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Conformational Changes in the Spike Glycoprotein of Murine Coronavirus Are Induced at 37°C either by Soluble Murine CEACAM1 Receptors or by pH 8

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The spike glycoprotein (S) of the murine coronavirus mouse hepatitis virus (MHV) binds to viral murine CEACAM receptor glycoproteins and causes membrane fusion. On virions, the 180-kDa S glycoprotein of the MHV-A59 strain can be cleaved by trypsin to form the 90-kDa N-terminal receptor-binding subunit (S1) and the 90-kDa membrane-anchored fusion subunit (S2). Incubation of virions with purified, soluble CEACAM1a receptor proteins at 37°C and pH 6.5 neutralizes virus infectivity (B. D. Zelus, D. R. Wessner, R. K. Williams, M. N. Pensiero, F. T. Phibbs, M. deSouza, G. S. Dveksler, and K. V. Holmes, J. Virol. 72:7237–7244, 1998). We used liposome flotation and protease sensitivity assays to investigate the mechanism of receptor-induced, temperature-dependent virus neutralization. After incubation with soluble receptor at 37°C and pH 6.5, virions became hydrophobic and bound to liposomes. Receptor binding induced a profound, apparently irreversible conformational change in S on the viral envelope that allowed S2, but not S1, to be degraded by trypsin at 4°C. Various murine CEACAM proteins triggered conformational changes in S on recombinant MHV strains expressing S glycoproteins of MHV-A59 or MHV-4 (MHV-JHM) with the same specificities as seen for virus neutralization and virus-receptor activities. Increased hydrophobicity of virions and conformational change in S2 of MHV-A59 could also be induced by incubating virions at pH 8 and 37°C, without soluble receptor. Surprisingly, the S protein of recombinant MHV-A59 virions with a mutation, H716D, that precluded cleavage between S1 and S2 could also be triggered to undergo a conformational change at 37°C by soluble receptor at neutral pH or by pH 8 alone. A novel 120-kDa subunit was formed following incubation of the receptor-triggered S_{A59}H716D virions with trypsin at 4°C. The data show that unlike class 1 fusion glycoproteins of other enveloped viruses, the murine coronavirus S protein can be triggered to a membrane-binding conformation at 37°C either by soluble receptor at neutral pH or by alkaline pH alone, without requiring previous activation by cleavage between S1 and S2.

As an initial step in virus replication, specialized attachment proteins on virions bind to specific receptors on host cell membranes. The specificity of virus-receptor interactions frequently determines which cells, tissues, and species are susceptible to virus infection. On binding to its specific receptor under optimal conditions of pH and temperature, the viral attachment glycoprotein undergoes one or more programmed conformational changes that exposes a hydrophobic fusion peptide which mediates fusion of the viral envelope with host cell membranes, releasing the viral nucleocapsid into the cytoplasm (24, 61). Class I viral fusion glycoproteins have the general structure of the HA protein of influenza A virus, consisting of an N-terminal receptor binding domain followed by an exposed protease cleavage site, a fusion domain containing several heptad repeats, and transmembrane and cytoplasmic domains (23).

So-called pH-independent fusion occurs when the viral envelope can fuse directly with the plasma membrane at neutral pH. Conformational changes in spike glycoproteins of some large minus-strand RNA viruses including paramyxoviruses such as simian virus 5 (SV5) and respiratory syncytial virus, and retroviruses including human immunodeficiency virus type 1 (HIV-1) and avian and murine leukemia viruses are triggered by binding at 37°C to specific receptors on the plasma membrane (1, 9, 24, 61). These pH-independent viruses cause cell-to-cell fusion, forming multinucleated syncytia in infected cell cultures and tissues when viral spike proteins expressed on the membrane of a cell are triggered to undergo a fusion-inducing conformational change by binding to a specific virus receptor on an adjacent cell. In contrast, the spike glycoproteins of other large, enveloped RNA viruses including influenza A virus, rabies virus, Ebola virus, and bunyavirus require an acidic environment to trigger the conformational changes in the viral fusion protein that lead to membrane fusion (16, 24, 44, 51, 61). Therefore, fusion of these viral envelopes occurs not at the plasma membrane but within the cell at endosomal membranes once the pH drops to 5.5. These pH-dependent or acid-dependent viruses do not induce the formation of multinucleated syncytia in cells and tissues at neutral pH.

This report describes conformational changes in the spike glycoprotein (S) of mouse hepatitis virus (MHV), a murine

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coronavirus, which are induced by incubation at 37°C either with specific virus receptor proteins at neutral pH or under alkaline conditions, at pH 8, without receptor proteins. Coronaviruses are large, enveloped viruses with plus-strand RNA genomes. The S glycoproteins of coronaviruses have both receptor binding and membrane-fusing activities. The S glycoprotein plays important roles in tissue tropism, cell fusion, and virulence. The receptors for MHV strains are murine glycoproteins in the carcinoembryonic antigen family of glycoproteins in the immunoglobulin (Ig) superfamily (10, 11, 62, 63). The MHV receptor glycoproteins are now called murine CEACAMs (CEA cell adhesion molecule; formerly called MHVR, biliary glycoprotein [Bgp], mmCGM, or CD66a) (4).

CEACAM1 is found in all mammals and plays important roles in differentiation, cell adhesion, immunoregulation, angiogenesis, tumor metastasis, and cell signaling (4, 22, 65). In rodents the CEACAM1 gene was duplicated, so that the CEACAM1 and CEACAM2 genes are adjacent on chromosome 7. CEACAM1 encodes the principal receptor glycoproteins for MHV, and the CEACAM2 protein has relatively weak MHV receptor activity (11, 39). There are two alleles for CEACAM1 in mice. Most strains of inbred mice are highly susceptible to MHV and are homozygous for the CEACAM1a allele. In contrast, adult SJL/J mice, which are highly resistant to infection with MHV (27, 53), are homozygous for the CEACAM1b allele (40). CEACAM1a and CEACAM1b differ principally in the N-terminal Ig-like domain (D1), to which the viral S glycoprotein binds (10). Alternative splicing of transcripts of CEACAM1a results in four isoforms composed of either two or four Ig-like domains linked by a transmembrane domain to either a long or a short cytoplasmic tail (36, 37). Each of the four isoforms of CEACAM1a can serve as a functional receptor for the hepatotropic and neurotropic A59 strain of MHV (MHV-A59) (10).

Isoforms of murine CEACAM1a, CEACAM1b, and CEACAM2 expressed as soluble anchorless proteins or Ig fusion proteins bind to MHV spike proteins at 4°C and neutralize the infectivity of virions at 37°C in a concentration-dependent manner (15, 42, 64). Soluble murine CEACAM1a proteins with four Ig-like domains (CEACAM1a[1–4]) or two Ig domains (CEACAM1a[1,4]) have 4- to 10-fold greater neutralizing activity for MHV-A59 than does the four-domain isoform of CEACAM1b (CEACAM1b[1–4]) (64). The two-domain isoform of CEACAM1b (CEACAM1b[1,4]) has little neutralizing activity. Unlike CEACAM1a glycoproteins, soluble CEACAM2[1,4] has little virus-neutralizing activity. MHV strains differ in their susceptibility to neutralization with these soluble receptor glycoproteins. The neurotropic MHV-4 (MHV-JHM) strain is neutralized by both two-domain and four-domain isoforms of CEACAM1a but not by isoforms of CEACAM1b (64). The molecular basis for selective receptor utilization by MHV strains and the mechanism for neutralization of MHV virions by soluble receptor glycoproteins have not been established.

When purified MHV-A59 virions are incubated at pH 8.0 and 37°C for 30 min, a conformational change in the S protein can be demonstrated by loss of an epitope recognized by one monoclonal antibody directed against S2 (60). In addition, viral infectivity is markedly reduced, a fraction of S1 detaches from the virions, and the S2-containing virions form large aggregates (57). Thus, the S glycoprotein of MHV can be induced to undergo a conformational change by incubation at 37°C either at pH 8.0 or with soluble CEACAM1a glycoproteins at neutral pH, and S proteins of various MHV strains differ in susceptibility to triggering of these conformational changes.

