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Amino acid substitutions and an insertion in the spike glycoprotein extend the host range of the murine coronavirus MHV-A59

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Abstract

The murine coronavirus [murine hepatitis virus (MHV)] is limited to infection of susceptible mice and murine cell lines by the specificity of the spike glycoprotein (S) for its receptor, murine carcinoembryonic antigen cell adhesion molecule 1a (mCEACAM1a). We have recently shown that 21 aa substitutions and a 7-aa insert in the N-terminal region of S are associated with the extended host range of a virus variant derived from murine cells persistently infected with the A59 strain of MHV (MHV-A59). We used targeted RNA recombination (TRR) to generate isogenic viruses that differ from MHV-A59 by the 21 aa substitutions or the 7-aa insert in S. Only viruses with both the 21 aa substitutions and the 7-aa insert in S infected hamster, feline, and monkey cells. These viruses also infected murine cells in the presence of blocking anti-mCEACAM1a antibodies. Thus, relatively few changes in the N-terminal region of S1 are sufficient to permit MHV-A59 to interact with alternative receptors on murine and non-murine cells.

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Keywords: Murine coronavirus; Extended host range; Spike glycoprotein; Targeted RNA recombination; Receptor jumping mutants; Persistent infection

Introduction

Viruses of the *Coronaviridae* family cause economically important respiratory, enteric, and systemic diseases of humans, livestock, pets, and laboratory rodents. Coronaviruses have large RNA genomes that probably exist as a heterogeneous RNA quasi-species due to an error-prone RNA-dependent RNA polymerase and a high rate of recombination (Lai and Holmes, 2001). Despite this genetic diversity, many coronaviruses cause disease in a single host species or several closely related host species (Siddell and Snijder, 1998). With the recent emergence of the severe acute respiratory syndrome coronavirus (SARS-CoV) that can cause lower respiratory tract disease in humans (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003), a better understanding of how coronaviruses jump to a new host species is clearly needed.

The limited host range of some coronaviruses is primarily determined by the interaction of the viral spike glycoprotein (S) with a specific glycoprotein receptor on host cell membranes. For example, cell lines from host species that are normally resistant to group I coronaviruses are rendered susceptible to infection by transfection with cDNA encoding the species-specific aminopeptidase N (APN) (Delmas et al., 1992; Tresnan et al., 1996; Yeager et al., 1992). The 180-kDa S glycoprotein of murine coronavirus [murine hepatitis virus (MHV)] is a type I viral fusion protein that mediates both receptor binding and fusion activities (Bosch et al., 2003; Gallagher and Buchmeier, 2001). S is posttranslationally cleaved by a cellular protease into 90-kDa S1 and S2 proteins that remain noncovalently associated on the MHV virion. Cleavage of S may enhance cell–cell fusion and viral infectivity, although the uncleaved S of MHV mutants can mediate cell–cell fusion and fusion with host cell membranes (Bos et al., 1997; Frana et al., 1985; Taguchi, 1993).

The A59 strain of MHV (MHV-A59) that lacks a functional hemagglutinin-esterase (HE) gene binds to murine cell lines and tissues, but not to a wide range of non-murine cell lines or tissues (Compton et al., 1992; Yokomori et al., 1991). The specificity of MHV strains for

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susceptible mice and murine cell lines is determined by the binding of S to the amino (N)-terminal Ig-like domain of the murine carcinoembryonic antigen cell adhesion molecule 1a (mCEACAM1a) (Beauchemin et al., 1999; Dveksler et al., 1993; Yokomori and Lai, 1992). Binding of the N-terminal domain of mCEACAM1a to S1 on MHV virions at 37 °C induces an irreversible conformational change in S2 that neutralizes virus and exposes a hydrophobic domain that presumably initiates fusion with host cell membranes (Gallagher, 1997; Matsuyama and Taguchi, 2002; Miura et al., 2004; Zelus et al., 2003). Mutational analysis and the crystal structure of mCEACAM1a[1,4] suggest that residues in the CC' loop and the C' β sheet in the N-terminal domain of mCEACAM1a comprise the docking site for MHV (Rao et al., 1997; Tan et al., 2002; Wessner et al., 1998).

Although no crystal structure has been determined for the S glycoprotein of any coronavirus, domains responsible for the receptor binding activity of some coronaviruses have been identified. The ectodomain of S is responsible for the specificity of MHV-A59 for murine cells and the specificity of feline coronavirus [feline infectious peritonitis virus (FIPV)] for feline cells (Haijema et al., 2003; Kuo et al., 2000). aa 417–547 of S of human coronavirus 229E (HCoV-229E) comprise a minimal receptor binding domain (RBD) for human APN *in vitro*, whereas aa 1–330 of S of MHV comprise the minimal RBD for mCEACAM1a *in vitro* and *in vivo* (Bonavia et al., 2003; Breslin et al., 2003; Kubo et al., 1994; Tsai et al., 2003). Variants that were generated during persistent MHV infection of murine cell cultures or co-cultures of murine and hamster cells infect a wide range of murine and non-murine cell lines (Baric et al., 1997, 1999; Schickli et al., 1997). The N-terminal region of S is associated with the extended host range of MHV/BHK, a virus variant generated during persistent MHV-A59 infection of murine 17 Cl 1 cells (Sawicki et al., 1995). All of the recombinant viruses between the parental MHV-A59 and MHV/BHK that could grow in hamster cells retained 21 aa substitutions and a 7-aa insert in this region of S, as well as genes upstream of the S gene of MHV/BHK (Schickli et al., 2004). The 7-aa insert could have been generated either by a replicase stuttering mechanism or by recombination between the S gene of MHV-A59 and an mRNA encoding a cellular phosphatidylinositol 3-kinase regulatory subunit.

To determine whether the 21 aa substitutions, the 7-aa insert, or both the 21 aa substitutions and the 7-aa insert were sufficient to extend the host range of MHV-A59, we used targeted RNA recombination (TRR) to generate isogenic viruses that differ from MHV-A59 at these residues in S. We demonstrated that both the 21 aa substitutions and the 7-aa insert in the N-terminus of S are needed to permit the interaction of MHV-A59 with alternative receptors on murine and non-murine cells. We also showed that the 21 aa substitutions and the 7-aa insert

did not prevent the interaction of MHV-A59 with its murine receptor, CEACAM1a.

Results

Generation of recombinant viruses

Mutations in the 5' of the S gene are associated with the extended host range of MHV/BHK, a virus variant generated from murine cells persistently infected with MHV-A59 (Sawicki et al., 1995; Schickli et al., 1997, 2004). Twenty-four point mutations and a 21-bp insert in the S gene result in 21 aa substitutions (Table 1) in the N-terminus and a 7-aa insert (TRTKKVP) at aa 494 in the S glycoprotein of MHV/BHK. To determine whether the 21 aa substitutions, the 7-aa insert, or both the 21 aa substitutions and the 7-aa insert in S are sufficient to expand the host range of MHV-A59, we used targeted RNA recombination (TRR) to introduce the mutations found in the S gene of MHV/BHK into the genome of MHV-A59. Donor RNAs, transcribed *in vitro* from mutant pMH54 constructs, were transfected into feline (Fcfw) cells that had been inoculated with the chimeric helper virus fMHV (Kuo et al., 2000). The infected and transfected Fcfw cells were immediately overlaid onto monolayers of murine (17 Cl 1) cells to select for isogenic recombinant viruses that had gained the ability to infect murine cells due to a crossover event upstream of the S gene (Navas and Weiss, 2003). For each mutant pMH54 construct: SA59+i, S21BHK, S21BHK+i, and S21BHK+i(R→L) (Fig. 1), three recombinant viruses (A, B, and C) were independently recovered and plaque-purified to control for adventitious mutations that might arise in the S gene or in the rest of the viral genome. In addition, in every experiment, wild-type pMH54 RNA was used to reconstitute wild-type MHV-A59 virus (SA59) in triplicate.

