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A conditional-lethal murine coronavirus mutant that fails to incorporate the spike glycoprotein into assembled virions

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Abstract

The coronavirus spike glycoprotein (S) mediates both the attachment of virus to the host cell receptor and membrane fusion. We describe here the characterization of a temperature-sensitive mutant of the coronavirus mouse hepatitis virus A59 (MHV-A59) having multiple S protein-related defects. The most remarkable of these was that the mutant, designated Albany 18 (Alb18), assembled virions devoid of the S glycoprotein at the nonpermissive temperature. Alb18 also failed to bring about syncytia formation in cells infected at the nonpermissive temperature. Virions of the mutant assembled at the permissive temperature were much more thermolabile than wild type. Moreover, mutant S protein that was incorporated into virions at the permissive temperature showed enhanced pH-dependent thermolability in its ability to bind to the MHV receptor. Alb18 was found to have a single point mutation in S resulting in a change of serine 287 to isoleucine, and it was shown by revertant analysis that this was the lesion responsible for the phenotype of the mutant.

Keywords: Coronavirus; Spike glycoprotein; Temperature-sensitive mutant; Virus assembly

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1. Introduction

Coronaviruses are a family of enveloped, positive-strand RNA viruses causing a number of diseases of medical and veterinary significance. The prototypic murine coronavirus mouse hepatitis virus (MHV) has been well-studied for a variety of reasons: it is not difficult to propagate in tissue culture; both it and its natural host are amenable to genetic analysis; and diverse strains of MHV provide excellent models for viral pathogenesis (Sturman and Holmes, 1983; Compton et al., 1993). The virion of MHV contains a nonsegmented, infectious RNA of nearly 31,500 nucleotides, which is associated with monomers of the nucleocapsid protein (N; 50 kDa) to form a helical nucleocapsid. This is enclosed within a lipid envelope that, for MHV strain A59, contains only three additional structural protein species. The most prominent of these, the spike glycoprotein (S; 180 kDa), forms the surface projections characteristic of members of the coronavirus family and mediates both virus attachment to the host cell receptor and membrane fusion. A second membrane glycoprotein (M; 23–25 kDa) has a small ectodomain, spans the viral envelope three times, and has a large carboxy-terminal tail located in the interior of the virion. A third, small membrane protein species (sM) is present in low stoichiometric amounts in the virion (Yu et al., 1994). Its function and membrane topology are currently unresolved.

The S protein is a bitopic membrane protein comprising a large amino-terminal ectodomain that accounts for most of its 1324 residues, a single transmembrane domain, and a short carboxy-terminal cytoplasmic tail. The ectodomain is modified by N-glycosylation, and acylation occurs near the transmembrane region (Ricard and Sturman, 1985). Following synthesis and folding, a process which involves extensive disulfide bond formation (Opstelten et al., 1993), S molecules oligomerize (Cavanagh, 1983; Vennema et al., 1990; Delmas and Laude, 1990) and proceed along the membrane protein secretory pathway. S protein is further processed into two 90 kDa subunits, S1 and S2, corresponding to the amino- and carboxy-terminal halves of the molecule, respectively (Ricard and Sturman, 1985; Sturman et al., 1985). The quantitative extent of this proteolytic cleavage is host cell-dependent (Frana et al., 1985). S protein associates with the M protein and assembles into virions, which bud intracellularly from a pre-Golgi compartment (Tooze et al., 1984; Tooze and Tooze, 1985). Since free M protein is not transported beyond the Golgi, it has been proposed that M is the determinant of the site of virion budding (Sturman and Holmes, 1985). A fraction of free S protein, however, is transported to the plasma membrane of the cell, where it causes cell–cell fusion resulting in formation of syncytia.

The S protein spike in the assembled virion initiates infection by binding to the receptor of the host cell. The cellular receptor for MHV was the first coronavirus receptor identified (Boyle et al., 1987; Williams et al., 1990) and is a member of the murine carcinoembryonic antigen family of glycoproteins in the immunoglobulin superfamily (Williams et al., 1991). The spike protein-receptor interaction is highly host species-specific, reflecting the considerable sequence divergence that exists among the S proteins of coronaviruses of different species. Even for a

particular member of the coronavirus family, the S protein can exhibit large intrastrain variation. Such differences in primary sequence, which occur mainly in the S1 portion of the molecule (Kusters et al., 1989; Parker et al., 1989; Gallagher et al., 1990; La Monica et al., 1991) are important for evasion of immune surveillance and are thought to play the major role in differences in tissue tropism and pathogenicity. An understanding of the S protein must balance this sequence diversity against the necessary conservation of essential structure that allows the functions of receptor binding and membrane fusion.