The present study was done to investigate the molecular mechanism mediating soluble murine CEACAM1a-induced neutralization of MHV and the effects of pH 8 on the viral S glycoprotein. We found that incubation of virions at 37°C either at pH 8.0 without receptor or at pH 6.5 with soluble receptor protein caused a marked increase in the hydrophobicity of the virions, as shown by association with liposomes in a flotation assay. This was associated with a conformational change in S2, which became susceptible to degradation by trypsin at 4°C. Murine CEACAM1a and CEACAM1b receptor proteins differed in their ability to induce conformational changes in the spike glycoprotein of MHV-A59. Thus, the spike glycoprotein of the plus-strand RNA coronavirus MHV can undergo conformational changes in response to receptor and/or pH 8.0 at 37°C in a manner that in some ways resembles the receptor-induced or low-pH-induced conformational changes in the class I fusion glycoproteins of some RNA viruses and retroviruses.

MATERIALS AND METHODS

Cells and viruses. The 17 Cl 1 line of spontaneously transformed BALB/c 3T3 cells was used for propagation and plaque assay of murine coronavirus MHV-A59 as previously described (50). The S_{A59}H716D mutant of MHV-A59 was constructed by targeted RNA recombination of MHV-A59 (30, 31, 35) (S. T. Hingley et al., personal communication). Recombinant MHV-A59 strains containing the MHV-A59 spike glycoprotein (S_{A59}R) or the MHV-4 spike glycoprotein (S_4R) in place of the MHV-A59 spike glycoprotein were constructed by targeted RNA recombination and sequenced to ensure that no other mutations had been introduced (46).

To prepare radiolabeled virus, [^{3}H]uridine (New England Nuclear; 50 Ci/mmol) was added to the growth medium to a final concentration of 20 μCi/ml at 1 hr after inoculation with 3 to 10 PFU/cell. Virions were purified from supernatant medium 24 h after inoculation and concentrated by sucrose density gradient ultracentrifugation at pH 6.5 as previously described (13). We previously showed that the infectivity of viruses is stable at pH 6.5 but is reduced at pH 7.5 or 8 (57). The virus band at 1.16 to 1.18 g/ml was dialyzed against BTSG buffer (25 mM BisTris, 150 mM sodium chloride, 5% glycerol) (pH 6.5) and stored at −80°C. The purified [^{3}H]uridine-labeled MHV-A59 had a titer of 1×10^8 to 2×10^8 PFU/ml and a specific radioactivity of 0.1 cpm/PFU.

Soluble murine CEACAM1a, CEACAM1b, and CEACAM2 glycoproteins. Anchorless soluble murine CEACAM1a and CEACAM1b glycoproteins with four Ig-like domains (called smCEACAM1a[1–4] and smCEACAM1b[1–4] respectively) and soluble murine CEACAM2 with two Ig-like domains (called smCEACAM2[1,4]) were engineered to have a thrombin cleavage site followed by a six-histidine tag on the carboxyl terminus. These soluble glycoproteins were expressed in SF9 cells by using recombinant baculovirus and purified to apparent homogeneity from the supernatant medium by nickel affinity and ion-exchange chromatography as previously described (64).

Antibodies. Specific antisera that recognized the murine CEACAM glycoproteins or viral spike glycoproteins were used in solid-phase and immunoblotting experiments. Polyclonal rabbit antibody 649 was raised by immunization of a rabbit with purified smCEACAM1a[1–4]. Anti-CEACAM1a MAb-CC1 recognizes an epitope in the N-terminal domain (D1) of murine CEACAM1a proteins and blocks binding of MHV-A59 virions to these receptor proteins (12). AO4 is a polyclonal goat antibody directed against spikes purified by sucrose density gradient ultracentrifugation from MHV-A59 virions disrupted with 1% NP-40 (56). Monoclonal antibodies A1.9, which detects an epitope in S1 of MHV-A59 (18), and 5B19 and 5B97.3, which detect epitopes in S2 of MHV-A59 (59), were kindly provided by John Fleming, University of Wisconsin, and Michael Buchmeier, Scripps Institute. Control antibodies included normal rabbit and goat sera and an IgG1 monoclonal antibody against an irrelevant antigen, cholera toxin.

Immunoblots performed as previously described (64) were visualized with the Renaissance ECL kit (New England Nuclear).

Preparation of unilamellar vesicles (liposomes). Equimolar amounts of phosphatidylethanolamine, phosphatidylcholine, and cholesterol (Avanti Polar Lipids, Alabaster, Ala.) were dried from chloroform into a thin film and rehydrated in BTSG buffer at a final concentration of 15 mM. Aliquots were stored under N_2 at −80°C. After three freeze-thaw cycles, liposomes were extruded by 21 passes through a 100-nm-pore-size polycarbonate membrane as specified by the manufacturer (Avanti Polar Lipids) and stored under N_2 at 4°C. The liposomes were used within one week of extrusion.

Liposome flotation assay. Purified virions (2×10^6 PFU) were incubated at 4 or 37°C for 30 min with 20 μl of liposomes (15 mM) with either purified soluble murine CEACAM glycoproteins (1.5 μM) at pH 6.5 or buffer alone at pH 6.5. In parallel reactions, 1/10 volume of 1 M Tris (pH 8) was added to the virus-liposome mixture to bring the pH of the reaction mixture to pH 8.0. Immediately after incubation, reaction mixtures were diluted with 400 μl of ice-cold 50% sucrose in BTSG buffer (pH 6.5), layered beneath a 4.6-ml 10 to 40% sucrose step gradient, and ultracentrifuged at 150,000 × *g* for 2.5 h at 4°C. Ten fractions (0.5 ml) were collected from the top, and 0.1 ml of each fraction was applied under vacuum to an Immobilon-P membrane in a 96-well dot blot apparatus. The sheet was blocked overnight with 5% milk in Tris-buffered saline (TBS), probed with goat AO4 anti-spike at a dilution of 1:1000, and visualized with a Renaissance ECL kit.

Electron microscopy. MHV-A59 virions in BTSG buffer (pH 6.5) were incubated with liposomes for 30 min at 4 or 37° C, with or without smCEACAM1a[1–4], placed on carbon-coated Formvar-covered copper grids, and fixed with 1% glutaraldehyde. The grids were negatively stained with 2% phosphotungstic acid and examined under a Phillips 400 electron microscope.

Protease sensitivity assay. Sucrose density gradient-purified virions (2×10^6 PFU) were incubated for 30 min at 4 or 37°C at pH 6.5 with smCEACAM glycoproteins (1.5 μM) or at pH 6.5 or pH 8.0 without smCEACAM proteins and then rapidly chilled to 4°C. Trypsin-TPCK (Worthington Biochemical, Freehold, N.J.) was added to a final concentration of 10 μg/ml, and samples were incubated at 4°C for 20 min. Soybean trypsin inhibitor (50 μg/ml; Worthington Biochemical) and Laemmli buffer were added to stop protease activity and solubilize proteins. Viral proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8% polyacrylamide), immunoblotted with goat AO4 anti-spike antibody, anti-S1 MAb-A1.9, anti-S2 MAb-5B1.9 or MAb-5B97.3, or a control MAb, and visualized as previously described (64).

RESULTS

We previously showed that incubation at 37°C and pH 8.0 (57) or with purified, soluble murine CEACAM1a glycoproteins at 37°C and pH 6.5 neutralizes the infectivity of MHV-A59 virions in a concentration-dependent manner (64). From 2×10^{-10} to 8×10^{-10}M CEACAM1a[1–4] or CEACAM1a[1,4] neutralizes the infectivity of approximately 50% of 5,000 PFU of MHV-A59 at 37°C, while 5×10^{-9} M receptor protein neutralizes 100% of viral infectivity. For the experiments in this study, the ratio of smCEACAM1 glycoprotein to infectious virus used in each sample was held at 2×10^{-6} M/2×10^6 PFU. This would provide more than enough of the smCEACAM1a[1–4] or smCEACAM1a[1,4] glycoprotein to neutralize the virus. However, this ratio of smCEACAM2[1,4] protein to virus would not neutralize MHV-A59, and this ratio of smCEACAM1b[1–4] to virus would not neutralize MHV-4 (64).