All of the 17 Cl 1 cell monolayers overlaid with pMH54-transfected, fMHV-inoculated Fcfw cells exhibited extensive cytopathic effects (CPE) at 48 h (h). In contrast, 17 Cl 1 cell monolayers overlaid with mock-transfected, fMHV-inoculated Fcfw cells exhibited no detectable CPE. Following TRR, all recombinant viruses were amplified, purified,

Table 1
Comparison of amino acids in the N-terminus of the spike glycoprotein of MHV-A59 with MHV/BHK^a

E43D	T212I	L300S	Y381H	I486K
T155M	D234E	L308V	Y450H	
G133D	Q267R	R338K	V474A	
T156N	F270L	E347K	S476A	
G210D	N271Y	R363S	D485G	

^a MHV-A59 (GenBank accession no. AY497328); MHV/BHK (GenBank accession no. AY497331).

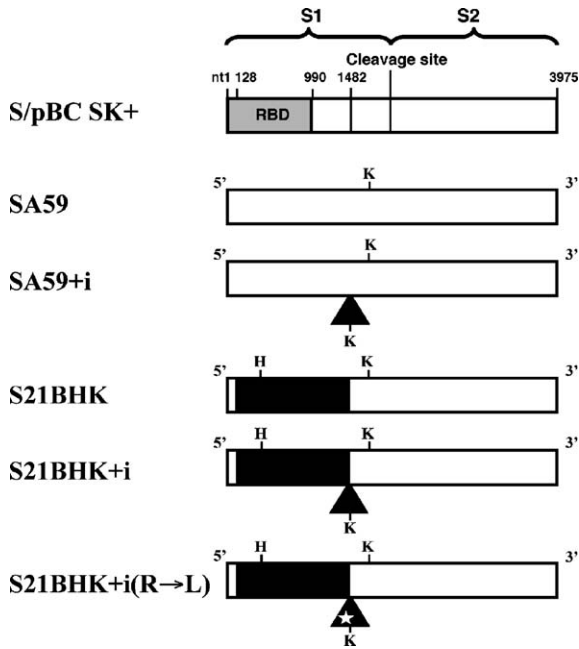


Fig. 1. Composition of S constructs used to introduce mutations into MHV-A59 using targeted RNA recombination. Mutations in the S gene of MHV/BHK were engineered into the S/pBC SK+ plasmid. These mutations result in 21 aa substitutions (Table 1) in the regions shaded in black, a 7-aa insert, TRTKKVL, (black triangles), and a R496L aa substitution (white star). The minimal receptor binding domain (RBD) for murine CEACAM1a is shaded in gray. The restriction enzyme sites *Hind*III (H) and *Kpn*I (K) used to screen recombinant viruses are also indicated.

and propagated in 17 Cl 1 cells. Most of the recombinant viruses formed homogeneous plaques on 17 Cl 1 cell monolayers, although all three recombinant viruses derived from the S21BHK construct formed both clear and turbid plaques (data not shown).

Restriction enzyme digestion and sequence analysis of recombinant viruses

To determine whether all three replicates of each recombinant virus contained S1 derived from MHV/BHK, we used restriction enzyme digestion of RT-PCR products of S genes from infected cells. RNA from 17 Cl 1 cells inoculated with MHV/BHK or the recombinant viruses or from mock-inoculated 17 Cl 1 cells was reverse transcribed and amplified by PCR. Amplification products were screened for the presence of diagnostic restriction enzyme sites *Hind*III and *Kpn*I in the S gene (Fig. 1). Digestion of amplification products with *Hind*III and *Kpn*I generated identical DNA fragment patterns for all three replicates of each recombinant virus (Fig. 2). These results strongly suggest that S1 of all of the recombinant viruses had the engineered mutations introduced during TRR.

To identify viruses that were free of adventitious mutations in the S gene, we sequenced the S genes of a subset of the recombinant viruses. Point mutations were found in the S genes of the S21BHK A, B, and C, and S21BHK+i(R→L) A viruses (Table 2). The S gene of S21BHK A had F845L and K997R substitutions in S2, the S gene of S21BHK B had a T423N substitution in S1, and the S gene of S21BHK C had a conservative I1043L substitution in S2. The S genes of the SA59 A, SA59+i A, S21BHK+i A, and S21BHK+i(R→L) C viruses were free of adventitious mutations. Although the S gene of S21BHK+i(R→L) B was free of adventitious mutations, the yield of this virus from 17 Cl 1 cells was over a 1000-fold lower than that of MHV-A59, and the S21BHK+i(R→L) A and C viruses, indicating that mutations outside of the S gene of S21BHK+i(R→L) B inhibited viral replication.

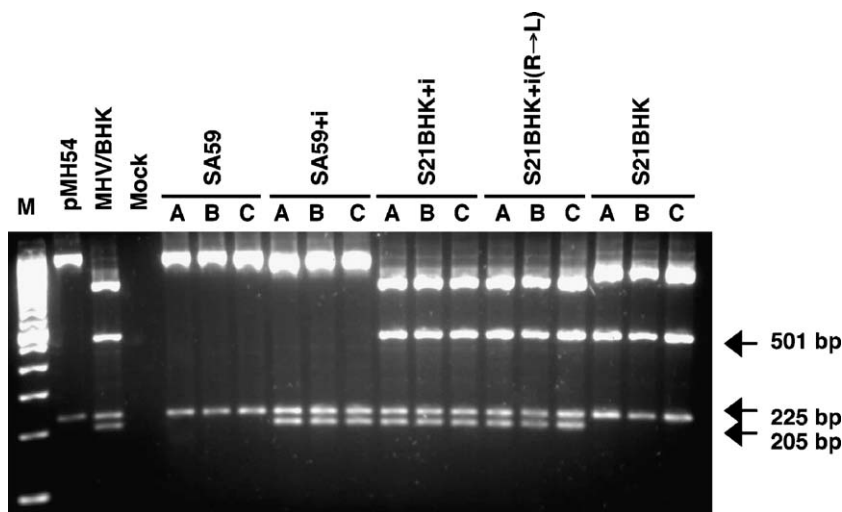


Fig. 2. Restriction enzyme digestion analysis of recombinant viruses. The 5' of the S genes of MHV/BHK, the recombinant viruses (A, B, and C), and the pMH54 plasmid were amplified using primers S(AvrII)+ and A59.C7. The amplification products were incubated with the restriction enzymes *Hind*III and *Kpn*I, and the DNA fragments were separated on a 4% agarose gel. Mutations derived from MHV/BHK generate a 501-bp fragment when cut with *Hind*III and a 205-bp fragment when cut with *Kpn*I.

Table 2
Adventitious mutations found in the S genes of recombinant viruses^a

Virus	Mutations ^b (nt)	Substitutions (aa)
SA59 A	-	-
SA59+i A	-	-
S21BHK A	{ T2545A A3000G	{ F845L K997R
S21BHK B	C1278A	T423N
S21BHK C	A3137T	I1043L
S21BHK+i A	-	-
S21BHK+i(R→L) A	C3543T	A1178V
S21BHK+i(R→L) B	-	-
S21BHK+i(R→L) C	-	-

^a S gene sequences are numbered according to GenBank accession no. AY497328.

