells.

brane anchor and cytoplasmic tail derived from herpes simplex virus type 1 (HSV) glycoprotein D (gD-1; Fig. 4A). Transfection of vTF7.3-infected cells with YS24 failed to induce any visible syncytia in cultures. Quantitation of the fusion activity of YS24 verified that this A59:HSV chimera was unable to induce detectable levels of fusion (Fig. 4B). The inability of the A59:HSV chimeric spike protein to mediate membrane fusion was unexpected since the gD-1 domains had previously been reported to support fusion when cloned into the VSV G protein (Odell *et al.*, 1997).

The previous experiments suggested that the loss of fusion activity was most likely due to substitution of the transmembrane anchor and not the cytoplasmic tail. This hypothesis was tested by constructing two additional chimeras: KC23, which possesses the native A59 transmembrane anchor and the amino-terminal 11 amino acids of the cys domain joined to the gD-1 cytoplasmic tail; and KC22, which has the gD-1 transmembrane anchor joined to the A59 cytoplasmic tail (Fig. 4A). Fusion activity correlated with the presence of the A59 membrane-spanning domain and the upstream portion of the cys domain in KC23 (Fig. 4B). The level of fusion observed with KC23 was reduced approximately 40% relative to that of wild-type spike, which is nearly identical to the

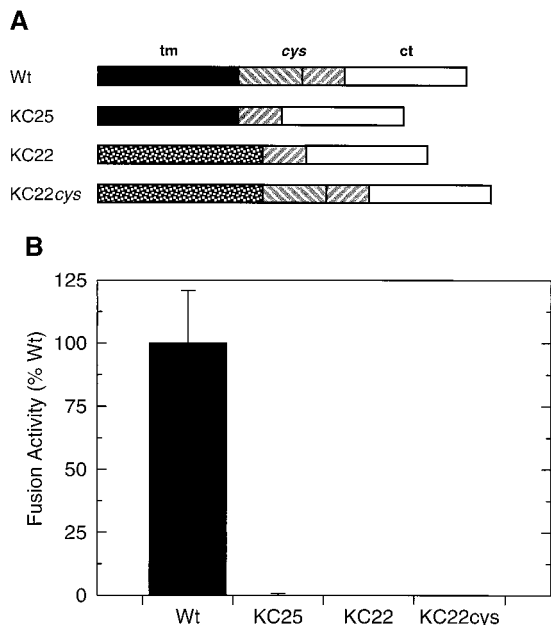
reduction in fusion observed in the absence of the A59 cytoplasmic tail ( $\Delta$ 25, Fig. 2).

*The cys domain is required for membrane fusion.* Since deletion of the cytoplasmic tail from the spike did not prevent fusion activity while the substitution of the transmembrane anchor in chimeric protein KC22 was fusion-defective, we concluded that the transmembrane domain contains one or more regions that are critical for fusion. The obvious candidate is the cys domain that overlaps the junction between the hydrophobic membrane-spanning domain and the cytoplasmic tail. To ascertain whether a requirement for the cys domain was responsible for the fusion phenotypes of the A59:HSV chimeras, we produced a spike protein that was entirely A59 in origin except that the first 11 amino acids of the cys domain (all amino acids lying upstream of the cytoplasmic tail) were removed. This construct, KC25, retains the hydrophobic portion of the membrane-spanning domain adjoined to the A59 cytoplasmic tail, including the last 7 amino acids of the cys domain (Fig. 5A). We also constructed an additional chimera based on the fusion-defective chimera KC22, in which the missing 11 amino acids of the cys domain were inserted between the end of the gD-1 transmembrane anchor and the A59 cytoplasmic tail, thus restoring the entire cys domain (Fig.



**FIG. 4.** Spike fusion activity requires the A59 membrane-spanning and cys domains. (A) Diagram of the chimeric transmembrane and cytoplasmic domains. Shown are the transmembrane anchors (tm) from the A59 spike (■) and gD-1 (▣), the cysteine-rich domain (cys, ▨), and the cytoplasmic tail (ct) of the spike protein (□) and gD-1 (▣). The cys domain is shown as two boxes to indicate the putative border between the transmembrane domain and the cytoplasmic tail. (B) Fusion activities (mean  $\pm$  standard deviation) of the spike chimeras quantitated using the  $\beta$ -galactosidase-based fusion assay. The data have been normalized to the fusion activity of the wild-type spike protein and show the activity measured 4 h after addition of reporter cells.