A hydrophobic domain that interacts with liposomes is exposed by incubation of virions at 37°C with soluble receptor at pH 6.5 or at pH 8.0 without receptor. Current models for the molecular mechanism of receptor-dependent, pH-independent fusion with the plasma membrane of the envelopes of paramyxoviruses, avian and murine retroviruses, and HIV-1 suggest that binding of the spike protein to its specific receptor (and coreceptor for HIV-1) on the plasma membrane induces conformational changes within the spike protein that expose a previously hidden hydrophobic fusion domain that inserts into the plasma membrane to initiate fusion (61).

To determine if incubation at 37°C and pH 8.0 or at pH 6.5 with smCEACAM1a[1–4] glycoprotein that neutralized the infectivity of MHV-A59 (64) was associated with the exposure of a hydrophobic domain on the coronavirus virion, we used liposome flotation assays (9, 25, 49). MHV virions were incubated for 30 min at 4 or 37°C with soluble receptor proteins at pH 6.5 or without receptor proteins at pH 6.5 or 8.0 in the presence of unilamellar liposomes consisting of phosphatidylcholine, phosphatidylethanolamine, and cholesterol (1:1:1) and then layered beneath a sucrose density gradient. Any virions in which a hydrophobic domain was exposed would bind to the liposomes and float with the liposomes to the top of the gradient during ultracentrifugation, while virions without an exposed hydrophobic domain would remain near the bottom of the gradient. The locations of virions and smCEACAM1a[1–4] glycoprotein in the gradient were determined by immunoblotting gradient fractions with polyclonal antibody AO4 to MHV-A59 spikes or polyclonal rabbit 649 anti-CEACAM1a antibody, respectively (Fig. 1A) or by monitoring the radiolabel in experiments using [^{3}H]uridine-labeled virions (Fig. 1B).

MHV-A59 virions incubated with or without smCEACAM1a [1–4] for 30 min at 4°C and pH 6.5 did not associate with liposomes, and the virions remained at the bottom of the gradient. However, virions incubated at 37°C and pH 6.5, with smCEACAM1a[1–4] became hydrophobic, associated with liposomes, and moved to the top of the gradient, as shown by the presence of the viral S antigen in fractions near the top of the gradient (Fig. 1A). Shifting the temperature of incubation from 4 to 37°C also led to association of the virions with liposomes (data not shown). A fraction of the receptor glycoprotein moved from the bottom to the top of the gradient in association with the virions and liposomes, as shown by detection with anti-CEACAM1a antibody 649 (Fig. 1A). MHV-A59 virions incubated with liposomes at 4 or 37°C and pH 6.5 in the absence of receptor protein remained at the bottom of the gradient (data not shown). Figure 1B shows that not only the viral glycoproteins but also the intact virions moved to the top of the gradient with liposomes following incubation of [^{3}H]uridine-labeled virions with liposomes at 37°C and pH 6.5. In contrast, incubation of the radiolabeled virions with liposomes and smCEACAM1a[1–4] at pH 6.5 and 4°C apparently did not expose a hydrophobic domain, since virions did not associate with liposomes, but remained at the bottom of the gradient. Electron microscopy of virions incubated with smCEACAM1a [1–4] at pH 6.5 showed that virions bound to liposomes at 37°C (Fig. 2) but not at 4°C (data not shown). Virions did not bind to liposomes in the absence of smCEACAM1a[1–4] (data not shown). Fusion of viral envelopes with the protein-free liposomes was not observed. Thus, immunoblotting, radioisotopic labeling, and electron microscopy showed that virions became hydrophobic and bound to liposomes after incubation with smCEACAM1a[1–4] at pH 6.5 and 37°C but not 4°C.

Interestingly, incubation of MHV-A59 virions at pH 6.5 and either 4 or 37°C with the same amount of smCEACAM2[1,4] caused only minimal association of virions with liposomes (Fig. 1A). This observation correlates well with the failure of this ratio of smCEACAM2[1,4] to virus to neutralize MHV-A59 virions (64). Thus, the liposome flotation assay demonstrated

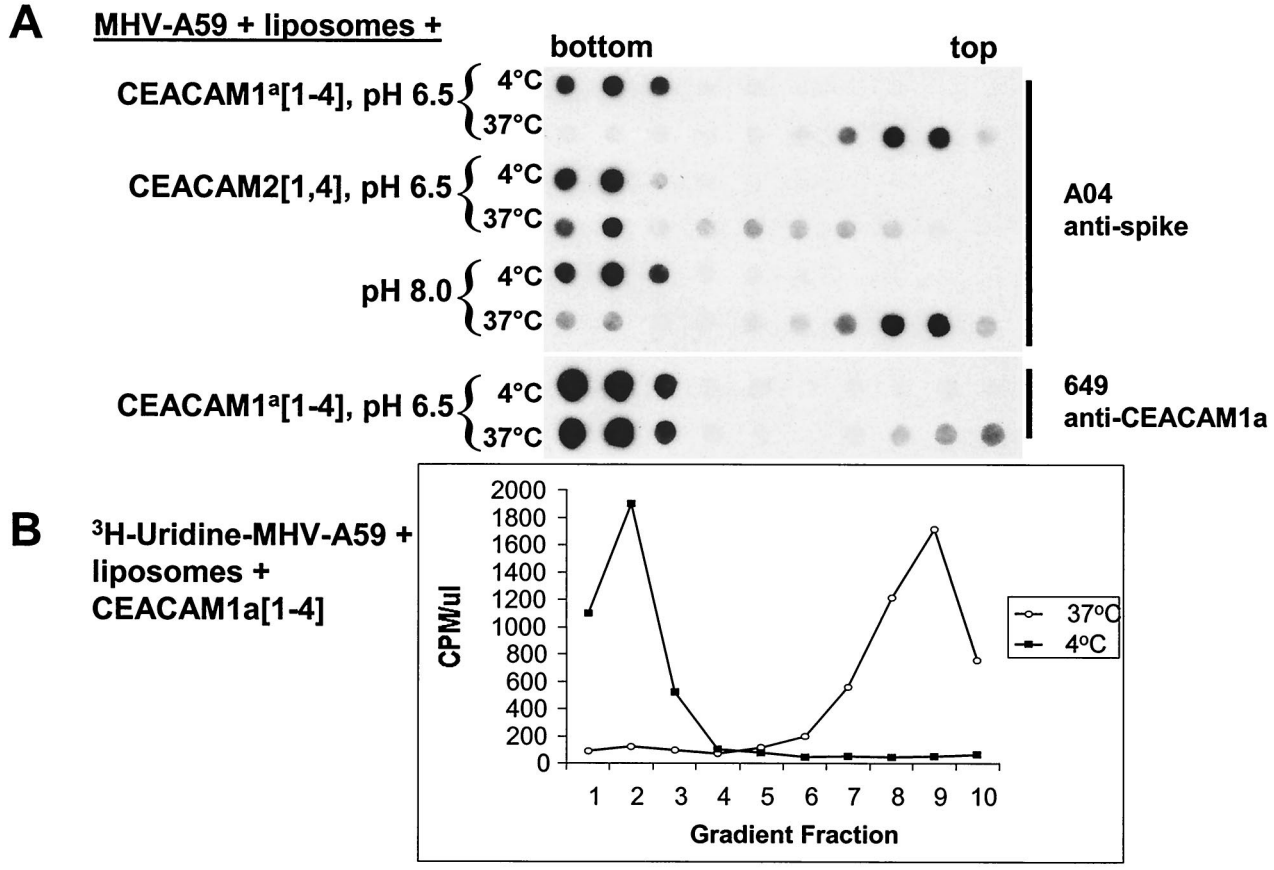

FIG. 1. Soluble CEACAM glycoproteins or alkaline pH at 37°C causes association of virions with liposomes. (A) MHV-A59 virions were incubated with liposomes for 30 min at 4 or 37°C and pH 6.5 with soluble murine CEACAM1a[1–4] or soluble murine CEACAM2[1,4] or with buffer at pH 8.0. The mixtures were chilled to 4°C, loaded into the bottom of sucrose density gradients, and ultracentrifuged as described in Materials and Methods. Gradient fractions were applied to polyvinylidere difluoride membranes; viral spike antigen was detected with goat anti-S antibody, and receptor antigen was detected with goat anti-murine CEACAM1a antibody. (B) In parallel experiments, the association of [^{3}H]uridine-labeled MHV-A59 virions with liposomes after incubation at pH 6.5 for 30 min at 4 or 37°C was determined by measuring the amount of radiolabel in each gradient fraction.