^b S genes of all recombinant viruses contained engineered mutations introduced using targeted RNA recombination. – indicates no adventitious mutations.

Growth of recombinant viruses in murine cells

Some extended host range variants of MHV form small plaques on 17 Cl 1 cell monolayers compared to MHV-A59 (Baric et al., 1997; Schickli et al., 1997). We examined the plaque morphologies of the recombinant viruses that differed from MHV-A59 by the 21 aa substitutions, the 7-aa insert, or both the 21 aa substitutions and the 7-aa insert in S. The SA59, SA59+i, S21BHK, and S21BHK+i(R→L) viruses formed plaques with fuzzy, scalloped borders similar to MHV-A59, whereas the S21BHK+i viruses formed plaques with sharp, smooth borders similar to MHV/BHK (Fig. 3). The three recombinant SA59 viruses formed large plaques like MHV-A59, although each of the three replicates of the SA59+i, S21BHK, and S21BHK+i viruses formed plaques intermediate in size between those of MHV-A59 and MHV/BHK. The plaque morphologies shared by the three replicates of each mutant virus indicate that the engineered mutations in S1 were responsible for the observed phenotypes of these viruses. Although the S21BHK+i(R→L) A and C viruses formed plaques intermediate in size between those of MHV-A59 and MHV/BHK (Fig. 3), S21BHK+i(R→L) B formed plaques smaller than those of the S21BHK+i(R→L) A and C viruses (data not shown). This result supports the idea that mutations outside of the S gene of S21BHK+i(R→L) B reduced the growth of this virus in murine cells. Consequently, S21BHK+i(R→L) B was not studied further.

Differences in plaque morphology could be caused by alterations in binding or fusion due to mutant S genes or by differences in replication due to mutations in other viral genes. The host range variant MHV/BHK is delayed in viral RNA synthesis and expression of viral nucleocapsid protein (N) compared to MHV-A59 (Schickli et al., 1997). To ascertain whether the 21 aa substitutions, the 7-aa insert, or both the 21 aa substitutions and the 7-aa insert in S delayed or decreased MHV-A59 virus production, we examined the growth of the recombinant viruses in 17 Cl 1

cells. All 14 of the recombinant viruses formed syncytia with strong, cytoplasmic expression of N and caused extensive CPE in 17 Cl 1 cells at 8 h p.i., similar to MHV-A59 (Fig. 4A). When inoculated at a multiplicity of infection (MOI) of 10, as determined by plaque assay on 17 Cl 1 cell monolayers, the SA59 A, SA59+i A, S21BHK C, S21BHK+i A, and S21BHK+i(R→L) C viruses exhibited one-step growth kinetics in 17 Cl 1 cells similar to MHV-A59 (Fig. 4B). These results suggest that both the 21 aa substitutions, the 7-aa insert, in S altered the plaque-forming ability of MHV-A59 on murine cell monolayers by affecting either the binding or fusion activities of S.

Interaction of recombinant viruses with murine CEACAM1a

The extended host range variant MHV/BHK bound mCEACAM1a in a virus overlay protein blot assay (data not shown). However, unlike MHV-A59, MHV/BHK is not efficiently neutralized by soluble mCEACAM1a[1,4] at 37 °C (Schickli et al., 1997). To investigate whether the 21 aa substitutions, the 7-aa insert, or both the 21 aa substitutions and the 7-aa insert in S inhibited the neutralization of MHV-A59 by mCEACAM1a, we incubated the recombinant viruses with purified, anchorless mCEACAM1a[1,4]. The SA59 A, SA59+i A, S21BHK C, S21BHK+i A, and S21BHK+i(R→L) C viruses were neutralized by mCEACAM1a[1,4] at 37 °C like MHV-A59 (Fig. 5). These results show that the 21 aa substitutions, the 7-aa insert, or both in S did not inhibit the neutralization of MHV-A59 virions by mCEACAM1a.

The neutralization of the mutant viruses by soluble mCEACAM1a[1,4] at 37 °C suggested that soluble mCEACAM1a bound S1 on the mutant virions and induced an irreversible conformational change in S2-like wild-type MHV-A59 (Gallagher, 1997; Matsuyama and Taguchi, 2002; Zelus et al., 1998, 2003). To further characterize the interaction between the mutant viruses and mCEACAM1a, we examined the binding of recombinant S proteins to soluble mCEACAM1a[1,4]. Full-length, anchored wild type, and mutant S proteins were generated using a vaccinia virus expression system. The amount of S proteins in clarified cell extracts was normalized by immunoblotting with polyclonal anti-S A04 serum. Cell extracts were incubated with immobilized mCEACAM1a[1,4] that had been incubated with the blocking anti-mCEACAM1a MAb-CC1 or an isotype-matched control MAb. The binding of both wild type and mutant S proteins to mCEACAM1a was inhibited by MAb-CC1 (Fig. 6). The MAb-CC1 blockade of mCEACAM1a[1,4] did not prevent the binding of polyclonal anti-CEACAM1a 649 serum to mCEACAM1a (data not shown), indicating that additional epitopes on mCEACAM1a were not sterically inhibited by MAb-CC1. These results show that 21 aa substitutions, the 7-aa insert, or both in S did not prevent the binding of MHV-A59 to mCEACAM1a.

Virus variants generated during persistent infection of murine cells with MHV-A59 infect murine cells in the