**FIG. 5.** The *cys* domain is necessary for spike fusion activity but is not sufficient to restore fusion activity to a fusion-defective A59:HSV chimera. (A) Diagram of the *cys* deletion mutant and the spike chimeras. Shown are the transmembrane anchors (tm) of the A59 spike (■) and gD-1 (▣), the cysteine-rich domain (*cys*, ▨), and the cytoplasmic tail (ct) of the spike protein (□) and gD-1 (▣). The *cys* domain is shown as two boxes to emphasize the border between the transmembrane domain and the cytoplasmic tail. (B) Fusion activities (mean  $\pm$  standard deviation) of the spike mutants quantitated using a  $\beta$ -galactosidase-based fusion assay. The data have been normalized to the fusion activity of the wild-type spike protein and show the activity measured 4 h after the addition of reporter cells.

5A). The rationale for analysis of this mutant was that if the fusion phenotype of KC22 was due to loss of the *cys* domain rather than to substitution of the transmembrane anchor, the replacement of the *cys* domain should restore fusion activity to the chimera.

The fusion activity of these two mutants, as well as KC22, is shown in Fig. 5B. Removal of the majority of the *cys* domain from the wild-type spike reduces fusion activity over 99%, as shown by the lack of detectable activity by KC25. However, the restoration of the *cys* domain in KC22cys failed to even partially restore fusion activity. Together, these results clearly show that the *cys* domain is necessary for the wild-type spike to function properly in membrane fusion, but is not sufficient to restore activity to fusion-defective A59:HSV chimeras. We conclude that fusion requires the *cys* domain and all or part of the transmembrane domain. In this regard, Bos *et al.* (1995) demonstrated that the spike protein of A59 loses syncytial activity if the entire *cys* domain and cytoplasmic tail are replaced by the vesicular stomatitis virus G protein cytoplasmic tail. These investigators hypothesized the need for flexibility in the transmembrane anchor and suggested that the *cys* domain might provide this property.

*Mutation of conserved cysteines in the transmembrane anchor and cytoplasmic tail.* To examine the role that the *cys* domain might have in either the structure or the syncytial activity of the spike protein, we made a series of point mutations at each of the four conserved cysteine residues present in the domain (amino acids 1287, 1292, 1296, and 1304; Fig. 1). Since more than half of the *cys* domain may constitute part of the membrane-spanning domain and, therefore, may potentially interact with the hydrophobic environment of the membrane (Bos *et al.*, 1995), we made three independent mutants for each conserved cysteine in an attempt to control for changes in hydrophobicity. Based on the normalized consensus hydrophobicity scale of Eisenberg (1984), a mutation from cysteine to tyrosine is expected to result in little change ( $-0.03$ ) while changes to serine and tryptophan decrease ( $-0.47$ ) and increase ( $+0.52$ ) local hydrophobicity, respectively.

These 12 mutants were examined in two independent experiments for their ability to induce cell fusion using the  $\beta$ -galactosidase fusion assay described previously. Table 1 shows the level of fusion induced by each of the mutants normalized to the activity of the wild-type spike protein. We found that mutation of any single cysteine residue diminished the level of fusion, but in only a few instances was fusion reduced to nearly undetectable levels. Cysteine 1287 (C1287) was the most sensitive to hydrophobic substitution and less affected by neutral or hydrophilic changes. Substitution of C1287 to a tryptophan nearly inhibited cell fusion, though the level of activity was still greater than that of the negative control (Student's *t* test,  $P < 0.05$ ). In contrast, C1292 and C1296 were least sensitive to the neutral or hydrophobic substitution of tyrosine or tryptophan, respectively. Mutation of either residue to a more hydrophilic serine reduced fusion over 90%. Finally, mutation of C1304 reduced the level of fusion by a comparable amount with all three substitutions. Of the four cysteines that were targeted, C1304 was affected the least by mutation, and the levels

**TABLE 1**  
Fusion Activity of Spike Proteins with Mutation of Conserved Cysteine Residues

Mutation <sup>a</sup>	Experiment No.	Cysteine residue				
		WT	C1287	C1292	C1296	C1304
C $\rightarrow$ S	I	100 <sup>b</sup>	44	4	9	73
	II	100	9	0	4	55
C $\rightarrow$ Y	I	100	7	18	35	65
	II	100	22	31	72	91
C $\rightarrow$ W	I	100	4	32	28	49
	II	100	6	40	49	52

<sup>a</sup> Cysteines (C) were mutated to serine (S), tyrosine (Y), or tryptophan (W).

<sup>b</sup> Percentage activity relative to wild-type spike.

of fusion that were observed were not dissimilar to those seen when the entire cytoplasmic tail was truncated. Clearly, mutation of any one of the four conserved cysteine residues can affect the level of fusion induced by the spike protein, suggesting that these cysteines play an important role in spike function.