As part of a genetic approach to the study of coronavirus structural proteins we have isolated a temperature-sensitive mutant of MHV-A59 that assembles virions devoid of spikes at the nonpermissive temperature. In this report we characterize the phenotype of this mutant and examine its molecular basis.

2. Materials and methods

2.1. Cells and viruses

Murine fibroblast cell lines 17 clone 1 (17Cl1), L2, and Sac$^-$ have been described previously (Frana et al., 1985). Stocks of wild-type MHV-A59, the mutant Alb18 and its revertants, and vesicular stomatitis virus (VSV) (New Jersey serotype) were grown in 17Cl1 cells; infectious titer was measured by plaque assay in L2 cells at both 33° and 39°C. Due to the high selective advantage of revertants of Alb18, only low passage stocks of Alb18 with efficiencies of plaquing (39°C/33°C) of less than 10^{-4} were used in experiments.

Eight independent spontaneous revertants (designated Alb18Rev) were isolated from Alb18. Alb18Rev1 arose in a stock that had been grown for 54 h at 39°C and was plaque purified four times. Alb18Rev2–Alb18Rev8 were each isolated as a plaque formed at 39°C from a virus stock begun from an individual plaque of Alb18 obtained at 33°C.

2.2. Labeling and analysis of virion proteins

For labeling of virion proteins, confluent monolayers of 17Cl1 or Sac$^-$ cells were infected at a multiplicity of 5 PFU/cell. Following absorption for 1 h at 33°C, cells were re-fed with MEM containing 1/10 of the normal level of cystine (2.4 mg/l), 10% fetal bovine serum, and 2.0 μCi/ml [^{35}S]cysteine (Amersham). Cultures were incubated at 33°C or 39°C until 95% of the wild type-infected cells at 39°C had formed syncytia (24 h for 17Cl1 cells or 16 h for Sac$^-$ cells).

Protein sample analysis was performed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Tube gels were fractionated, solubilized and quantitated by liquid scintillation counting as described previously (Sturman, 1977). For Western blotting, proteins separated on slab gels were electroblotted to a PVDF membrane (Millipore) using a tank type transblotter (BioRad). Nonspecific binding to membranes was blocked by incubation in 0.1 M

Tris-HCl (pH 7.5) containing 3% nonfat milk; primary antiserum was used at a dilution of 1:250 in 0.1 M Tris-HCl (pH 7.5). Goat anti-S polyclonal antibody AO4 (Boyle et al., 1987) was generously provided by Dr. Kathryn Holmes (Uniformed Services University of the Health Sciences). Staining of bound antibody was performed with a biotin avidin horseradish peroxidase kit (Vector Labs).

2.3. Solid phase receptor binding assay

The binding of virion S protein to the MHV receptor was assayed essentially as described previously (Boyle et al., 1987). Intestinal brush border membranes from BALB/c mice (kindly provided by Dr. Mark Frana and Dr. Kathryn Holmes, Uniformed Services University of the Health Sciences) were separated by SDS-PAGE, transblotted to PVDF, and blocked as for Western blots. Prior to the binding assay, purified virus samples were heat-inactivated at the indicated pH for 24 h at 40°C (Koetzner et al., 1992); control samples were held on ice at pH 6.5. Strips cut from the blot were incubated with virus samples for 1 h at room temperature and were then stained with goat anti-S protein antiserum AO4 and developed as for Western blots.

2.4. Cloning of cDNA and sequencing

Alb18 genomic RNA was extracted from purified virions as described previously (Koetzner et al., 1992). Libraries of cDNA clones of the M and S genes of Alb18 were generated by a modification of the procedure of Gubler and Hoffman (Gubler and Hoffman, 1983) using primers complementary to nt 580–597 of the N gene (for M first strand cDNA) or to nt 1625–1641 of the S gene and to nt 5–24 beyond the S gene stop codon (for S first strand cDNA). An additional set of cDNA clones corresponding to nt 1136–2770 of the S gene was obtained by reverse transcription followed by PCR using a primer pair bordering this region; PCR was performed as described previously (Koetzner et al., 1992).

DNA sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977) using modified T7 DNA polymerase (Sequenase, U.S. Biochemical). A set of 31 oligonucleotide primers spanning the S gene and adjacent regions and a set of 7 oligonucleotide primers spanning the M gene and adjacent regions were used. In addition to sequencing of cDNA clones, direct sequencing of reverse transcription-PCR products was performed to verify portions of the Alb18 S and M genes and to sequence the relevant portions of the S genes of Alb18Rev1–Alb18Rev8.