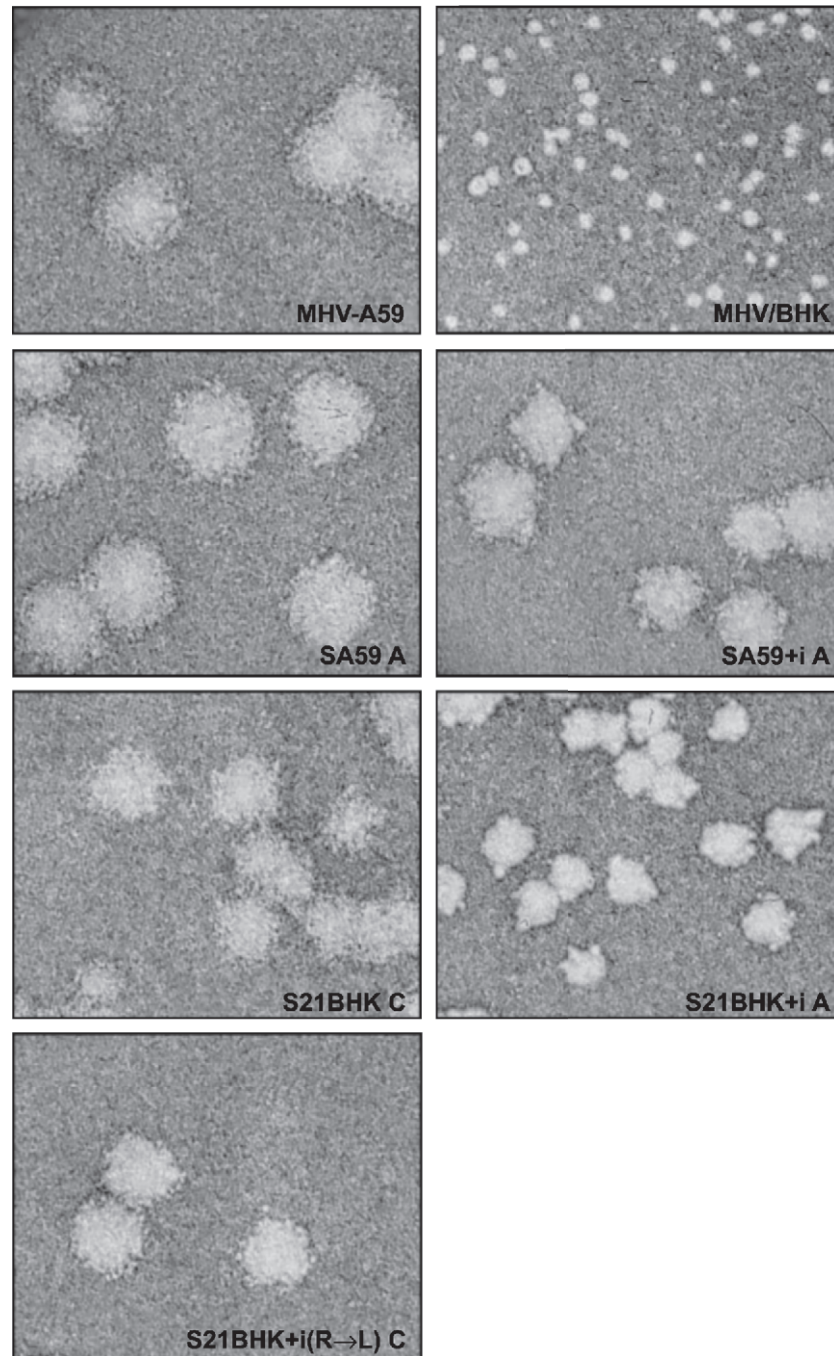


Fig. 3. Plaque phenotypes of recombinant viruses on murine 17 Cl 1 cell monolayers. Neutral red-stained plaques of MHV-A59, MHV/BHK, or the recombinant viruses at 72 h post inoculation (p.i).

presence of the anti-receptor MAb-CC1 that binds to an epitope on mCEACAM1a that overlaps, but is not identical to, the virus binding site (Chen and Baric, 1996; Schickli et al., 1997; Wessner et al., 1998). MAb-CC1 binds to mCEACAM1a with extremely high affinity and prevents MHV infection in vitro and in vivo (Smith et al., 1991; Williams et al., 1990). To further analyze the interactions of the recombinant viruses with mCEACAM1a, we examined the yields of the recombinant viruses from 17 Cl 1 cells treated with

MAb-CC1. The SA59 A, SA59+i A, and S21BHK C viruses did not infect 17 Cl 1 cells in the presence of 0.05 mg/ml of MAb-CC1 (Fig. 7A). In contrast, the S21BHK+i A and S21BHK+i(R→L) C viruses infected 17 Cl 1 cells even in the presence of 10-fold more MAb-CC1 (Fig. 7B). The S21BHK+i A and S21BHK+i(R→L) C viruses also infected 17 Cl 1 cells in the presence of polyclonal anti-mCEACAM1a 649 serum (Fig. 7B), supporting the idea that these mutant viruses interact with an alternative receptor on murine

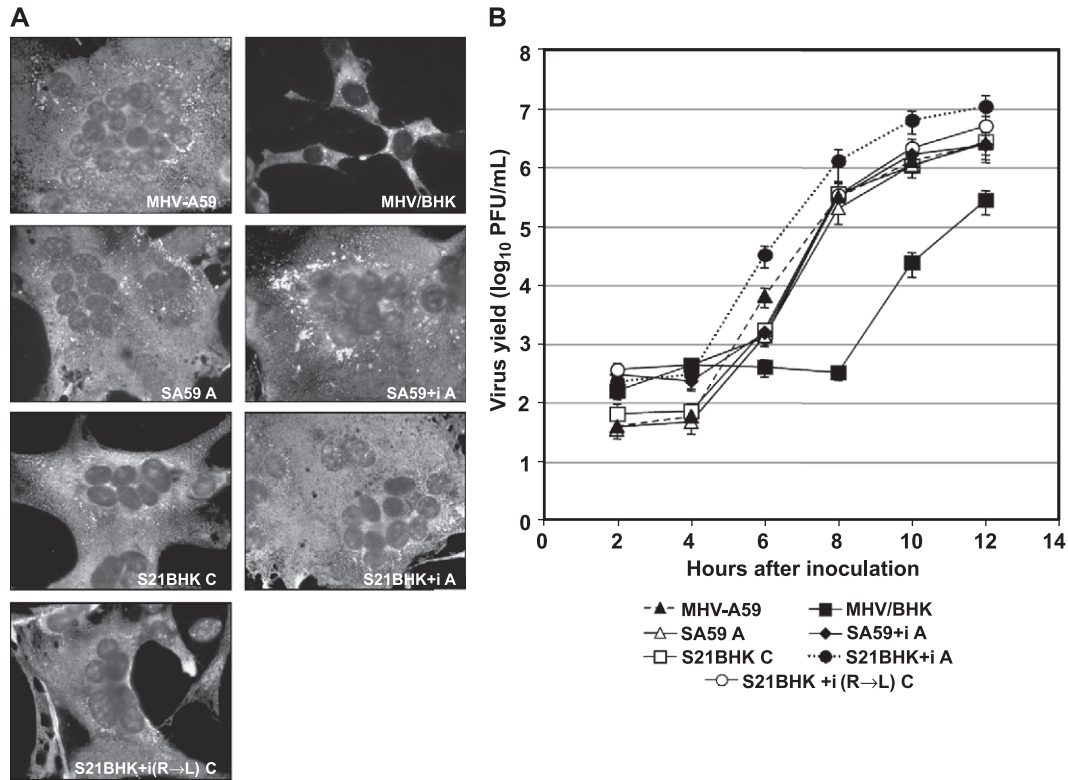


Fig. 4. Growth of recombinant viruses in murine 17 Cl 1 cells. (A) Expression of viral nucleocapsid protein (N) of MHV-A59, MHV/BHK, or the recombinant viruses was detected at 8 h p.i. with anti-N MAb. Magnification $\times 400$. (B) Yields of viruses released into tissue culture supernatants. Average virus yields \pm SEM of two independent experiments are shown.

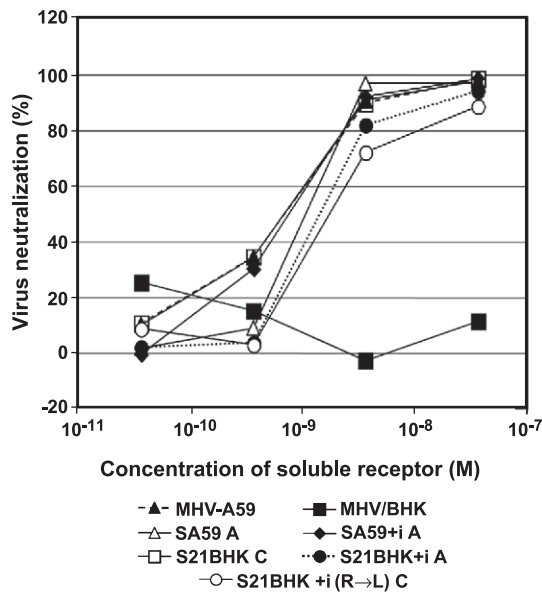


Fig. 5. Neutralization of recombinant viruses by soluble, murine CEACAM1a. MHV-A59, MHV/BHK, or the recombinant viruses (5000 PFU) were pre-incubated with serial dilutions of recombinant mCEACAM1a[1,4]. Virus survival was determined and percentage of neutralization calculated as described in Materials and methods. Percentage of neutralization shown is representative of two independent experiments.