that the receptor-induced changes in MHV-A59 virions that led to increased hydrophobicity and association of virions with liposomes were specific for murine CEACAM1a proteins.

Based on our previous observation that MHV-A59 virions aggregated after incubation at pH 8.0 and 37°C in the absence of receptor (57, 60), we tested whether MHV-A59 virions would become hydrophobic after incubation at pH 8.0 in the absence of receptor glycoprotein. Blotting with anti-S antibody also showed the temperature dependence of the conformational change. Virions incubated with liposomes at pH 8.0 and 4°C in the absence of receptor glycoprotein did not associate with liposomes, but virions incubated with liposomes at pH 8.0 and 37°C became hydrophobic and floated with the liposomes to the top of the gradient (Fig. 1A).

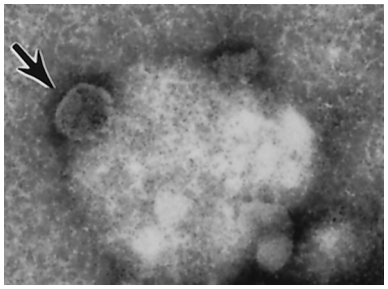

FIG. 2. Binding of MHV-A59 virions to liposomes after incubation at 37°C with soluble CEACAM1a[1–4] glycoprotein. Virions were incubated with soluble receptor protein for 30 min at 37°C and pH 6.5, immediately placed on a carbon-coated Formvar-covered grid, fixed in 1% glutaraldehyde, and negatively stained with 2% phosphotungstic acid. The arrow shows a virion adsorbed to a liposome. Magnification, ×79,400.

Protease sensitivity assays demonstrate conformational changes in S2 induced at 37°C by soluble murine CEACAM1a proteins at pH 6.5 or by pH 8.0 without receptor. To determine whether the temperature-dependent neutralization of MHV-A59 that is induced either by pH 8.0 (57) or by incubation with smCEACAM1a receptor glycoproteins at pH 6.5 (64) was associated with conformational changes in the viral spike glycoprotein, we examined the susceptibility to trypsin degradation at 4°C of virion-associated spike glycoprotein after virions had been incubated at pH 6.5 with soluble receptor at 4 or 37°C (9, 17). We had previously shown that treatment of MHV-A59

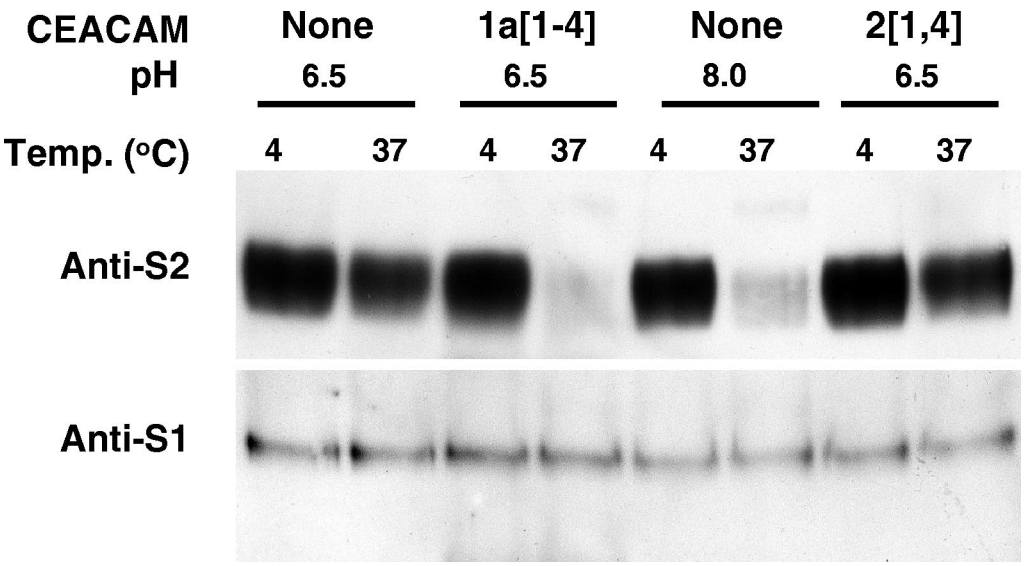

FIG. 3. Conformational changes in the S2 domain of MHV-A59 spike glycoprotein induced at 37°C by CEACAM1a at pH 6.5 or by the presence of pH 8.0 without receptor. A trypsin sensitivity assay was used to demonstrate conformational changes in the viral spike protein. Gradient-purified MHV-A59 virions were incubated at 4 or 37°C for 30 min at pH 6.5 with either soluble murine CEACAM1a[1–4] or CEACAM2[1,4] or without receptor proteins at pH 6.5 or 8.0. The samples were then incubated at 4°C for 20 min with trypsin-TPCK, subjected to SDS-PAGE, and immunoblotted with MAbs specific for the receptor binding S1 domain of the spike (MAb A1.9) or for the carboxyl-terminal S2 domain of the spike (MAb 5B93.7) which has been associated with membrane fusion.

virions with trypsin-tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) at 4°C cleaves the 180-kDa spike protein to form two 90-kDa subunits, the N-terminal S1 subunit, which binds to receptors, and the C-terminal S2 subunit, which is associated with membrane fusion. S1 and S2 remain noncovalently associated on virions and are resistant to further proteolysis despite the presence of numerous potential trypsin cleavage sites in both proteins (55). Immunoblotting with anti-S1 or anti-S2 MAb showed that incubation of virions at pH 6.5 and 4 or 37°C followed by treatment with trypsin-TPCK for 20 min at 4°C and pH 6.5 completely cleaved the 180-kDa spike protein to S1 and S2 without further cleavage of these two 90-kDa glycoproteins (Fig. 3). In the absence of trypsin treatment, virions incubated at pH 6.5 and 4 or 37°C and analyzed by immunoblotting showed some uncleaved 180-kDa S protein as well as a 90-kDa band containing both S1 and S2 (data not shown). Incubation of MHV-A59 virions at pH 6.5 and 37°C with soluble murine receptor glycoproteins CEACAM1a[1–4] (Fig. 3) or the corresponding two-domain isoform smCEACAM1a[1,4] (data not shown) followed by trypsin-TPCK digestion at 4°C resulted in degradation of the S2 protein but not of the S1 protein. Incubation of MHV-A59 virions with smCEACAM1a[1–4] receptor glycoprotein at 4°C did not make the S2 protein susceptible to degradation by trypsin-TPCK at 4°C (Fig. 3).