3. Results

3.1. Phenotype of Alb18

The mutant Alb18 was identified among a collection of mutants obtained by nitrous acid mutagenesis of wild-type (heat-resistant) MHV-A59 (Sturman et al.,

1987). In this search mutants were selected for alterations in or lack of cytopathic effect on 17Cl1 cells at the nonpermissive temperature (39°C) as compared with that occurring at the permissive temperature (33°C). At temperatures of 33°C through 36°C, it was found that Alb18 infection resulted in polykaryon formation by 24 h post-infection. At 37°C or above, however, no polykaryons were observed. This suggested that there was a sharp transition temperature above which Alb18 became unable to replicate.

Roughly half of the mutants obtained in the search produced small plaques at the nonpermissive temperature; we have previously characterized two members of this set as N gene mutants (Koetzner et al., 1992; Masters et al., 1994). The remaining half of the collection comprised tight temperature-sensitive mutants, having efficiencies of plaquing (39°C/33°C) on the order of 10^{-4}. For these, in all cases examined, plaques formed at the nonpermissive temperature were found to be revertants. Alb18 fell in this latter set of mutants but was unique in being RNA^+, i.e., able to synthesize viral RNA at the nonpermissive temperature (Sturman et al., 1987). Moreover, preliminary results indicated that Alb18 synthesized viral structural proteins at the nonpermissive temperature. These considerations led us to examine the possibility that Alb18-infected cells produced non-infectious virions at the nonpermissive temperature.

Alb18-infected 17Cl1 cells incubated at either the permissive or nonpermissive temperature were metabolically labeled with [^{35}S]cysteine, and virus released into the culture supernatant was purified by sucrose gradient centrifugation (Sturman et al., 1980). Material obtained from the 39°C culture behaved identically to material from the 33°C culture (and to wild-type controls) throughout this standard virus purification procedure. Remarkably, when analyzed by SDS-PAGE it was found that Alb18 virions formed at the nonpermissive temperature were devoid of both the 180 kDa and the 90 kDa forms of the S protein (Fig. 1). These virions contained the other two viral structural proteins, N and M, in the same relative amounts and with the same electrophoretic mobilities as those of virions obtained at the permissive temperature. In Fig. 1, since equal amounts of radioactivity of each sample were analyzed, the 39°C sample contained proportionately more of the N and M proteins.

A similar experiment was performed using Sac^- cells to test whether this result was host cell line-specific and to simplify the analysis of S, since all S protein in virions released from Sac^- cells is cleaved to the 90 kDa form (Frana et al., 1985). In addition, to address the possibility that loosely associated S protein on Alb18 virions produced at 39°C might have been stripped from virus particles during the multiple steps of the purification procedure, culture medium was instead collected and pelleted directly for analysis by both Western blotting and autoradiography. As shown in Fig. 2A, Western blotting detected equivalent amounts of 90 kDa S protein in virus released from Alb18-infected Sac^- cells at 33°C and from wild type-infected cells at either 33°C or 39°C. In contrast, virus released from Alb18-infected cells at 39°C contained no 90 kDa S protein, nor was there S-specific immunoreactive material of any other molecular weight. Examination of the same blot by autoradiography confirmed this result (Fig. 2B). Because the samples in

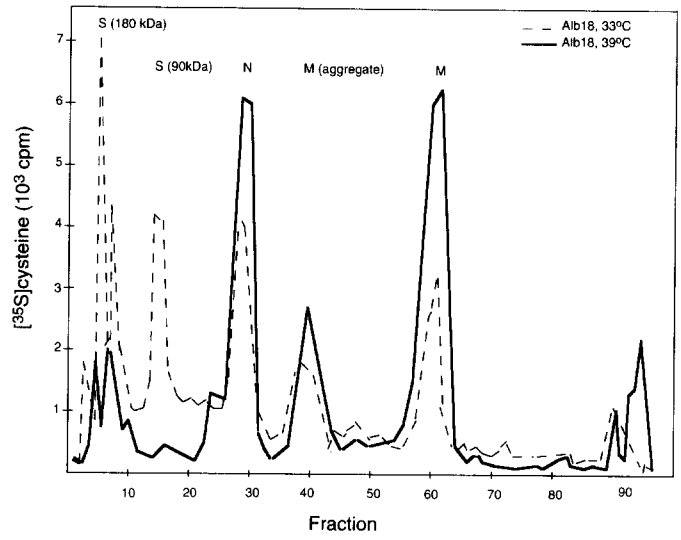

Fig. 1. SDS-PAGE profiles of Alb18 virions. Alb18 grown in 17Cl1 cells at 33°C (broken line) or 39°C (solid line) was metabolically labeled with [^{35}S]cysteine, and released virus was purified by sucrose gradient centrifugation (Sturman et al., 1980). Equal amounts of radioactivity for each sample were analyzed by electrophoresis on cylindrical 10% polyacrylamide gels, followed by fractionation and quantitation by liquid scintillation counting.

this experiment had not been purified, there were a number of additional nonviral proteins present in the pelleted material, but no 90 kDa S protein could be detected in the Alb18 at 39°C sample, nor were any of the additional bands unique to this sample. Analysis of supernatant fractions by immunoprecipitation failed to detect the presence of soluble S protein (data not shown). These results confirmed the conclusion that Alb18 failed to incorporate S protein into assembled virions at the nonpermissive temperature.