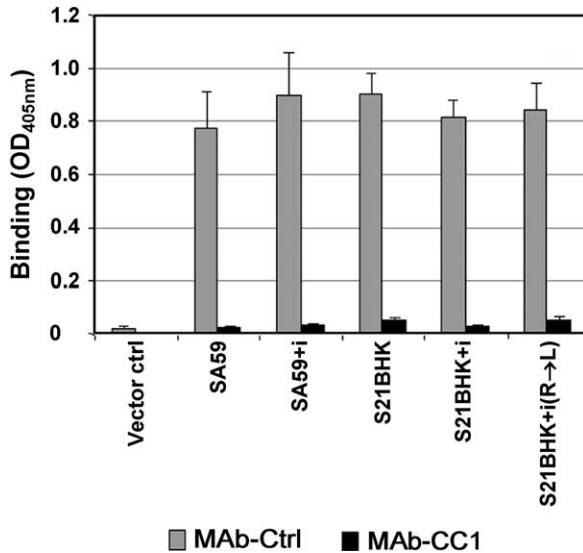


Fig. 6. Binding of recombinant S proteins to soluble, murine CEACAM1a. Immobilized mCEACAM1a[1,4] was incubated with 0.05 mg/ml of anti-mCEACAM1a MAb-CC1 or an isotype-matched MAb-Ctrl, and then incubated with full-length S proteins. Binding of S proteins was detected by immunoperoxidase labeling using polyclonal anti-S AO4 serum. Binding was calculated from the absorbance of mCEACAM1a incubated with S proteins minus the absorbance of mCEACAM1a alone. Average binding \pm SEM of two independent experiments is shown.

cells and not with alternative epitopes on mCEACAM1a. The yield of S21BHK+i(R→L) C was always lower than that of S21BHK+i A in the blockade experiments (Figs. 7A and B), suggesting that R496 in the insert may facilitate the interaction of the mutant viruses with an alternative receptor on murine cells.

Entry of recombinant viruses into non-murine cells

Since extended host range variants of MHV infect a wide range of non-murine cell lines (Baric et al., 1999, 1997; Schickli et al., 1997), we investigated the ability of the recombinant viruses with the 21 aa substitutions, the 7-aa insert, or both the 21 aa substitutions and the 7-aa insert to infect non-murine cell lines. Although Syrian hamster (BHK) cells are not susceptible to MHV-A59 infection, BHK cells stably transfected with mCEACAM1a[1–4] (called BHK+mCEACAM1a) are productively infected by MHV-A59 (Dveksler et al., 1991). MHV-A59, MHV/BHK, and the 14 recombinant viruses were inoculated at an MOI of 10, as determined by plaque assay on murine 17 Cl 1 cells. Although MHV-A59, MHV/BHK, and the recombinant viruses infected BHK+mCEACAM1a cells, only MHV/BHK and the S21BHK+i viruses infected BHK cells (Fig. 8). The S21BHK+i viruses induced syncytia in BHK cells, but MHV/BHK did not. The S of the S21BHK+i viruses was cleaved into 90-kDa S1 and S2 proteins like wild-type MHV-A59, although the S of MHV/BHK was uncleaved (data not shown). S21BHK+i A also infected Chinese hamster (CHO), feline (Fcowf), and monkey (Vero 76) cells, although the SA59

A, SA59+i A, S21BHK C, and S21BHK+i(R→L) C viruses did not infect any of the non-murine cells tested. Thus, both the 21 aa substitutions and the 7-aa insert in the N-terminus of S are sufficient to extend the host range of MHV-A59.

Growth of recombinant viruses in non-murine cells

The virus variant MHV/pi23 that was derived from passage 23 of murine cells persistently infected with MHV-A59 infects hamster BHK cells as shown by immunofluorescence assay (Schickli et al., 2004). However,

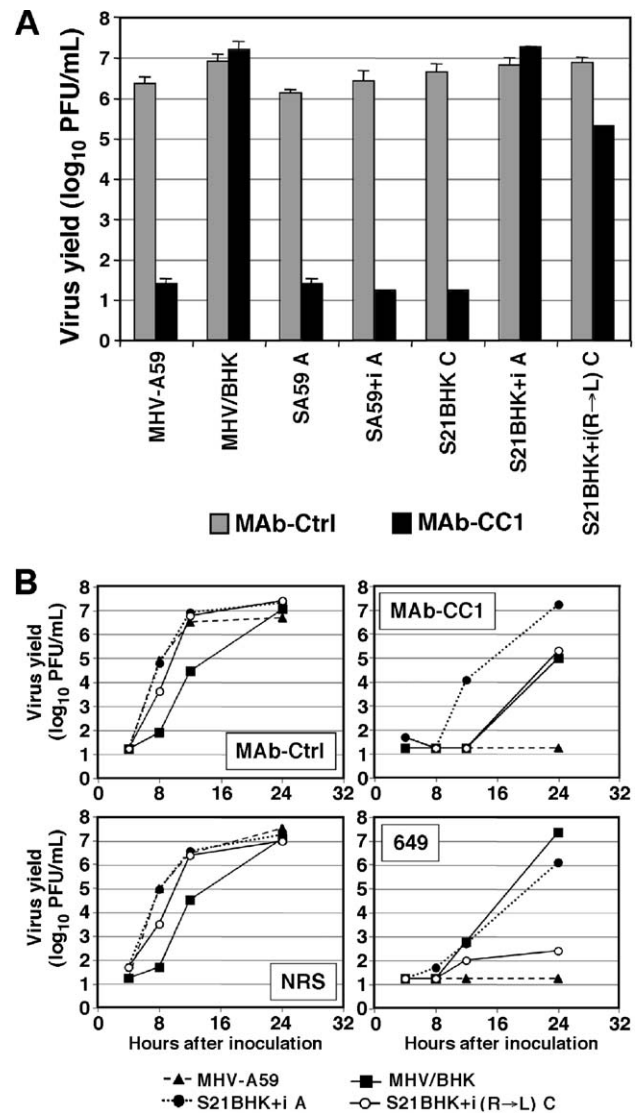


Fig. 7. Growth of recombinant viruses during anti-receptor antibody blockade of murine 17 Cl 1 cells. (A) Yields of MHV-A59, MHV/BHK, or the recombinant viruses at 24 h p.i. from cells treated with 0.05 mg/ml of anti-mCEACAM1a MAb-CC1 or an isotype-matched MAb-Ctrl before, during, and after inoculation. Average virus yields \pm SEM of two independent experiments are shown. (B) Yields of viruses from cells treated with 0.5 mg/ml of MAb-CC1 or MAb-Ctrl, or with polyclonal rabbit anti-mCEACAM1a serum (649) or normal rabbit serum (NRS) before, during, and after inoculation. Virus yields shown are representative of two independent experiments.