The receptor-induced conformational changes in the viral S2 protein at 37°C and pH 6.5, which made S2 susceptible to trypsin degradation, were specific for CEACAM1a receptor glycoproteins. Incubation of MHV-A59 virions at pH 6.5 and either 4 or 37°C with soluble CEACAM2[1,4], which was previously shown to neutralize MHV-A59 virions very poorly (64), made only a small amount of the viral S2 protein susceptible to trypsin cleavage (Fig. 3). The protease sensitivity assay also detected a conformational change in S of MHV-A59 that was induced by pH 8.0 and 37°C and permitted the degradation of S2 on subsequent incubation with trypsin at 4°C (Fig. 3).

These experiments showed that binding of smCEACAM1a receptor glycoproteins to the N-terminal S1 subunit of the spike glycoprotein (12) at pH 6.5 and 37°C specifically induced a major conformational change in S2. It is likely that the triggered S2 could not be detected by immunoblotting because the protein was degraded by incubation with trypsin at 4°C, but an alternative explanation of the data would be that triggering by receptor or pH 8.0 caused a conformational change that destroyed the epitope in S2 recognized by the MAb. Apparently the same conformational change in S2 could be induced in the absence of any receptor glycoprotein by incubation of virions at pH 8.0 and 37°C. No conformational changes in S were detected after incubation of virions at 4°C with soluble receptor or at pH 8.0. The conformational changes in S induced at 37°C by soluble receptor or pH 8.0 were apparently irreversible, since cooling the treated virions to 4°C and incubating for a prolonged time did not prevent subsequent degradation of S2 by trypsin-TPCK at 4°C.

S proteins of different MHV strains vary in susceptibility to triggering of conformational changes by CEACAM1a or CEACAM1b proteins. We previously showed that MHV-A59 is neutralized at 37°C and pH 6.5 by purified, soluble CEACAM1a[1–4] more efficiently than by CEACAM1b[1–4] and that MHV-4 (MHV-JHM) is neutralized by purified, soluble CEACAM1a but not by soluble CEACAM1b glycoproteins (64). To explore possible virus strain-specific differences in receptor- induced conformational changes in the spike protein, we used two isogenic recombinant MHV-A59 strains that differed only in their spike glycoprotein. Using targeted RNA recombination with the Alb-4 mutant of MHV-A59 (30, 35), the spike gene from either MHV-A59 or MHV-4 was substi-

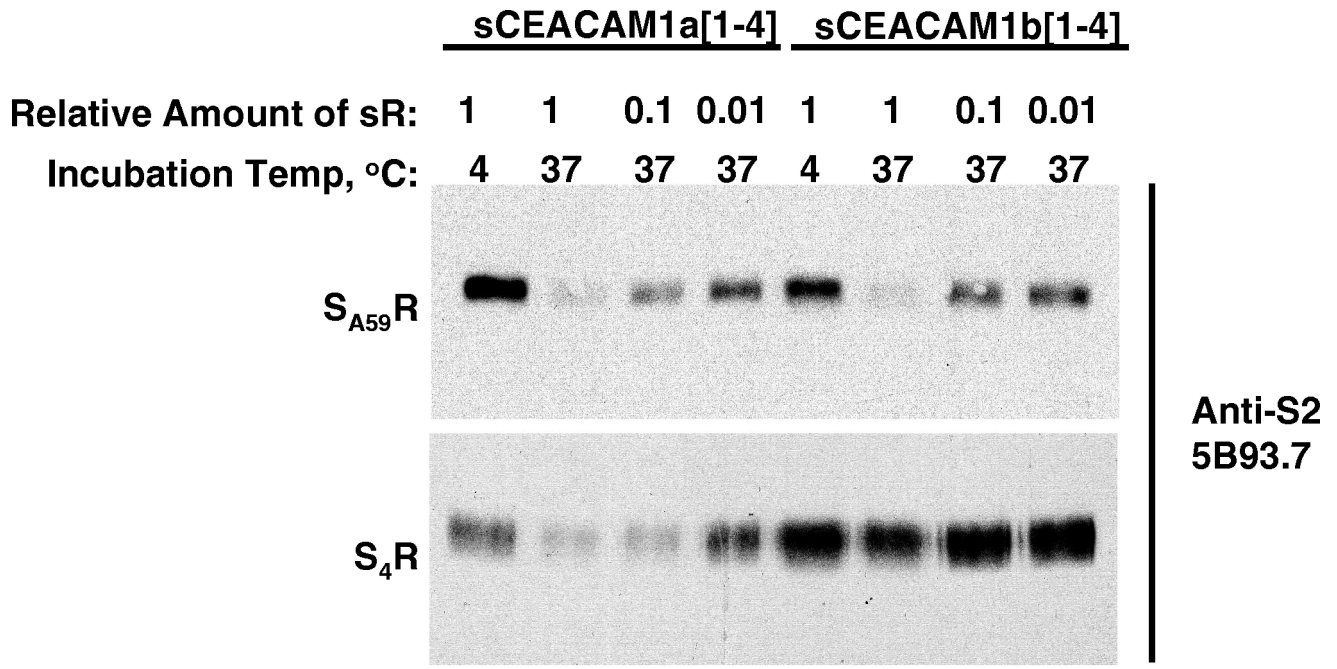

FIG. 4. Recombinant MHV-A59 viruses expressing the spike glycoproteins of MHV-A59 or MHV-4 differ in susceptibility to conformational changes in S2 induced by soluble murine CEACAM1a or CEACAM1b glycoproteins. Isogenic recombinant MHV-A59 viruses that express either the spike glycoprotein of MHV-A59 ($S_{A59}R$) or MHV-4 (S_4R) were prepared by targeted RNA recombination (30). The virions were incubated with different concentrations of purified, soluble murine CEACAM1a[1–4] (cloned from MHV-susceptible mice) or CEACAM1b[1–4] receptor glycoproteins (cloned from MHV-resistant SJL mice [10]) at pH 6.5 for 30 min at the temperatures indicated and then incubated for 20 min at 4°C with trypsin. The S2 glycoproteins were detected by immunoblotting with anti-S2 MAb 5B93.7.

tuted for the spike gene in the genome of MHV-A59 (46). The recombinant MHV-A59 strain with the MHV-A59 spike protein is called $S_{A59}R$, and the recombinant MHV-A59 strain with the MHV-4 spike protein in the MHV-A59 genome is called S_4R. The $S_{A59}R$ and S_4R viruses were incubated with different amounts of purified smCEACAM1a[1–4] or smCEACAM1b[1–4] at 4 or 37°C for 30 min before being exposed for 20 min to trypsin-TPCK at 4°C. The viral proteins were then analyzed by SDS-PAGE and immunoblotted with anti-S2 MAb 5B93.7. As anticipated, at 37°C and pH 6.5, smCEACAM1a[1–4] induced a conformational change in the MHV-A59 spike in the $S_{A59}R$ virus that made the S2 protein susceptible to degradation by trypsin-TPCK (Fig. 4). smCEACAM1b[1–4] also triggered the conformational change in S2 at 37°C but did so less efficiently than did smCEACAM1a [1–4] (Fig. 4). In marked contrast, although the S_4R virus that contains the spike protein from MHV-4 (MHV-JHM) was also efficiently triggered by incubation with smCEACAM1a[1–4] at 37°C and pH 6.5, no conformational change was induced in the MHV-4 spike protein in S_4R virions by smCEACAM1b[1–4] at 37°C and pH 6.5. Thus, the different neutralization activities of smCEACAM1a[1–4] and CEACAM1b[1–4] for MHV-A59 and MHV-4 virions at 37°C and pH 6.5 that were described previously (64) correlate well with the differences in the abilities of these receptor proteins to trigger conformational changes in the S2 glycoproteins of recombinant viruses containing the S proteins of the two virus strains.