*Intracellular transport and surface expression of mutant spike glycoproteins.* The loss of fusion activity exhibited by several of the mutants could have been due to an absence of cell surface spike. This possibility was examined by expressing wild-type or mutant spike in DBT cells and detecting the protein in the cytoplasm or on the cell surface by immunofluorescence. Both wild-type and mutant spike proteins were expressed normally in the cytoplasm of cells without alterations in distribution or localization (Fig. 6). More importantly, all three of the transmembrane chimeras shown in Fig. 6 were expressed on the cell surface. We also have examined the surface expression of KC25, the *cys* deletion mutant, and found that it is also expressed on the cell surface (data not shown). While it is not possible to determine from these data the relative levels of spike found on the cell surface, it is clear that none of the mutants examined are markedly impaired in surface expression.

The conclusion that the fusion-defective phenotype of the mutant spike proteins reported here is not due to aberrant transport and/or surface expression is supported by pulse-chase experiments that show that the mutants acquire an endoglycosidase H-resistant phenotype with essentially wild-type kinetics (data not shown). In addition, immunoprecipitation of spike proteins following a 30-min pulse-labeling period and a subsequent chase revealed that both the fusion-positive and the fusion-negative chimeras are cleaved proteolytically into 90-kDa subunits (data not shown). Together, these data argue that the chimeric spike proteins are synthesized, transported to the Golgi where they are modified and processed, and then transported to the cell surface in a manner similar if not identical to that of the wild-type spike protein. Therefore, the inability of these mutants to induce membrane fusion does not appear to be due to problems in transport to and expression on the cell surface.

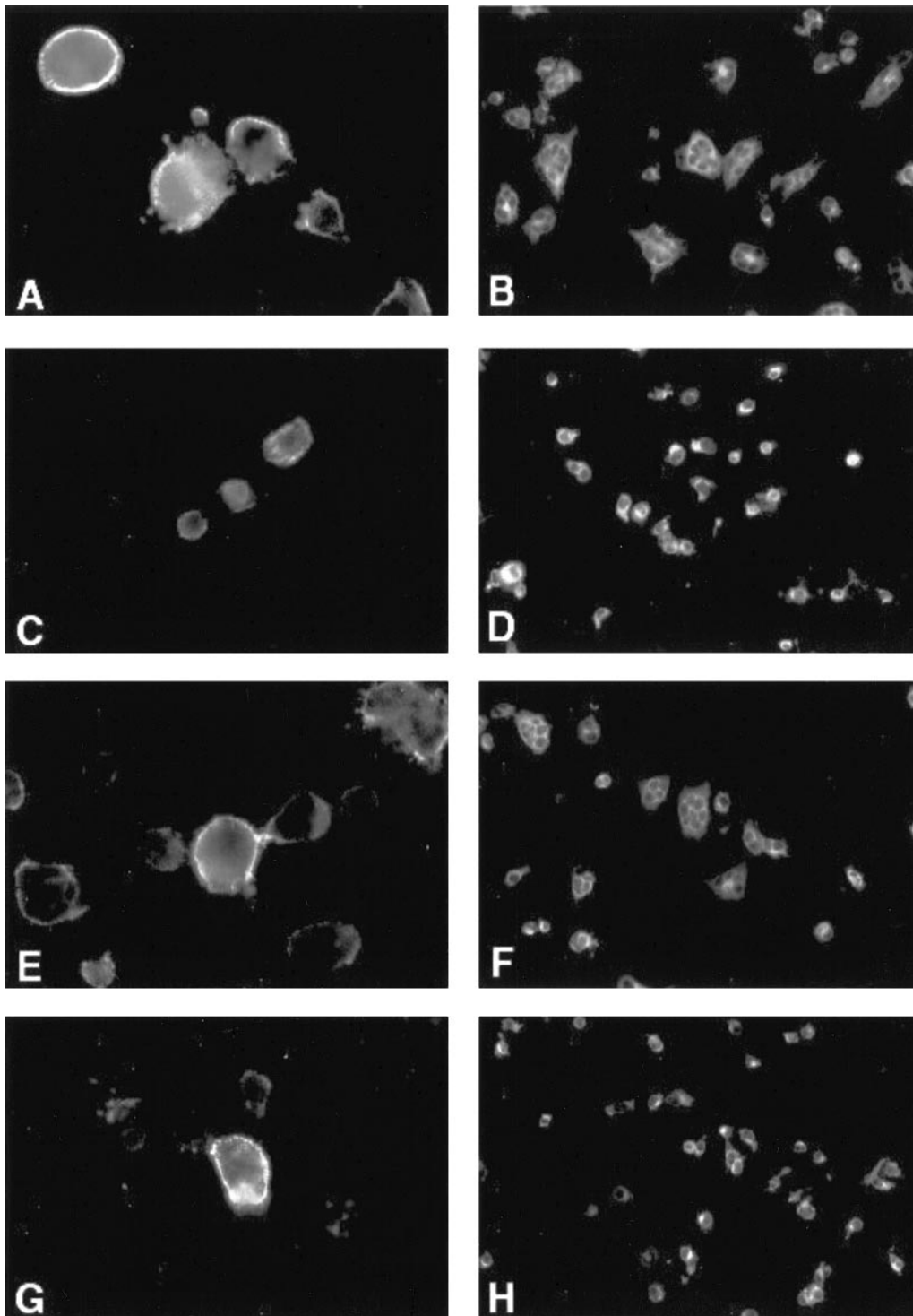
*Fusion-defective chimeras mediate lipid mixing.* Membrane fusion is a two-step process that initially involves the formation of a hemifusion intermediate, which is characterized by the mixing of lipids between the outer leaflets of the opposing membranes. To ascertain whether the fusion-defective mutants were capable of promoting lipid mixing, DBT cells were infected with vTF7.3 and subsequently transfected with spike cDNAs, labeled with 5-chloromethylfluorescein diacetate (CMFDA), and then incubated with normal DBT cells that had been labeled previously with the lipophilic dye octadecyl rhodamine B (R18). Hemifusion induced by fusion-defective spike molecules should result in the transfer of