MHV/pi23 does not form plaques on BHK cell monolayers or productively infect BHK cells. We examined the plaque-forming ability of all 14 of the recombinant viruses on Syrian (BHK) and Chinese (CHO) hamster cells. Both the S21BHK+i and S21BHK+i(R→L) viruses formed plaques on BHK and CHO cell monolayers, although the SA59, SA59+i, and S21BHK viruses did not form plaques on either BHK or CHO cell monolayers (data not shown). The relative infectivities of the S21BHK+i A and S21BHK+i(R→L) C

Table 3

Relative infectivity of viruses in murine and hamster cells (PFU/ml)^a

Virus	Cell line		
	Murine 17 Cl 1	Hamster	
		BHK	CHO
MHV-A59	6.4×10^7	<3.3	<3.3
MHV/BHK	6.9×10^6	6.3×10^5	7.4×10^3
SA59 A	8.9×10^6	<3.3	<3.3
SA59+i A	9.1×10^6	<3.3	<3.3
S21BHK C	1.7×10^7	<3.3	<3.3
S21BHK+i A	3.0×10^7	4.5×10^4	2.6×10^4
S21BHK+i(R→L) C	2.4×10^7	3.1×10^4	1.3×10^4

^a Viruses were propagated in murine 17 Cl 1 cells. Average PFU/ml was derived from two independent plaque assays.

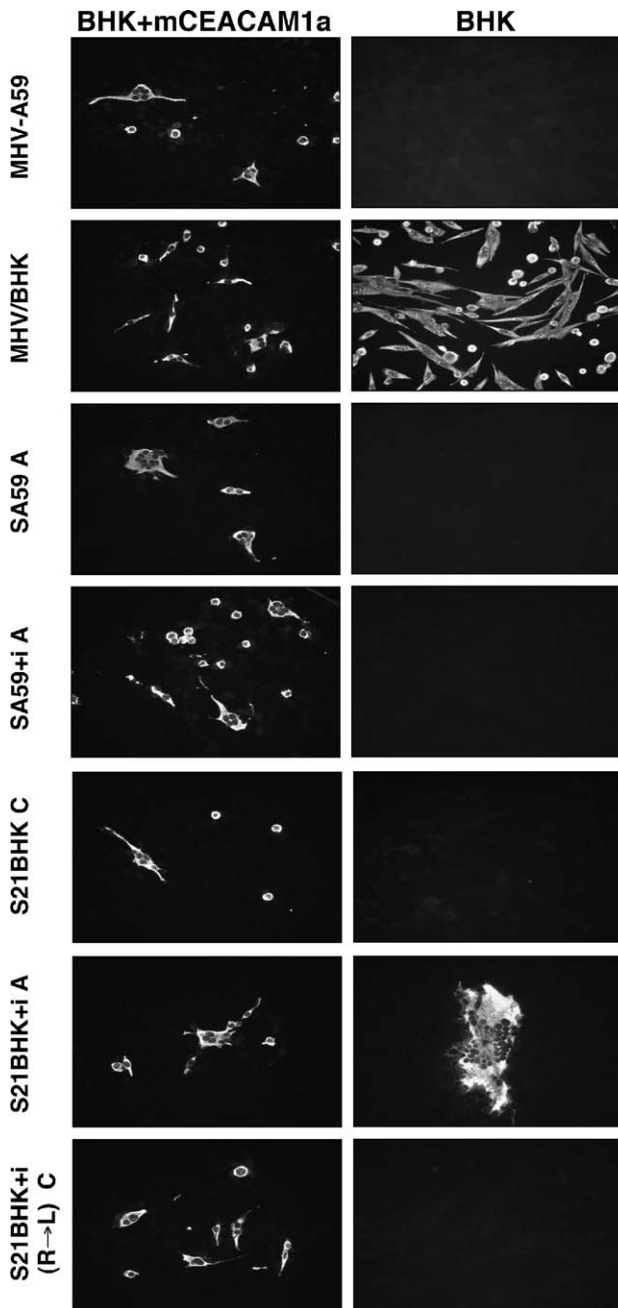


Fig. 8. Infection of hamster cells by recombinant viruses. Expression of viral N in hamster (BHK) cells and hamster cells stably transfected with mCEACAM1a[1–4] (BHK+mCEACAM1a) inoculated with MHV-A59, MHV/BHK, or the recombinant viruses were detected at 24 h p.i. as described in Fig. 4. Magnification $\times 200$.

viruses was 1000-fold lower in BHK and CHO cells than murine 17 Cl 1 cells, although the relative infectivity of MHV/BHK was 10-fold lower in BHK cells and 1000-fold lower in CHO cells than 17 Cl 1 cells (Table 3). The plaques of the S21BHK+i(R→L) viruses were turbid and diffuse in contrast to the clear plaques of MHV/BHK and the S21BHK+i viruses (data not shown). These results suggest that R496 in the insert may facilitate the interaction of the mutant viruses with an alternative receptor on non-murine cells.

To ascertain whether the 21 aa substitutions and the 7-aa insert in S permit MHV-A59 to productively infect non-murine cells, we measured the yield of S21BHK+i A from BHK cells and BHK cells stably transfected with mCEACAM1a[1–4] (BHK+mCEACAM1a). When inoculated at an MOI of 0.1, as determined by plaque assay on BHK cells, BHK cells yielded 3.5×10^4 PFU/ml of S21BHK+i A virions into the tissue culture supernatant at 16 h p.i. (Fig 9A). Unlike MHV/BHK, the yield of S21BHK+i A decreased after 16 h p.i., suggesting that the S21BHK+i viruses did not undergo a second round of replication in BHK cells under liquid medium. However, BHK+mCEACAM1a cells yielded 2.8×10^6 PFU/ml of S21BHK+i A virions at 72 h p.i. (Fig. 9A). When inoculated at an MOI of 0.1, as determined by plaque assay on BHK cells, the percentage of BHK cells infected by S21BHK+i A decreased from 0.1% at 24 h p.i. to undetectable levels at 72 h p.i. (Fig. 9B). In contrast, the percentage of BHK cells infected with MHV/BHK increased from 0.1% at 24 h p.i. to 100% at 72 h p.i. These results suggest that although both the 21 aa substitutions and the 7-aa insert in S permit MHV-A59 to enter and productively infect BHK cells, one or more of the additional 35 aa substitutions in the S of MHV/BHK may be needed to permit multiple cycles of infection in BHK cells under liquid medium.

Discussion

Extended host range variants of MHV are generated during persistent infection of murine cell cultures or cocultures of murine and hamster cells, and these viruses have

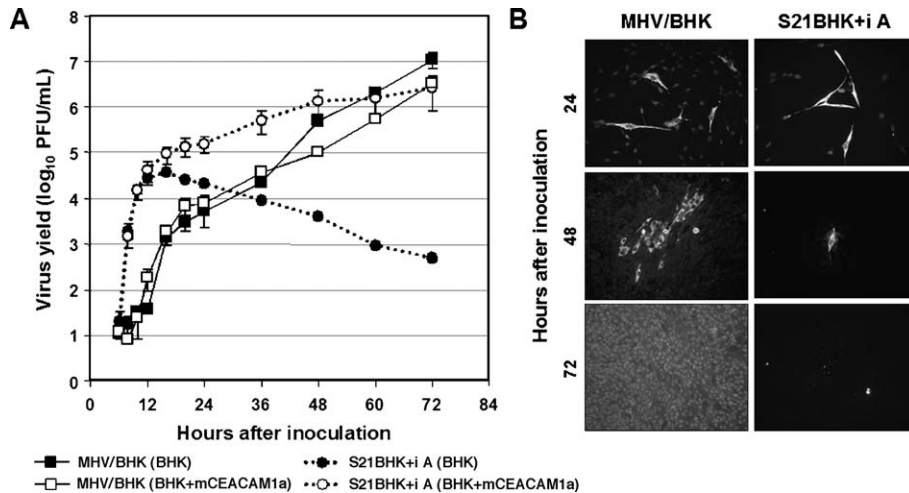


Fig. 9. Growth of mutant viruses in hamster cells. (A) Yields of MHV/BHK or S21BHK+i A from hamster (BHK) cells or hamster cells stably transfected with mCEACAM1a[1–4] (BHK+mCEACAM1a). Virus titers were determined using murine 17 Cl 1 cell monolayers. Average virus yields \pm SEM of two independent experiments are shown. (B) Spread of MHV/BHK or S21BHK+i A in BHK cell monolayers. Expression of viral N was detected as described in Fig. 4. Magnification $\times 200$.