Significance of S1-S2 cleavage for receptor-induced or pH 8-induced conformational changes in S2 at 37°C. For influenza A virus, cleavage of the HA_0 glycoprotein to generate the HA_1 and HA_2 subunits is a prerequisite for the subsequent pH 5.5-induced conformational change that exposes the hydrophobic fusion peptide at the N terminus of HA_2. The cleavage of the MHV spike glycoprotein between the S1 and S2 domains is an important determinant of the extent of virus-induced cell fusion (13, 20) but is not essential for virus infectivity (5). A recombinant virus (S_{A59}H716D) created by targeted RNA recombination has the entire genome of MHV-A59 except for a single H716D substitution adjacent to the S1-S2 cleavage site in the viral spike glycoprotein (20). As a result of this mutation, the 180-kDa spike glycoprotein on S_{A59}H716D virions is resistant to cleavage by trypsin. We used the S_{A59}H716D virus to investigate whether cleavage of the viral spike protein is a prerequisite for temperature-dependent, receptor- or pH 8-induced conformational changes in S2.

A liposome flotation assay of the S_{A59}H716D mutant virus (Fig. 5) showed several differences from MHV-A59. Incubation at 37°C and pH 6.5 without receptor caused a small increase in hydrophobicity of virions relative to virions held at 4°C. In contrast, wild-type MHV-A59 virions remained at the bottom of the gradients following incubation at pH 6.5, at both 4 and at 37°C (data not shown). Significantly increased hydrophobicity of S_{A59}H716D virions resulted from incubation at 37°C either with smCEACAM1a[1–4] at pH 6.5 or at pH 8.0 without receptor protein (Fig. 5).

Surprisingly, a trypsin sensitivity assay of the S_{A59}H716D mutant virus (Fig. 6) showed that after incubation at pH 6.5 and 4 or 37°C, a minor fraction of the spike glycoprotein of the S_{A59}H716D virus could be proteolytically cleaved by trypsin at 4°C to yield a novel 120-kDa subunit that was detected by

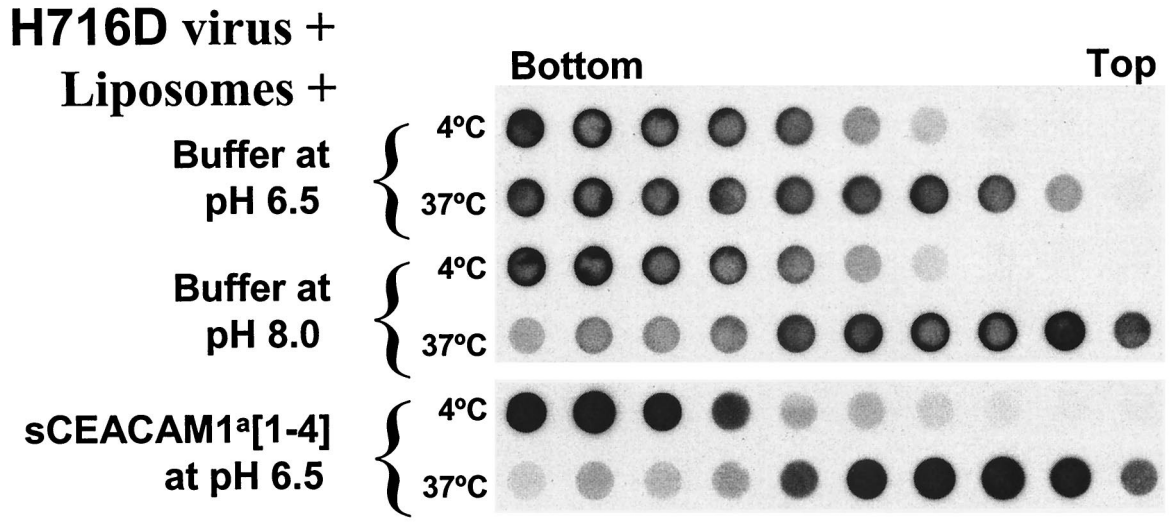

FIG. 5. A mutant MHV-A59 strain, S_{A59}H716D, that is not cleaved between S1 and S2 nevertheless exposes a hydrophobic domain and associates with liposomes after incubation at pH 6.5 with soluble CEACAM1a[1–4] glycoprotein or alkaline pH at 37°C but not at 4°C. A mutant MHV-A59 virus with a single mutation, H716D, in the viral spike glycoprotein that prevents protease cleavage at the S1-S2 junction was constructed by targeted RNA recombination (20, 26, 30, 35; Hingley, personal communication). S_{A59}H716D virions were incubated with liposomes for 30 min at 4 or 37°C and pH 6.5 with soluble murine CEACAM1a[1–4] or with buffer at pH 6.5 or 8.0 and then analyzed by sucrose density gradient ultracentrifugation as described in the legend to Fig. 1. Viral spike antigen in each gradient fraction was detected with polyclonal goat A04 anti-S antibody.

MAbs to both S1 and S2. When S_{A59}H716D virions were incubated at 37°C and pH 6.5 with smCEACAM1a[1–4] or at pH 8.0 without receptor, the S glycoprotein became highly susceptible to cleavage with trypsin-TPCK at 4°C to yield the 120-kDa glycoprotein, without further degradation of S2 as seen with wild-type MHV-A59 and S_{A59}R, (Fig. 3 and 4, respectively). These data, together with the liposome flotation data for S_{A59}H716D (Fig. 4), show that treatment of S_{A59}H716D virions at 37°C with smCEACAM1a[1–4] receptor at pH 6.5 or at pH 8.0 in the absence of receptor triggered a limited conformational change in the uncleaved 180-kDa viral spike glycoprotein. This limited conformational change ex-

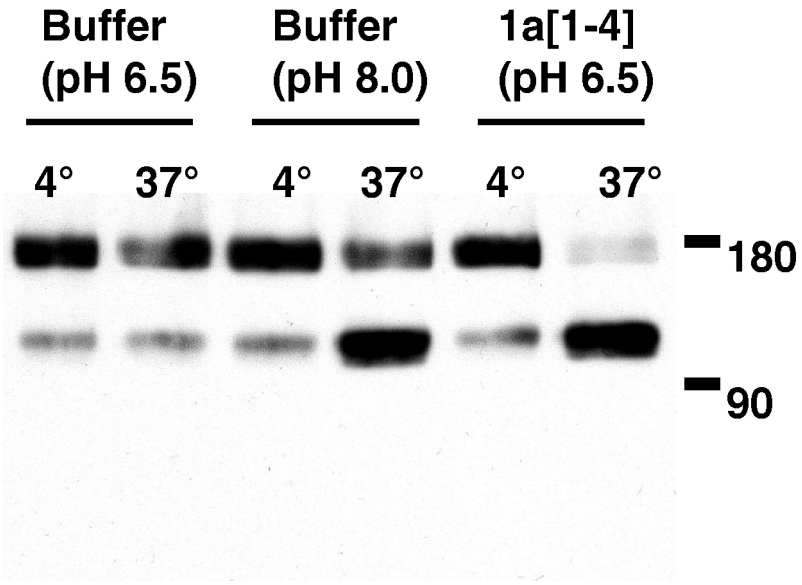

FIG. 6. The S2 glycoprotein of mutant MHV-A59 virus S_{A59}H716D undergoes a conformational change and becomes susceptible to a unique trypsin cleavage event after incubation at 37°C with soluble murine CEACAM1a[1–4] at pH 6.5 or at pH 8.0 in the absence of receptor. Gradient purified S_{A59}H716D virions were incubated at 4 or 37°C for 30 min at pH 6.5 with soluble murine CEACAM1a[1–4] or without receptor proteins at pH 6.5 or 8.0. The samples were incubated at 4°C for 20 min with trypsin-TPCK at 10 μg/ml, subjected to SDS-PAGE, and immunoblotted with anti-S2 MAb 5B19.5. Untreated virions contained a novel subunit of S with an apparent molecular mass of 120 kDa. Incubation of the S_{A59}H716D virions with soluble murine CEACAM1a[1–4] at 37°C made the 180-kDa spike glycoprotein susceptible to cleavage by trypsin-TPCK at this novel site. To a lesser degree, incubation of virions at pH 8.0 and 37°C also increased the susceptibility of the S_{A59}H716D spike to trypsin cleavage.

posed a hydrophobic domain by which the S_{A59}H716D virions bound to liposomes and also exposed one or more trypsin cleavage sites in S that yielded the 120-kDa protein without complete degradation of S2 by trypsin-TPCK.