rhodamine to the fluorescein-positive, spike-expressing cells and result in doubly fluorescent cells. Cells transfected with the wild-type spike gene caused cell-cell fusion and readily induced the transfer of R18 to the CMFDA-labeled, spike-expressing cells as would be expected during cell-cell fusion. More importantly, both CMFDA and R18 can be observed in individual cells that have not yet fused to form heterokaryons (Figs. 7A and 7B, arrow) as well as in syncytia (Figs. 7A and 7B, asterisk). Expression of the *cys*-deletion mutant KC25 in DBT cells failed to induce visible cell-cell fusion, consistent with data presented in Fig. 5. However, the expression of KC25 did promote lipid mixing (Figs. 7C and 7D), indicating that loss of the full-length *cys* domain prevents a step of membrane fusion occurring after formation of the hemifusion intermediate. Lipid mixing was not observed in control cells that were infected with vTF7.3 and subsequently transfected with the expression plasmid lacking a spike coding sequence (Figs. 7E and 7F). Thus, the fusion-defective spike protein lacking the *cys* domain was able to promote hemifusion but was blocked in its ability to generate a fusion pore between the two cells.

## DISCUSSION

The A59 spike glycoprotein mediates fusion of the viral envelope with the plasma membrane following receptor binding. The current model of fusion (reviewed in Hernandez *et al.*, 1996; Lamb, 1993; White, 1992) proposes that the fusion protein undergoes a conformational change after binding to the host cell receptor that exposes a hydrophobic fusion peptide or fusion domain. The fusion peptide promotes a state of hemifusion, which is characterized by the merger of the outer (but not inner) leaflets of the opposing membrane bilayers. Formation of a fusion pore between two cells (or virus and cell) occurs when the two inner leaflets of the membranes fuse. Several studies have shown that the membrane anchor of the fusion protein must span both leaflets of the membrane for pore formation to occur (Kemble *et al.*, 1994; Melikyan *et al.*, 1995, 1997a).

In this study, we examined the role of the cytoplasmic tail and transmembrane anchor of the spike protein in fusion activity. Initial experiments focused on the cytoplasmic tail and showed that partial truncation of the cytoplasmic tail increased fusion activity up to twofold. However, deletion of the entire cytoplasmic domain reduced fusion activity approximately 40%. Immunofluorescence staining of cells expressing the various spike truncation mutants showed that all were expressed on the cell surface. The changes in fusion activity may be due to modulation of the inherent fusion activity of the spike or, alternatively, simply to alterations in the level of the spike protein present on the surface of transfected cells. In this latter regard, it is interesting to note that an