aa substitutions in their S glycoproteins (Baric et al., 1997, 1999; Schickli et al., 2004). However, the role of aa substitutions in S in the host range of MHV had not been previously studied using isogenic recombinant viruses that differ only in S. This study demonstrated that both the 21 aa substitutions and the 7-aa insert found in N-terminal region of S of MHV/BHK are sufficient to permit the interaction of MHV-A59 with alternative receptors on murine and non-murine cells. Interestingly, although 12 of the 21 aa substitutions lie within of the N-terminal 330 aa of S that comprise the minimal receptor binding domain (RBD) for mCEACAM1a *in vitro*, 9 of the 21 aa substitutions and the 7-aa insert lie outside of the RBD (Kubo et al., 1994; Tsai et al., 2003). Viruses with both the 21 aa substitutions and the 7-aa insert (S21BHK+i) infected murine cells in the presence of blocking anti-mCEACAM1a MAb-CC1 or polyclonal 649 serum that inhibit MHV-A59 infection of murine cells (Fig. 7). The S21BHK+i viruses also infected Syrian and Chinese hamster, feline, and monkey cell lines that are normally resistant to MHV-A59 infection. The alternative receptor(s) used by the S21BHK+i viruses and MHV/BHK have not yet been identified. However, the extended host range variant MHV-H2 generated during persistent MHV infection of co-cultures of murine and hamster cells can utilize human carcinoembryonic antigen (hCEA) as a receptor (Baric et al., 1997; Hensley and Baric, 1998).

This study demonstrates that the 7-aa insert (TRTKKVP) at aa 494 in S is necessary, but not sufficient to permit MHV-A59 to interact with alternative receptors on murine and non-murine cells. Although adventitious mutations were present in the S genes of all three replicates of the S21BHK virus, the identical growth phenotypes of the independently generated replicates strongly suggests that the 21 aa substitutions in S1 derived from MHV/BHK, rather than adventitious mutations

in the S gene, are responsible for the phenotypes of these viruses in murine and non-murine cells.

Viruses with the 21 aa substitutions (S21BHK) or the 7-aa insert (SA59+i) in S could only utilize the murine CEACAM1a receptor since they did not infect murine cells in the presence of the blocking anti-mCEACAM1a MAb-CC1 (Fig. 7). In addition, the S21BHK and SA59+i viruses did not infect any of the non-murine cell lines tested. However, the 7-aa insert is not necessary for the interaction of the S21BHK+i viruses with mCEACAM1a. The S21BHK and S21BHK+i viruses grew equally well in murine cells and were similarly neutralized by soluble mCEACAM1a at 37 °C (Figs. 4 and 5). In addition, both recombinant S proteins with the 21 aa substitutions and recombinant S proteins with both the 21 aa substitutions and the 7-aa insert bound to soluble mCEACAM1a (Fig. 6).

R496 in the insert may play an important role in the interaction of the 7-aa insert with host cell membranes. Viruses with an additional R496L substitution in the insert [S21BHK+i(R \rightarrow L)] infected murine cells in the presence of blocking anti-mCEACAM1a MAb-CC1 or polyclonal 649 serum, yet the yields of the S21BHK+i(R \rightarrow L) viruses released during antibody blockade were always lower than those of the S21BHK+i viruses (Fig. 7). In addition, although the relative infectivities of the S21BHK+i and S21BHK+i(R \rightarrow L) viruses on hamster cell monolayers under agar were similar, the S21BHK+i(R \rightarrow L) viruses did not infect hamster cells under liquid medium (Table 3 and Fig. 8). Agar may serve to decrease diffusion of S21BHK+i(R \rightarrow L) viruses away from host cell membranes and may promote increased interaction of between the mutant viruses and a putative alternative receptor(s).

Low-affinity interactions between a region of concentrated positive charge in the 7-aa insert and negatively charged moieties on host cell membranes, such as sialic acid or

heparan sulfate, may facilitate the interaction of S21BHK+i viruses with alternative receptors on murine and non-murine cells. Like the 7-aa insert, the HE glycoprotein expressed on the envelope of most group II coronaviruses may enhance viral infectivity by concentrating the virus at host cell membranes. A MAb directed against the HE of bovine coronavirus (BCoV) partially inhibits virus infectivity, although expression of recombinant HE of BCoV is not sufficient to permit a MHV pseudotype virus to infect human HRT-18G cells that are normally permissive for BCoV infection (Deregt et al., 1989; Popova and Zhang, 2002; Schultze and Herrler, 1992).

The S21BHK+i viruses have an inefficient interaction with alternative receptors on non-murine cells, while maintaining a wild-type interaction with murine CEACAM1a. The S21BHK+i viruses had 1000-fold lower relative infectivity in hamster cells than murine cells (Table 3). However, S21BHK+i virions bound mCEACAM1a in a virus overlay protein blot assay (data not shown) and were neutralized by soluble mCEACAM1a[1,4] at 37 °C like MHV-A59 (Fig. 5). In addition, recombinant S proteins with both the 21 aa substitutions and the 7-aa insert bound to soluble mCEACAM1a[1,4] using an epitope that is blocked by MAb-CC1 just like wild-type S proteins (Fig. 6). Although both the 21 aa substitutions and the 7-aa insert in S permit MHV-A59 to productively infect non-murine cells, additional aa substitutions in S or mutations in other viral genes may be needed for efficient infection of non-murine cells. The S21BHK+i viruses entered and productively infected hamster BHK cells, but, unlike MHV/BHK, the S21BHK+i viruses did not induce a second round of infection in BHK cells under liquid medium (Fig. 9B).

The use of isogenic recombinant viruses differing only in S has begun to elucidate the molecular determinants that permit MHV to utilize an alternative receptor(s) on murine and non-murine cells. MHV infection of murine cells is associated with the rapid reduction of mCEACAM1a expression levels (Chen and Baric, 1996; Rao and Gallagher, 1998; Sawicki et al., 1995). Mutations that enhance the affinity or avidity of S for mCEACAM1a or delay the triggering of S may be selected during persistent infection. These mutations in the S gene may also permit the interaction of MHV with alternative receptors on murine and non-murine cells. Although these variants may initially have inefficient interactions with alternative receptors, continued selection in persistently infected murine or non-murine cells may select for additional mutations in the S gene that optimize the interaction of MHV with alternative receptors. During continued passage in non-murine cells, extended host range variants of MHV may lose the ability to interact with mCEACAM1a and infect murine cells (Hensley et al., 1998).