3.2. Sequence analysis of the S and M genes of Alb18

The phenotype of Alb18 strongly suggested that the lesion in this mutant resided in either the S protein or the M protein. In cells infected with MHV the M protein localizes in the endoplasmic reticulum (ER) and Golgi region (Tooze et al., 1984; Tooze and Tooze, 1985), and M is believed to be responsible for determining the assembly and budding of progeny virions (Sturman and Holmes, 1985). To examine the possibility that Alb18 contained a defect in the M protein leading to an altered ability to associate with S protein, the M gene of Alb18 was cloned and sequenced. No nucleotide difference was found between the M gene of Alb18 and that of wild-type virus in either the coding region or in the upstream intergenic region. This finding precluded a causative role for M protein in the phenotype of Alb18.

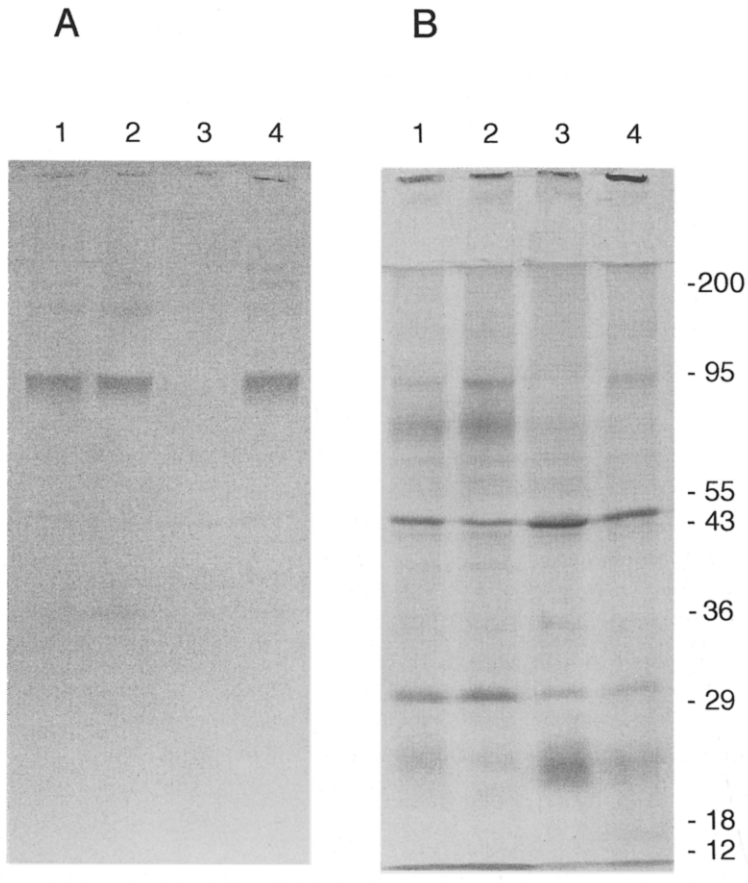

Fig. 2. Analysis of Alb18 and wild-type virus released from infected Sac$^-$ cells. Alb18 (lanes 1 and 3) or wild-type virus (lanes 2 and 4) was grown in Sac$^-$ cells at either 33°C (lanes 1 and 2) or 39°C (lanes 3 and 4). Culture medium containing [^{35}S]cysteine-labeled released virus was clarified by low-speed centrifugation, and then virus was directly pelleted at 76,000 × g through a cushion of 20% sucrose in 50 mM Tris-maleate (pH 6.5), 100 mM NaCl, 1 mM EDTA and equal amounts of radioactivity for each sample were analyzed by SDS-PAGE. (A) Western blot probed with polyclonal goat antiserum AO4. (B) Autoradiograph of the blot shown in panel A. Molecular weight standards (kDa) are indicated at the right.