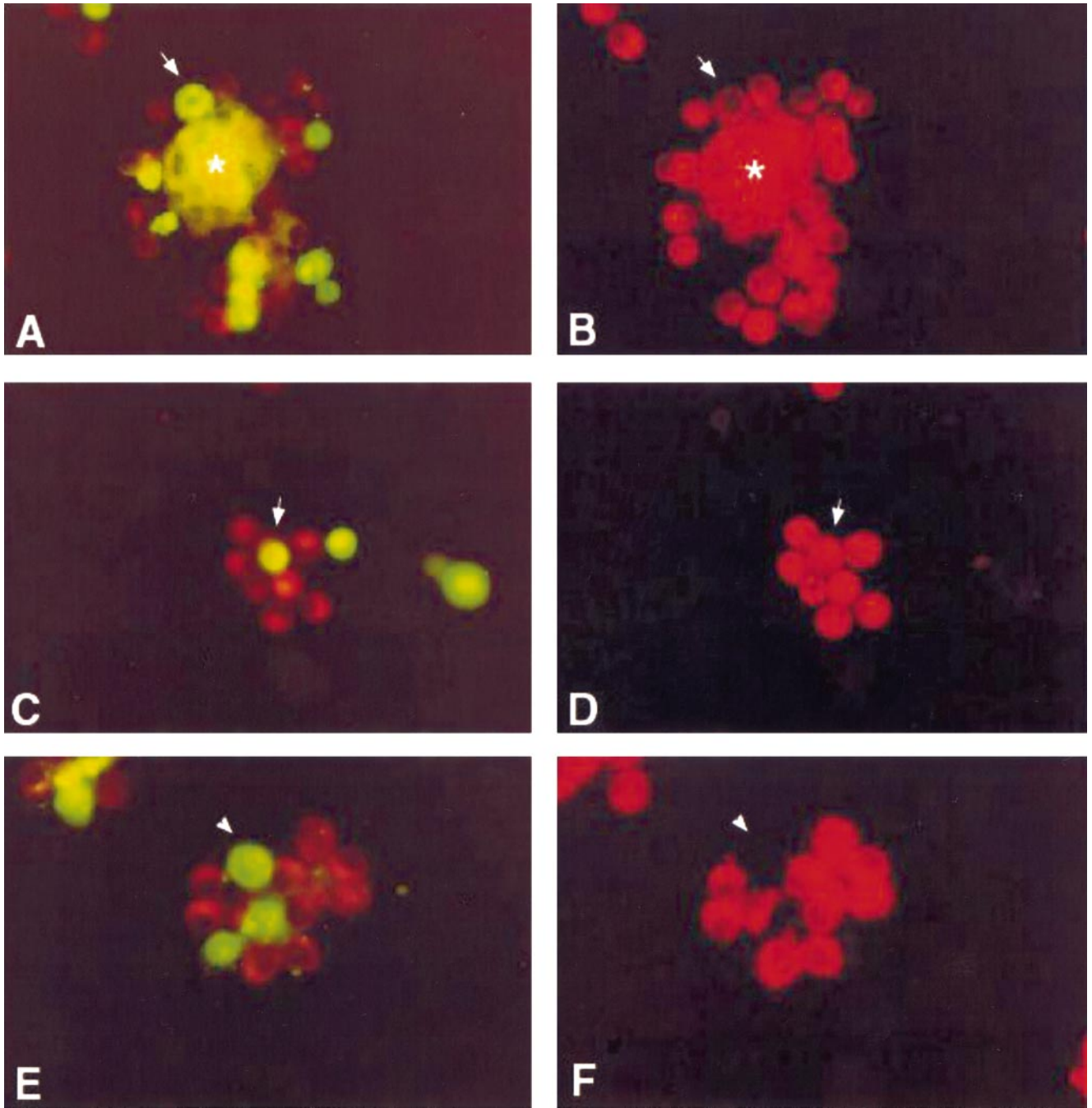


**FIG. 6.** A59 spike chimeras are expressed on the cell surface. DBT cells expressing spike proteins were prepared for immunofluorescence staining at 8 h posttransfection. For cell surface staining (A, C, E, and G), the cells were fixed with 4% paraformaldehyde prior to incubation with the anti-spike primary antiserum. A duplicate set of cells was permeabilized with methanol prior to incubation with primary antiserum to detect intracellular spike proteins (B, D, F, H). Anti-spike antibodies were detected using FITC-conjugated rabbit anti-goat serum. Shown are cells transfected with the wild-type spike (A and B), chimera KC22 (C and D), chimera KC23 (E and F), and chimera YS24 (G and H). Surface staining was photographed at 400 $\times$  and cytoplasmic staining at 200 $\times$ .

interaction between lentivirus envelope glycoproteins and clathrin-associated adaptor complexes AP-1 and AP-2 was shown to markedly affect the subcellular dis-

tribution and cell surface expression of the viral glycoproteins (Berlioz-Torrent *et al.*, 1999). The cellular adaptor proteins recognize and bind a tyrosine-based sorting