DISCUSSION

The experiments described above using liposome flotation and protease sensitivity assays demonstrated that the S glycoprotein of MHV-A59 was induced to undergo an apparently irreversible conformational change in the carboxyl-terminal S2 domain either by incubation at 37°C, but not at 4°C, with soluble murine CEACAM1a[1–4] receptor glycoprotein at pH 6.5 or by treatment with pH 8.0 in the absence of receptor glycoprotein. This conformational change made the virions more hydrophobic and exposed trypsin-sensitive sites in S2 that are normally not accessible to protease in the spike protein on the viral envelope. The same conditions of pH, temperature, and receptor were previously found to be associated with loss of viral infectivity, dissociation of some S1 protein from the virions, clumping of virions, and loss of a MAb epitope in S2 (57, 60). The results presented here suggest that this conformational change in S can also be triggered at neutral pH and 37°C by binding of MHV spikes on the viral envelope to CEACAM1a isoforms anchored in the plasma membrane, leading to fusion of the viral envelope with the plasma membrane and initiation of virus infection. The same molecular interactions between S on the surface of an infected cell and murine CEACAM1a isoforms on an adjacent cell could lead to cell-to-cell fusion.

Only the amino-terminal 330 amino acids of S1 is required for binding to murine CEACAM1a glycoprotein (58). The S2 domain is associated with membrane fusion (33, 34). Binding of the receptor to S1 at 37°C and pH 6.5 induces a conformational change in S2 that is associated with increased hydrophobicity and exposure of trypsin cleavage sites in S2. The accompanying paper (59a) shows that the N-terminal receptor-binding domain of S1 determines the specificity of receptor-induced conformational change in the MHV S protein. Receptor binding also induces a conformational change in S1 and, in some MHV strains, facilitates separation of S1 from S2 (32). Thus, concerted actions of S1 and S2 are required for temperature-dependent murine CEACAM1a-induced triggering of the fusion-active conformation of the viral spike. Incubation of MHV-A59 virions at pH 8.0 and 37°C in the absence of the receptor apparently triggers the same conformational change in the S2 protein. In contrast, at pH 6.0 and 37°C, viral infectivity is quite stable in the absence of receptor (54), and MHV-induced cell-to-cell fusion is inhibited at pH 6. It is not yet clear whether the immediate effect of incubation at pH 8 and 37°C is on S1 or S2, although the ultimate result is to trigger a conformational change in S2. Since the interaction at 37°C of MHV-A59 virions with murine CEACAM1a glycoproteins triggers the spike glycoprotein at neutral pH, cell fusion occurs at neutral pH in MHV-infected CEACAM1a-expressing murine cell lines. Multinucleate syncytia are also observed on the tips of the villi in intestines of suckling mice infected with MHV (3). In the small intestine, where the pH is approximately 8.0, MHV spike glycoproteins on virions or infected cell membranes could possibly undergo the conformational change that leads to membrane fusion even if abundant CEACAM1a were not expressed on apical membranes of the epithelial cells (19). Thus, in mouse strains that do not express CEACAM1a, some "receptor-independent" infection might occur in the small intestine, mediated by conformational change in S2 triggered by pH 8. However the receptor-independent spread of infection by cell fusion mediated by pH 8-activated S2 would probably be inefficient because the triggered spike protein would be susceptible to rapid degradation by trypsin and other proteases in the lumen of the small intestine. The MHV-4 (MHV-JHM) strain causes receptor-independent cell fusion at neutral pH in hamster cell cultures, suggesting that the spikes can spontaneously assume a fusion-active state. The fusion-active state may be transient, however, if the spike protein is degraded or undergoes further conformational changes. Mutations that stabilize the noncovalent association between S1 and S2 on virions restore the receptor dependence of cell fusion (29).

To our knowledge, MHV S protein is the only viral spike protein that can be triggered at 37°C to undergo a conformational change in the fusion domain (S2) either by the virus receptor protein at pH 6.5 or by pH 8.0 in the absence of receptor. Fusion glycoproteins of other viruses that cause pH independent cell-cell fusion are activated by incubation with receptor at neutral pH but apparently not by high pH in the absence of receptor. For structural studies to characterize the fusion-active state of the MHV spike glycoprotein, it will be useful to induce the conformational change associated with membrane fusion by incubation at pH 8.0 and 37°C. The striking conformational changes in the influenza A virus HA glycoprotein that are associated with membrane fusion were revealed by X-ray crystallography of HA treated with acidic pH (7, 28, 48, 61). Although low pH can trigger conformational changes in the spike proteins of many viruses that enter by the endocytic route, the molecular mechanisms of acid-triggered conformational change are not yet fully understood (16, 21, 51, 52). The novel high-pH triggering of coronavirus S protein provides a new and different model for pH-induced conformational change in a viral spike protein.

Although the MHV-A59 spike protein binds to its CEACAM1a receptor glycoprotein at 4°C, the triggering of the receptor-induced conformational change is temperature dependent. Other class I viral fusion proteins and many cellular proteins that undergo conformational changes also require temperatures above 20°C to provide the energy for the conformational change (17, 25, 28).

The S proteins of various MHV strains differ markedly in many ways, including binding to isolated murine CEACAM1a versus CEACAM1b glycoproteins in solid-phase assays or virus overlay protein blots (6, 15, 42), ability to utilize anchored CEACAM1a versus CEACAM1b proteins as receptors (8, 41), strength of interactions between S1 and S2 (29), pH of viral entry (14); length of deletions in the hypervariable region of S1 (43, 47), susceptibility to protease cleavage between S1 and S2 on virions (20), rate and extent of S-induced cell-cell fusion (13), host range (2, 50), tissue tropism (31, 38), and susceptibility to neutralization by soluble murine CEACAM1a, CEACAM1b, and CEACAM2 glycoproteins (64). These phenotypes probably result from mutations in S that affect the binding of the N-terminal 330 amino acids of S1 to murine