The entire S gene of Alb18 as well as 150 nt upstream was next analyzed by sequencing of overlapping cDNA clones prepared from genomic RNA. In the coding region of the Alb18 S gene, a single nucleotide difference was found in comparison to the S gene of wild-type virus: nt 860 was changed from G to T. In the encoded amino acid sequence, this resulted in a change of residue 287 from serine to isoleucine (Fig. 3). Two additional nucleotide changes were found in the Alb18 S coding region in comparison to the previously published sequence for MHV-A59 (Luytjes et al., 1987; GenEMBL accession Nr. P11224). The first, a

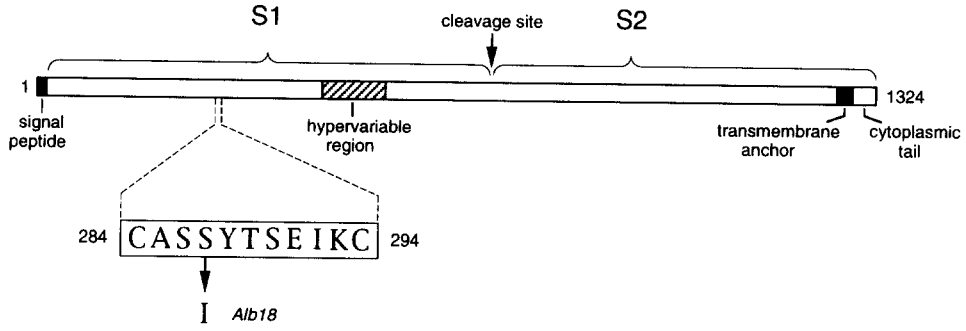

Fig. 3. Schematic of the MHV S protein showing the amino acid residue that is mutated in Alb18. The signal peptide and transmembrane domain of S are represented by solid rectangles; the stippled rectangle indicates the hypervariable region defined by interstrain sequence comparison (Parker et al., 1989). The proteolytic cleavage site separating the S1 and S2 portions of the molecule is marked with an arrow.

change of A to G at nucleotide 293, resulted in a change from asparagine to serine at residue 98; the second, a change of G to A at nt 3045, was silent. Both of these changes were also found in the wild-type virus sequence, however, and thus were of no significance to the phenotype of Alb18. The same two nucleotide differences in our laboratory wild-type MHV-A59 strain have been reported by Gombold et al. (1993). The 150 nt upstream of the S coding region were found to be identical to the previously published MHV-A59 sequence (Luytjes et al., 1988).

3.3. Revertant analysis

To determine the significance of the single coding change that was found in the Alb18 S gene, an analysis of revertants was carried out. Eight independent, spontaneously arising revertants of Alb18 were isolated on the basis of their ability to form plaques at the nonpermissive temperature. Revertants arose at a frequency on the order of 10^{-5}, and all formed wild-type-sized plaques at 39°C. In addition, all of the revertants resembled wild-type virus in thermal stability. Alb18 virus produced at the permissive temperature was 10-fold more thermolabile than wild-type virus when incubated at 40°C, pH 6.5, for 24 h (Table 1). By contrast, the thermolability of each of the revertants under the same conditions was indistinguishable from that of wild-type virus (Table 1). This showed that temperature-sensitivity and thermolability of virions were two consequences of the same mutation in Alb18.

The region encompassing nt 860 of the S gene was sequenced in all of the revertants. For one of these, Alb18Rev6, nt 860 was found to have exactly reverted from T to G, returning codon 287 to that of the wild-type serine. For each of the seven other revertants, nt 860 was changed from T to C, resulting in a conservatively substituted threonine residue at position 287 (Table 1). These results clearly

Table 1
Properties of revertants of Alb18

Virus	Codon 287	Amino acid 287	Thermal inactivation [a]		
			Titer (PFU/ml), 0 h	Titer (PFU/ml), 24 h	Fold reduction in titer
Wild type	AGT	Ser	$1.9 \cdot 10^8$	$4.1 \cdot 10^7$	4.1
Alb18	ATT	Ile	$8.8 \cdot 10^6$	$2.1 \cdot 10^5$	42.0
Alb18Rev1	ACT	Thr	$2.9 \cdot 10^7$	$7.7 \cdot 10^6$	3.8
Alb18Rev2	ACT	Thr	$2.3 \cdot 10^6$	$6.4 \cdot 10^5$	3.6
Alb18Rev3	ACT	Thr	$3.8 \cdot 10^7$	$8.9 \cdot 10^6$	4.3
Alb18Rev4	ACT	Thr	$1.5 \cdot 10^7$	$4.5 \cdot 10^6$	3.2
Alb18Rev5	ACT	Thr	$3.3 \cdot 10^7$	$8.2 \cdot 10^6$	4.1
Alb18Rev6	AGT	Ser	$5.7 \cdot 10^7$	$1.5 \cdot 10^7$	3.9
Alb18Rev7	ACT	Thr	$1.3 \cdot 10^7$	$3.7 \cdot 10^6$	3.4
Alb18Rev8	ACT	Thr	$7.5 \cdot 10^7$	$1.8 \cdot 10^7$	4.2

[a] Thermal inactivation was carried out for 24 h at 40°C and pH 6.5 as described previously (Koetzner et al., 1992). Titers of surviving virus were determined on L2 cells at 33°C.

established that the nucleotide change found at codon 287 was the lesion responsible for the phenotype of Alb18.