**FIG. 7.** Fusion-defective chimeric spike proteins mediate lipid mixing. vTF7.3-infected DBT cells were transfected with plasmids encoding the wild-type spike (A and B), the *cys* domain mutant KC25 (C and D), or the empty expression plasmid pYS78 (E and F). After transfection, the cells were stained with the green fluorescent dye CMFDA. A fivefold excess of normal DBT cells previously labeled with R18 was added to the CMFDA cells and examined by fluorescence microscopy for transfer of the red fluorescent R18 to CMFDA-labeled cells. A, C, and E show fluorescence due to fluorescein (CMFDA), and B, D, and F show fluorescence due to the rhodamine (R18). Cells transfected with wild-type spike formed syncytia as indicated by the asterisk. The arrows in A, B, C, and D point to cells that are dually labeled. The arrowheads in E and F point to cells from the control sample that are stained with fluorescein but not R18.

signal present in the cytoplasmic tail of the viral glycoproteins. Such an interaction could potentially regulate the level of the MHV spike protein on the cell surface. Although the observed changes in fusion activity for the three truncation mutants are statistically significant compared to wild-type (Student's *t* test,  $P < 0.05$ ), it remains

to be determined whether a twofold reduction or enhancement in fusion activity is biologically significant and affects the infection of cells or spread of virus.

Mutations in the cytoplasmic tail of viral fusion proteins have in some cases been shown to affect membrane fusion activity. For many retroviruses, deletion of

all or part of the cytoplasmic tail enhances membrane fusion, as demonstrated for simian immunodeficiency virus (Ritter *et al.*, 1993), HIV (Dubay *et al.*, 1992; Wilk *et al.*, 1992), Mason–Pfizer monkey virus (Brody *et al.*, 1994), and murine leukemia virus (Januszski *et al.*, 1997; Ragheb and Anderson, 1994a; Yang and Compans, 1996, 1997). In the last case, deletion of the C-terminal 16 amino acids of the env glycoprotein removes the R peptide, which is responsible for the inhibition of fusogenic activity (Ragheb and Anderson, 1994a; Rein *et al.*, 1994; Yang and Compans, 1996). In contrast, the F protein of SV5 (Bagai and Lamb, 1996) and parainfluenza type 3 (PIV3; Yao and Compans, 1995) and possibly the G protein of VSV (Odell *et al.*, 1997) appear to require the cytoplasmic domain for efficient membrane fusion. In at least one case, this requirement is strain specific. Truncation of the PIV3 F protein cytoplasmic tail markedly reduces fusion while truncation of the parainfluenza virus type 2 F protein does not (Yao and Compans, 1995). The role of the cytoplasmic tail in fusion is not clear, but studies of the influenza hemagglutinin have shown that mutations in the cytoplasmic tail can affect fusion pore “flickering” without affecting the subsequent enlargement of the fusion pore (Melikyan *et al.*, 1997b).

Alterations of the A59 spike protein transmembrane domain showed pronounced effects on membrane fusion. Substitutions of the entire transmembrane domain with the analogous domain from gD-1 abrogated membrane fusion, regardless of the origin of the cytoplasmic tail. As noted below, we believe that this is due partly to the disruption of the *cys* domain. The loss of fusion in the gD-1 transmembrane substitution mutants was unexpected since the gD-1 transmembrane anchor supports fusion when cloned into the VSV G protein (Odell *et al.*, 1997). Similar results for the influenza hemagglutinin further support the idea that the specific sequence of the transmembrane anchor is not critical for function (Schroth-Diez *et al.*, 1998).

During fusion, the transmembrane anchor participates in the formation of the fusion pore (Kemble *et al.*, 1994; Melikyan *et al.*, 1995, 1997a); Ragheb and Anderson, 1994b; Salzwedel *et al.*, 1993), a function that is not considered to depend on the amino acid sequence of the anchor. However, in the case of the A59 spike protein, it would appear that full fusion (i.e., fusion pore formation) is dependent on the sequence of all or part of the hydrophobic membrane-spanning domain. Two possibilities are being considered. First, the amino-terminal one-half of the A59 transmembrane anchor is highly conserved among coronaviruses (Fig. 1), suggesting that this part of the anchor is functionally important. For example, if this region of the anchor is involved in membrane destabilization during fusion, the spike may be unable to resolve the hemifusion intermediate in its absence. The second possibility is that the body of the hydrophobic membrane-spanning domain harbors a “glycine hinge” similar

to that described for the VSV G protein (Cleverley and Lenard, 1998). The presence of the VSV glycine hinge was shown to markedly enhance the efficiency of membrane fusion by the G protein. The hinge is postulated to destabilize the hemifusion intermediate and thus aid in the formation of the fusion pore.