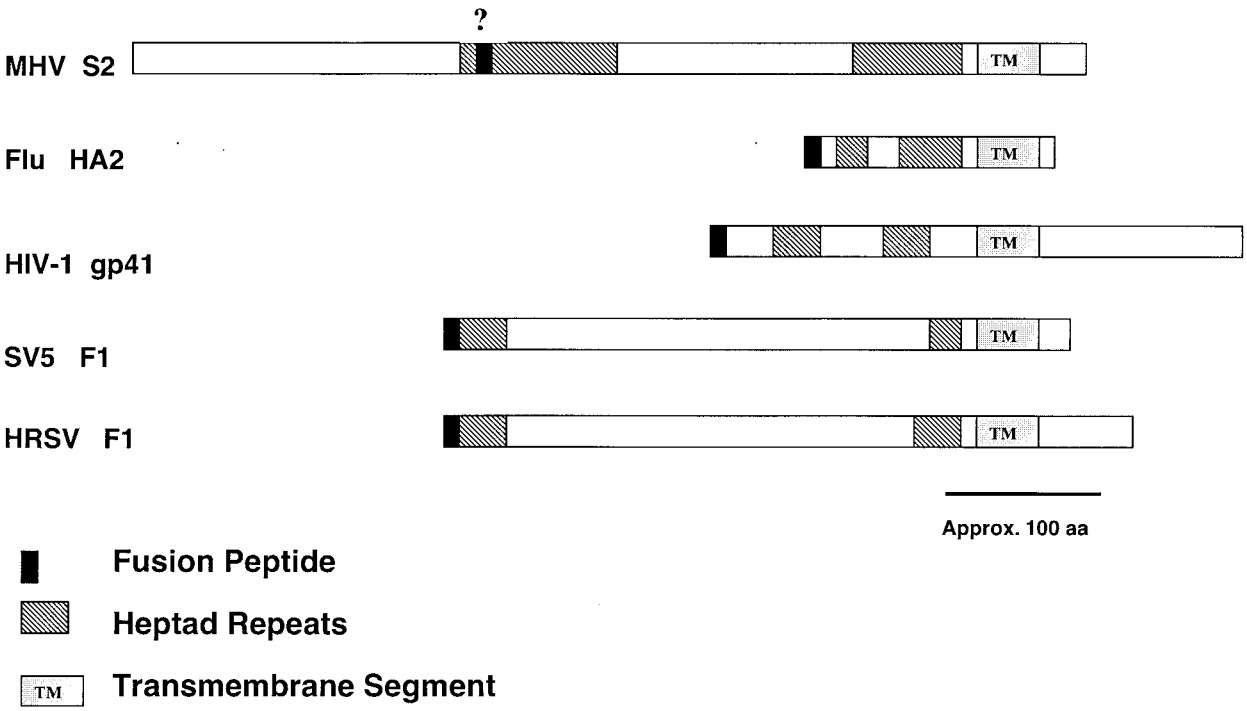

FIG. 7. Comparison of the structures of the fusion domains of spike proteins from several enveloped viruses. The spike glycoproteins of many enveloped viruses have a single protease-susceptible cleavage site (or region) between the N-terminal receptor-binding domain and the carboxyl-terminal fusion domain that is anchored in the viral envelope. Cleavage at this site may activate the membrane-fusing activity of the viral glycoprotein, which is mediated by a hydrophobic fusion peptide that is hidden in the spike on the virion until binding of the RBD to its cognate receptor or a pH change, or both, triggers a specific conformational change in the fusion domain that leads to fusion of the viral envelope with the host plasma membrane or endosomal membrane. The fusion domain of MHV spike (S2) is much larger (90 kDa) than fusion domains of the spikes of many other enveloped viruses such as influenza A virus (Flu), paramyxovirus SV5, Ebola virus, respiratory syncytial virus (HRSV), and HIV-1. The putative fusion peptide of MHV-A59 is not at the N terminus of S2 but lies within the first amphipathic helix. The functions of the large N-terminal region of murine coronavirus S2 are not well understood.

CEACAM glycoproteins (58), cleavage between the S1 and S2 subunits, and/or the subsequent conformational changes of S2 leading to membrane fusion that are demonstrated in this study. Receptor selectivity at the level of binding and/or receptor-induced conformational change differs significantly between MHV-A59 and MHV-4 (64). In addition, MHV strains isolated from persistently infected cell lines have S mutations that restrict protease cleavage between S1 and S2 and limit virus-induced cell fusion (20). Thus, disparate interactions of S proteins of various MHV strains with different CEACAM isoforms on murine tissues in vivo may result in different tissue tropisms and patterns of disease for the virus strains.

To explore the importance of cleavage between S1 and S2 in receptor-induced or pH 8-induced conformational changes in S, we studied a recombinant MHV-A59 strain that contains the H716D mutation that blocks protease cleavage of S so that virions of S_{A59}H716D contain the uncleaved 180-kDa S glycoprotein but little or no 90-kDa S1 or S2 (20; S. Hingley, personal communication). This virus causes delayed and reduced fusion of murine cells relative to MHV-A59. The liposome flotation assay on S_{A59}H716D virions revealed that interaction of S_{A59}H716D virions with smCEACAM1a[1–4] at pH 6.5 and 37°C or incubation of virions at pH 8.0 for 30 min caused a conformational change in the spike that led to increased hydrophobicity of the virions. In addition, a protease sensitivity assay showed that pH 8 or soluble receptor induced a novel trypsin cleavage pattern of the mutant S glycoprotein on S_{A59}H716D virions that resulted in a ≈120-kDa cleavage product. Cleavage of S on S_{A59}H716D virions also occurs during virus maturation. In S_{A59}H716D virions purified from 17 Cl 1 cells, a small amount of the 120-kDa cleavage product was detected (Fig. 6). Thus, although the S glycoprotein on S_{A59}H716D virions was triggered by binding to murine CEACAM1a[1–4] or pH 8 and 37°C to undergo a conformational change, this change was qualitatively different from that of the S protein of wild-type MHV-A59. The virions did show increased hydrophobicity, suggesting that a fusion peptide had been exposed by the treatment with receptor or pH 8. Because there was little or no cleavage between S1 and S2 on S_{A59}H716D virions, the presence of soluble receptor or pH 8.0 at 37°C led to a conformational change that permitted trypsin cleavage at a site that is not normally accessible to protease on the viral envelope at pH 6.5. The observed differences between the receptor-induced or pH 8-induced conformational changes in S_{A59}H716D virions with respect to wild-type MHV-A59 may be responsible for the mutant virus phenotype of little and late cell fusion. Thus, efficient cleavage between S1 and S2 on MHV-A59 virions may be required for the complete receptor-induced or pH 8-induced conformational change in S2 that leads to rapid and extensive membrane fusion and, presum-

ably, to virus entry. However, in mutants where cleavage between S1 and S2 does not occur, pH 8 or receptor binding to S1 can trigger a limited conformational change in S2 that allows aberrant cleavage and permits virus infection.

The fusion peptide(s) of the MHV S2 protein has not yet been unequivocally identified, although it is clear that there is not a hydrophobic peptide at the N terminus of S2 that would correspond to the fusion peptides of the SV5, influenza A virus, Ebola virus, and HIV-1 fusion proteins (34). Several candidate fusion peptides within the S2 subunit of MHV have been suggested, and mutational analysis has shown that amino acids in the coiled-coil domain of S2 can inhibit receptor-induced cell fusion (33, 34).

The spike glycoproteins of MHV strains have many structural and functional similarities to the class I fusion proteins of the large enveloped viruses such as influenza A virus, HIV-1, SV5, avian leukemia virus, and respiratory syncytial virus (Fig. 7). In the oligomeric spikes of MHV-A59 on the viral envelope, the S protein is cleaved at one site by protease, yielding S1 and S2 proteins that remain noncovalently attached. After S1 binds to the receptor protein on the cell membrane or to soluble receptor, the S2 protein undergoes a profound conformational change that activates membrane fusion. However, in several important ways, the MHV S protein differs from class I fusion proteins of all other viruses described to date (61). The coronavirus S protein is much larger than the spikes of the minus-strand RNA viruses or retroviruses, and it has an internal fusion peptide rather than an N-terminal fusion peptide on the fusion subunit. A long domain at the N terminus of the S2 subunit before the first predicted heptad repeat has no homolog in other class I viral fusion proteins. This region may be important for the stability of the S1-S2 complex on the virions, which varies considerably among MHV strains (29). Incubation of MHV-4 virions at pH 7 with a soluble CEACAM1a-IgGFc fusion protein neutralizes viral infectivity and readily dissociates S1 from virions (15). In contrast, the S1 protein of the mutant JHMX is not as readily released from virions under these conditions. Isogenic recombinant viruses containing chimeric spike proteins of MHV-4 and MHV-A59 replicated well in vitro but were attenuated in vivo relative to viruses with the parental spike proteins (45). These studies suggest that interactions between multiple regions of the MHV spike are required for efficient infection in the central nervous system. Conformational changes in the S protein of MHV-A59 can be triggered either by CEACAM1a receptor protein at 37°C and pH 6.5 or by pH 8 in the absence of receptor, while the fusion proteins of other viruses are triggered either by receptor binding at neutral pH or by acidic pH at 37°C. The structural and functional similarities between coronavirus spike proteins and class I fusion proteins of other enveloped RNA viruses suggest either that there may be a distant evolutionary relationship between these viruses or that class I viral fusion proteins evolved independently from one or more cellular fusion proteins (51).

Further studies of the structure and receptor-induced or high-pH-induced conformational changes of coronavirus spike proteins will provide insight into the molecular mechanisms associated with coronavirus spike-receptor interactions, virus entry, and virus-induced cell fusion.

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