3.4. Lack of molecular reversibility of the effects of temperature

To learn whether the temperature-sensitivity of Alb18 could be reversed, temperature shift experiments were performed. 17Cl1 cells were infected with Alb18 or wild-type virus at either the permissive temperature (Fig. 4A and B) or the nonpermissive temperature (Fig. 4C and D). At 8, 16, or 20 h post-infection, samples of virus released into the culture medium were taken for determination of infectious titer, and then the cultures were shifted to the nonpermissive or permissive temperature, respectively. Samples were again taken at 24 h, and their infectious titers were compared with those of unshifted control cultures. In cells infected with Alb18, shifting the temperature to 39°C after infection at 33°C resulted in the immediate and complete inhibition of further release of infectious virus following the shift (Fig. 4B). This suggested that the mutant S protein underwent a change to an assembly-incompetent form as soon as the temperature was raised.

Conversely, for cells infected with Alb18 at the nonpermissive temperature, the yield of infectious virions after a shift to the permissive temperature was equivalent to the yield from an infection at the permissive temperature that had proceeded for the same amount of time (Fig. 4D). This showed that prior incubation at 39°C was not lethal to the intracellular virus and that replication could resume once infected cells were returned to 33°C. It also suggested that, following the downwards shift, newly synthesized S protein was required for the assembly of virions. If the population of Alb18 S protein previously synthesized at 39°C had acquired an assembly-competent form upon being shifted to 33°C, then we would have ex-

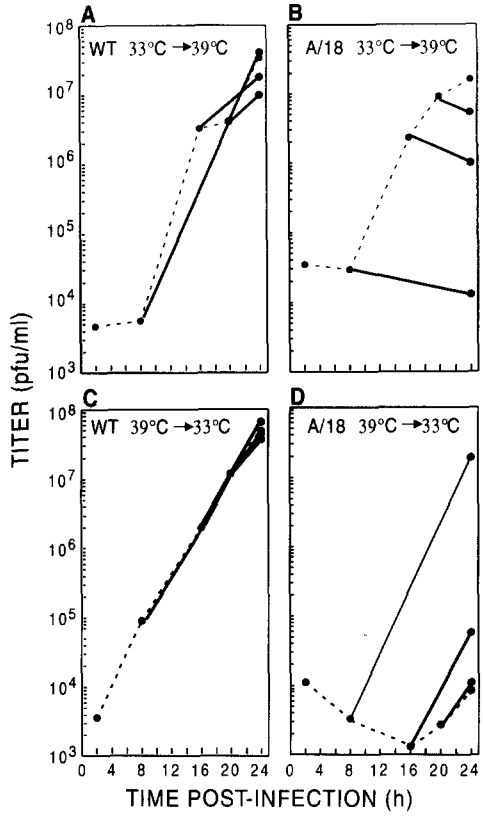

Fig. 4. Infectivity of virus released into culture medium after upwards or downwards temperature shift. 17Cl1 cells were inoculated with wild-type virus (A and C) or Alb18 (B and D) at a multiplicity of 1 PFU/cell for 1 h at 33°C. Following removal of inoculum, monolayers were washed and then incubated at either 33°C (A and B) or 39°C (C and D). At 8, 16 or 20 h post-infection samples were taken for titration of infectivity, and cultures were then shifted from 33°C to 39°C (A and B) or from 39°C to 33°C (C and D). Final infectivity samples were taken at 24 h post-infection. Infectious titer was determined by plaque assay in L2 cells at 33°C. Solid lines, shifted samples; broken lines, unshifted control samples.

pected to see a sharper burst of release of infectious virus assembled from previously synthesized components, and the final titers at 24 h for all shifted samples in Fig. 4D should have approached the same value. Further studies will be required to determine the molecular basis for the irreversibility of the defect in Alb18 S protein.

3.5. Partial dominance of the Alb18 mutation

Because the MHV S protein is an oligomer, it was of interest to examine the outcome of a mixed infection of Alb18 and wild-type virus. Coinfection of either 17Cl1 or Sac$^-$ cells with both Alb18 and wild-type virus, each at a multiplicity of 5

Table 2
Infectious yield from mixed infections [a]