This study also demonstrates an absolute requirement for the *cys* region and extends the results of Bos *et al.* (1995) that showed a loss of fusion activity when the cytoplasmic tail, including the cytoplasmic portion of the *cys* domain, was replaced with the VSV G protein cytoplasmic tail. We speculated that the inability of the gD-1 transmembrane anchor chimeras to induce fusion was due to loss of the *cys* domain. However, restoration of the *cys* domain to a fusion-defective chimera (i.e., KC22*cys*) did not restore activity and led to the conclusion that the body of the A59 transmembrane domain contains one or more functional regions necessary for membrane fusion. The importance of the *cys* domain is further supported by experiments in which each of the four conserved cysteines in the domain was individually mutated. With the exception of C1304, which probably lies in the cytoplasmic tail of the protein, mutation of any one cysteine drastically reduced or abrogated fusion. Most pronounced were mutation of C1292 and C1296, both of which were quite sensitive to hydrophilic substitutions. Similar studies by Bos *et al.* showed that a spike protein with mutation of cysteine 1295, cysteine 1296, and phenylalanine 1297 also exhibited reduced fusion activity. If the *cys* domain exists as a helix, C1292 and C1296 would lie on a strongly hydrophobic face of the spike transmembrane anchor, which could explain the marked reduction in fusion when hydrophilic mutations are made at these two positions.

It is interesting to note that a single cysteine residue (Cys 1163) found in the ectodomain of the S2 subunit of the JHM spike protein has been implicated as a possible reactive thiol involved in conformational changes that occur during the fusion process (Gallagher, 1996). However, the A59 spike protein lacks this unpaired cysteine residue and yet is able to undergo pH-independent fusion. Due to the location of Cys 1163, it is plausible that this cysteine may be involved in early conformational changes required to bring about hemifusion. However, the location of the four invariant cysteines (C1287, C1292, C1296, and C1304) within the membrane-spanning region and cytoplasmic tail of the S2 subunit, together with the results of lipid-mixing experiments, suggests that these four cysteines are important during the later stages of fusion.

One role for cysteine residues in this region of the spike is to serve as sites for the covalent attachment of palmitic acid (Ponimaskin and Schmidt, 1995; Rose *et al.*, 1984; Schlesinger *et al.*, 1993; Schmidt, 1989; Sefton and Buss, 1987). The spike protein is palmitylated on the carboxyl-terminal S2 fragment (Niemann and Klenk,

1981; Schmidt, 1982; Sturman *et al.*, 1985; van Berlo *et al.*, 1987). Palmitate is generally attached to viral fusion proteins through a thioester bond involving cysteines that reside near the inner leaflet of the plasma membrane (Ponimaskin and Schmidt, 1995; Rose *et al.*, 1984; Schlesinger *et al.*, 1993; Schmidt, 1989; Sefton and Buss, 1987). All of the cysteine residues in the spike that potentially could be acylated are in the *cys* domain, suggesting that mutations to this region may be inhibiting fusion by interfering with palmitoylation of the spike protein. Since palmitoylation is known to affect a variety of processes mediated by or involving fusion proteins, including infection of cells, membrane fusion, and virus assembly (Glick and Rothman, 1987; Jin *et al.*, 1996; Melikyan *et al.*, 1997b; Naim *et al.*, 1992; Schroth-Diez *et al.*, 1998; Zurcher *et al.*, 1994), studies are in progress to identify the sites of acylation and determine the role that palmitoylation may play in spike protein function.

## MATERIALS AND METHODS

*Virus and cells.* Mouse hepatitis virus, strain A59, was grown on mouse DBT cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Wild-type vaccinia virus (strain WR) and the T7 RNA polymerase recombinant vaccinia virus, vTF7.3 (Fuerst *et al.*, 1986, 1987), were grown on COS-7 cells in DMEM containing 2% FBS.

*PCR mutagenesis.* Mutations in the transmembrane anchor and cytoplasmic tail of the A59 spike protein were generated using PCR techniques previously described (Chen and Przybyla, 1994; Li and Wilkinson, 1997). Mutagenic primers were purchased from Operon Technologies (Alameda, CA). Briefly, PCRs were done using the wild-type spike gene cDNA as template, a mutagenic forward primer, and a universal reverse primer. The product from this reaction was purified and used as a reverse mega-primer in a second PCR that contained the wild-type spike gene cDNA as template and universal forward primer. The final PCR product from this three-primer procedure, comprising the entire transmembrane domain and cytoplasmic tail, was digested with *Nde*I and *Bam*HI and subcloned into the wild-type A59 spike gene cDNA. In some cases, a four-primer mutagenesis procedure was used. In this technique, separate forward and reverse mega-primers were made using the universal forward primer and a reverse mutagenic primer, and a forward mutagenic primer and a universal reverse primer, respectively. These two mega-primers were mixed and used in a PCR together with the universal forward and reverse primers, and the final PCR product was digested with *Nde*I and *Bam*HI and subcloned into the wild-type spike cDNA.