Virus	Titer (PFU/ml)	
	17Cl1 cells	Sac$^-$ cells
Experiment 1		
Alb18	$1.1 \cdot 10^5$	$2.0 \cdot 10^4$
Wild-type MHV	$9.6 \cdot 10^7$	$1.0 \cdot 10^7$
Wild-type MHV and Alb18	$4.2 \cdot 10^6$	$4.9 \cdot 10^5$
Experiment 2		
Alb18	$1.0 \cdot 10^5$	$2.8 \cdot 10^2$
Wild-type MHV	$5.5 \cdot 10^7$	$2.7 \cdot 10^6$
VSV	$7.8 \cdot 10^7$	$9.6 \cdot 10^8$
VSV and wild-type MHV	$5.1 \cdot 10^7$	$3.3 \cdot 10^8$
VSV and Alb18	$1.3 \cdot 10^8$	$8.9 \cdot 10^8$

[a] Confluent monolayers of 17Cl1 or Sac$^-$ cells were infected with each virus at a multiplicity of 5 PFU/cell. Following adsorption for 1 h at 33°C, cultures were shifted to 39°C, and released virus was harvested at 24–26 h (17Cl1 cells) or 16–19 h (Sac$^-$ cells) post-infection. In mixed infections with MHV (wild type or Alb18) and VSV, inoculation with MHV preceded inoculation with VSV by 3 h. Infectious titers were determined on L2 cells at 33°C. Values for Alb18 alone represent residual input virus infectivity, as determined in independent titrations.

PFU/cell, resulted in a 20-fold reduction of infectious yield at 39°C compared with that obtained with wild-type virus alone (Table 2, Experiment 1). The progeny of these mixed infections resembled wild-type virus in thermolability (data not shown). This suggested that at the nonpermissive temperature, Alb18 and wild-type S protein monomers were able to form mixed oligomers that were at least partially defective, and only those progeny having a sufficient concentration of wild-type homo-oligomeric S protein molecules were infectious.

An alternative explanation of the data was that inhibition of wild-type infection by Alb18 was the result of a general phenomenon such as nonspecific aggregation between Alb18 S protein and other proteins in the membrane of the ER, including wild-type S protein. To examine this possibility, mixed infections were carried out at 39°C with Alb18 (or wild-type MHV) and the rhabdovirus VSV. Although VSV assembles at the plasma membrane rather than at intracellular membranes, its envelope glycoprotein follows the same secretory pathway as MHV S protein. The infectious yield of VSV in either 17Cl1 or Sac$^-$ cells was not reduced by coinfection with Alb18 irrespective of whether both viruses were infected simultaneously (data not shown) or even if Alb18 infection was allowed to proceed for 3 h prior to VSV infection (Table 2, Experiment 2). Therefore, the inhibition of wild-type MHV by Alb18 was a specific effect.

3.6. Altered ability of virus-associated Alb18 S protein to bind to the MHV receptor

The ability of mutant virion S protein to bind to the MHV receptor was evaluated using an in vitro solid phase assay developed previously by Holmes and

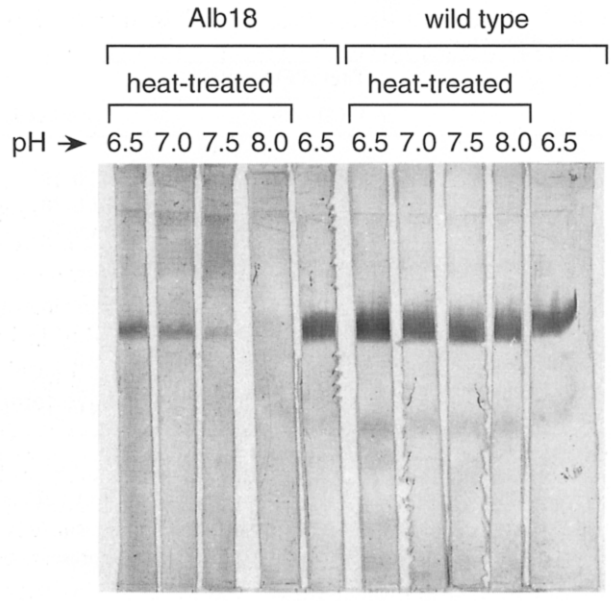

Fig. 5. Solid phase assay of MHV receptor-binding ability of virion S protein. Mouse intestinal brush border membrane proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels and were transferred to PVDF membranes. Blots were cut into strips and incubated with purified Alb18 or wild-type virus that had been inactivated for 24 h at 40°C at pH 6.5, 7.0, 7.5 or 8.0. Control (non-heat-inactivated) virus samples were held at 4°C at pH 6.5. Bound virus was detected by incubation with polyclonal goat antiserum AO4 followed by staining with horseradish peroxidase-coupled second antibody.

coworkers (Boyle et al., 1987). Receptor-containing intestinal membrane vesicles from BALB/c mice were solubilized and separated by SDS-PAGE followed by Western blotting. Virus samples were incubated with strips of the blot, and virions bound to the MHV receptor were visualized by staining with anti-S protein antisera. Owing to the absence of spikes on Alb18 virions released from cells at the nonpermissive temperature, it was only possible to analyze Alb18 virions produced at the permissive temperature. As shown in Fig. 5, Alb18 virions exhibited the same ability as wild-type virions to bind to receptor (compare non-heat-treated lanes for each). To determine the stability of the Alb18 spikes under conditions whose effects on wild type were already known (Sturman et al., 1990), virus samples were treated at pH 6.5, 7.0, 7.5 or 8.0 for 24 h at 40°C. Heat-treatment at pH 6.5 slightly decreased the ability of Alb18 virions to bind to receptor. This effect increased with increasing pH, and by pH 8.0 the receptor-binding ability of Alb18 was almost completely abrogated. By contrast, wild-type virions retained the ability to bind in this assay after all pH treatments. These data show that Alb18 virion spikes could be impaired by heat and mildly alkaline conditions much more readily than those of wild-type virions, indicating that the pH-dependent confor-

mational change in S protein (Sturman et al., 1990) is more pronounced or more easily triggered in the mutant S protein of Alb18.

4. Discussion

The single amino acid change of serine 287 to isoleucine in the spike glycoprotein of the MHV mutant Alb18 confers a phenotype in which multiple functions of S protein are affected. The most salient feature of this mutant is that at the nonpermissive temperature virions are assembled devoid of S protein. In addition, the lesion results in two other major impediments: the inability of infected cells to form syncytia at the nonpermissive temperature and the thermolability of S protein even after it has been assembled into virions at the permissive temperature.

The formation of spikeless virions by Alb18 indicates that S protein does not play an active role in MHV assembly, which is probably mediated by interactions between the M protein and the viral nucleocapsid (Sturman et al., 1980). This supports the same conclusion reached previously from studies demonstrating that spikeless virions are released by MHV-infected cells treated with tunicamycin, an inhibitor of N-linked glycosylation (Holmes et al., 1981; Rottier et al., 1981). Similar observations have also been made with human coronavirus OC43 (Mounir and Talbot, 1992) and turkey enteric coronavirus (Dea et al., 1989). The Alb18 phenotype recalls that of the well-studied VSV mutant tsO45, which also produces spikeless virions at its nonpermissive temperature (Gallione and Rose, 1985; Metsikkö and Simons, 1986, and references therein). It has been shown that, in virions of tsO45 assembled at the nonpermissive temperature, the ectodomain of the VSV spike glycoprotein G has been proteolytically removed, but an anchor consisting of the transmembrane and cytoplasmic portions of the molecule remains embedded in the virion membrane (Metsikkö and Simons, 1986). We were not able to detect an analogous carboxy-terminal fragment of the Alb18 S protein in purified spikeless virion preparations either directly or by immunoprecipitation with an anti-peptide antibody, which was raised against the carboxy terminus of S (data not shown). At present, however, we cannot completely rule out the possibility that the very cysteine-rich carboxy terminus of S is refractory to immunoprecipitation or standard conditions of SDS-PAGE, as is the VSV G protein fragment (Metsikkö and Simons, 1986). If S protein anchors were found in Alb18 virions formed at 39°C, this would require re-evaluation of the notion that S is only a passive participant in MHV assembly.

The locus of the mutation that brings about the multiple changes in the S protein of Alb18 is distinct from that of any previously mapped MHV S mutation (Gallagher et al., 1991; Wang et al., 1992; Gombold et al., 1993; Fu and Baric, 1994). Serine 287 falls a little more than a third of the distance from the mature amino terminus of the S1 half of the molecule (Fig. 3). It is highly conserved, and it lies between two cysteine residues that are absolutely conserved in all reported coronavirus S sequences. The multiple effects of the Alb18 mutation likely cannot be accounted for at the level of primary sequence. The loss of serine 287 does not

eliminate or create a consensus motif for N-linked glycosylation. Moreover, there is no evidence that two other potential post-translational modifications of serine, O-glycosylation or phosphorylation, occur in the MHV S protein; and a third, acylation, has been shown to occur in the S2, but not the S1, half of the molecule (Ricard and Sturman, 1985). It is noteworthy that the Alb18 mutation falls within the region to which the receptor-binding ability of MHV S has been recently mapped (amino acids 1–330; Kubo et al., 1994). Conceivably, the lesion in Alb18 S results in a temperature- and pH-dependent conformational change that affects assembly, receptor binding and fusion.

Further study of this mutant should provide useful insights into the structure and function of the MHV spike glycoprotein. In particular, it would be informative to use conformation-specific monoclonal antibodies to probe the structure of Alb18 S protein that is synthesized at the nonpermissive temperature. It would also be highly interesting if second-site revertants could be obtained to the Alb18 mutation, since these might yield evidence about intramolecular interactions in the S molecule.

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