*$\beta$ -Galactosidase-fusion assay.* The fusion assay described by Nussbaum *et al.* (1994) was adapted for use with the MHV spike protein. DBT cells were infected with

vTF7.3, a recombinant vaccinia virus expressing the T7 RNA polymerase (Fuerst *et al.*, 1986, 1987), at 10 PFU per cell for 1 h at 37°C. The monolayers were washed once with PBS and then transfected with 10  $\mu$ g of plasmid DNA containing the spike gene under control of the T7 RNA polymerase promoter and EMC IRES sequence. Reporter cells were infected with wild-type vaccinia, strain WR, and subsequently transfected with plasmid DNA containing the *lacZ* gene under control of the T7 promoter and EMC IRES sequence. Both groups of cells were removed with trypsin at 4 h after transfection and washed in DMEM–2% FBS. Equal numbers of spike-expressing cells and *lacZ* reporter cells were mixed and added to wells of a 96-well plate and incubated for 1 to 4 h at 37°C. After incubation, the cells were lysed by adding Triton X-100 in PBS to a final concentration of 1%.  $\beta$ -galactosidase activity was quantitated by mixing aliquots of the cell lysates with the  $\beta$ -galactosidase substrate CPRG (Boehringer Mannheim) and measuring the OD at 570 nm.

In some experiments, the spike gene plasmids were transfected into vTF7.3-infected DBT cells grown in 48-well plates. Fusion was measured by adding the *lacZ* reporter cells to the wells at a 1:1 ratio without trypsinizing or counting the spike-expressing cells.

*Immunoprecipitation.* DBT cells were infected with vTF7.3 for 60 min and then transfected with plasmid DNA encoding wild-type or mutant spike proteins. At 3 h posttransfection, the DNA was removed and the cells were refed with DMEM containing 2% FBS. At 7.5 h posttransfection, the cells were incubated in methionine-free DMEM for 30 min and then pulse-labeled with 200  $\mu$ Ci [<sup>35</sup>S]-methionine (Expre<sup>35</sup>S<sup>35</sup>S, Dupont NEN) for 60 min. In some cases, the cells were chased in DMEM–2% FBS containing a 10-fold excess of unlabeled methionine. The cells were lysed in 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1% Triton X-100 and clarified by centrifugation. The spike protein was immunoprecipitated using a polyclonal rabbit anti-MHV serum as described previously (Gombold *et al.*, 1993) and examined by SDS–PAGE.

To examine spike protein transport, wild-type or mutant spike proteins were immunoprecipitated as described above and treated with endoglycosidase H (BMB) prior to electrophoresis.

*Immunofluorescence.* Expression of the spike protein on the cell surface was detected by immunofluorescence essentially as described previously (Gombold *et al.*, 1993). Briefly, wild-type or mutant spike proteins were expressed in DBT cells grown in two-chamber LabTek (Nunc) slides using vTF7.3 as described above. Six to 8 h posttransfection, the cells were washed and incubated with AO4, a goat anti-spike serum generously provided by Dr. Kathryn Holmes (University of Colorado Health Science Center, Denver, CO), followed by FITC rabbit anti-goat F(ab')<sub>2</sub> fragments. To detect intracellular spike



proteins, the cells were permeabilized with cold ( $-20^{\circ}\text{C}$ ) methanol prior to incubation with AO4 serum.

**Lipid mixing assay.** The ability of spike mutants to induce hemifusion but not full fusion was determined using a modified lipid mixing assay (Bagai and Lamb, 1996; Cleverley and Lenard, 1998; Kemble *et al.*, 1994). DBT cells were infected with vTF7.3 and subsequently transfected with either wild-type or mutant Spike protein genes as described above. Four hours following transfection, the cells were washed with PBS and then incubated in PBS containing  $2\ \mu\text{M}$  CMFDA (Molecular Probes, Eugene, OR) at  $37^{\circ}\text{C}$ . A separate flask of uninfected, nontransfected, DBT cells was incubated in PBS containing  $2.0\ \mu\text{g}$  R18 per milliliter. Both groups of cells were washed extensively to remove unincorporated fluorescent label, trypsinized, and counted. The CMFDA-labeled (spike-expressing) cells were mixed with a five-fold excess of R18 labeled cells, incubated at  $37^{\circ}\text{C}$ , and then examined by fluorescence microscopy. Hemifusion would result in the transfer of rhodamine from the R18-labeled cells to the green fluorescent, CMFDA-labeled cells.

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