Viruses can emerge in a new host either due to social or ecological factors that perturb the environment or genetic changes in the virus that allow entry, replication, or dissemination in a new host (Ludwig et al., 2003; Wool-

house, 2002). Although the mechanisms underlying the 2002 emergence of SARS-CoV in humans have not yet been identified, the complete genome sequence of SARS-CoV (Marra et al., 2003; Rota et al., 2003) proved that mutation or recombination between previously known coronaviruses was not responsible for the emergence of this virus in humans. Instead, the emergence of SARS-CoV may reflect increased contact between animal reservoirs and human hosts at exotic food markets in China, genetic changes in or recombination between ancestral or previously unknown coronaviruses, or a combination of environmental and genetic changes (Cyranoski and Abbott, 2003; Holmes, 2003; Rest and Mindell, 2003; Stanhope et al., 2004; Stavrinos and Guttman, 2004). The work described in this study suggests that relatively few aa substitutions in S may be sufficient to permit the introduction of a coronavirus into a new host species. Viruses closely related to human SARS-CoV isolates have been isolated from Himalayan palm civets and a raccoon dog (Guan et al., 2003). One or more of the 10 aa substitutions in S that differ between the known animal and human SARS-CoV isolates may have extended the host range of the animal virus. Further studies are needed to examine the role of genetic change in the host range of SARS-CoV to evaluate the possibility of a reintroduction of SARS-CoV in humans from zoonotic reservoirs.

Materials and methods

Antibodies, cell lines, and viruses

A mouse monoclonal antibody (MAb) to the MHV nucleocapsid protein (N) (anti-N MAb) was kindly provided by Julian Leibowitz (Department of Pathology and Laboratory Medicine, Texas A&M University, College Station, TX). Mouse anti-mCEACAM1a MAb-CC1 and polyclonal rabbit anti-CEACAM1a 649 serum block MHV binding and infection of murine cells (Dveksler et al., 1991; Williams et al., 1990). A mouse MAb directed against the β subunit of cholera toxin (MAb-Ctrl) was used as an isotype-matched control for anti-N MAb and MAb-CC1. Polyclonal goat anti-S AO4 serum neutralizes MHV virions (Frana et al., 1985).

The 17 Cl 1 line of BALB/c 3T3 fibroblasts, *Felis catus* whole fetus (Fcwf) cells, baby hamster kidney BHK-21 (BHK) cells, BHK cells stably transfected with murine CEACAM1a cDNA (BHK+mCEACAM1a) (Dveksler et al., 1991), and African green monkey kidney (Vero 76) cells were propagated as previously described (Schickli et al., 1997). Chinese hamster ovary (CHO) cells [from American Type Culture Collection (ATCC), Rockville, MD] were propagated in Minimal Essential Medium alpha (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT), 2% antibiotic-antimycotic (PSF; GIBCO), and 10

mM HEPES buffer solution (GIBCO). *F. catus* lung epithelial (AK-D) cells (ATCC) were propagated in Ham's F12K medium (GIBCO) with 10% FBS and 2% PSF.

MHV-A59 and the host range variant MHV/BHK, derived from 17 Cl 1 cells persistently infected with MHV-A59, were propagated in 17 Cl 1 cells as previously described (Schickli et al., 1997). The S genes of MHV-A59 (GenBank accession no. AY497328), MHV/BHK (GenBank accession no. AY497331) and all recombinant viruses generated in this paper have three mutations compared to the S gene sequence published by Luytjes et al. (1987) and Schickli et al. (2004). The chimeric helper virus fMHV used in targeted RNA recombination (TRR) experiments in this paper was kindly provided by Paul Masters (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY) and propagated in Fcwf cells as previously described (Kuo et al., 2000). Virus titers were measured by plaque assay on 17 Cl 1, BHK, Fcwf, or AK-D cells as previously described (Gagneten et al., 1995; Kuo et al., 2000).

Generation of S constructs

The constructs (Fig. 1) used in the experiments in this paper were assembled in pBC SK+ (Stratagene, La Jolla, CA) and used to replace the S gene of pMH54. The transcription vector pMH54, containing the 3' -most 7.4 kb of the MHV genome, was provided by Paul Masters (Wadsworth Center for Laboratories and Research). S gene sequences in this paper were numbered according to GenBank accession no. AY497328 (Schickli et al., 2004).

Site-directed mutagenesis of cDNA templates was performed essentially as described previously (Wentworth and Holmes, 2001). Mutant templates were amplified with cloned Pfu DNA polymerase (PFU; Stratagene) using various primer pairs as specified by the manufacturer. Mutant templates were screened by restriction enzyme digestion or sequence analysis. Site-directed mutagenesis using primers S(*AvrII*)⁺ and S(*AvrII*)⁻ (Table 4) was used to introduce a silent *AvrII* site at nt 37 and 38 of the S/pBS SK+ (Stratagene) construct. The *AvrII* mutation was subcloned into pBC SK+ (Stratagene) from S/pBS SK+ using *Bam*HI sites to generate S/pBC SK+. Two fragments incorporating the 21-bp insert of MHV/BHK (ACTCG-GACTAAGAAGGTACCA) were amplified using primers A59.3 and A59.24 or A59.23 and A59.20 (Table 4 and Schickli et al., 2004). PCR amplifications were run for five cycles of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C using Pfu (Stratagene). The PCR products were mixed and amplified using primers A59.3 and A59.20 for 30 cycles as described above. The amplicon was subcloned into S/pBC SK+ using an *Af*III site at nt 332 and a *Bst*EII site at nt 1508 to generate SA59+i. The 5' region of the S gene of MHV/BHK was amplified using primers S(*AvrII*)⁺ and A59.6 (Table 4 and Schickli et al.,

Table 4

Oligonucleotides used for mutagenesis, RT-PCR, and sequencing

Primer	Sequence (5' -3') ^a
<i>Forward</i>	
A59.23	actcggactaagaaggtaccaAAGCCTAAGTCTGCTTTG
A59.30	ACTGGTGCTATGAGTTATCC
S(IGS)	ATTTTATGACGGATAGCGGTG
Span	CTACTCAGACTAAGCCTAAGTC
insert+	
S(<i>AvrII</i>) ⁺	ATTCTATTTTTGCCCTCTTGccTAGGGTATATTGG
Sstart	ATGCTGTTCGTGTTTATTC
<i>Reverse</i>	
4a(IGS)	ACGGCCATAGCTATGACTGC
A59.24	aggtacctcttagtccgagtAGTCTGAGTAGTGCAAGGGC
G133D-	GGAAGTATAGCCAAACAAGCTiCTATAACTATAGTAGG
Span	GACTTAGGCTTAGTCTGAGTAG
insert-	
S(<i>AvrII</i>) ⁻	ATCTAAAATCACCAATATACCCTAaggCAAGAGGGC

^a Nucleotides that differ from the S gene sequence of MHV-A59 (GenBank accession no. AY497328) are shown in lower case.

2004). The amplicon was subcloned into S/pBC SK+ using *AvrII* and *Bst*EII to generate S21BHK+i. Sequence analysis of S21BHK+i clones identified a clone with one coding mutation (G1487T) in the S gene generating S21BHK+i(R→L). Site-directed mutagenesis using primers Span insert+ and Span insert- (Table 4) was used to eliminate the 21-bp insert from S21BHK+i, and the construct was subcloned into S/pBC SK+ using *AvrII* and *Bst*EII to generate S21BHK. Following assembly in S/pBC SK+, all four S constructs were subcloned into pMH54 using *AvrII* and a *Xho*I site at nt 2486. The 5' end of each construct was sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility using ABI Prism kits (Applied Biosystems, Foster City, CA) as previously described (Wentworth and Holmes, 2001). Eight primers: S(IGS), A59.3, A59.4, A59.6, A59.7, A59.8, A59.13, and A59.14 (Table 4 and Schickli et al., 2004) were used to generate overlapping sequence for each construct.

Targeted RNA recombination

Mutations were introduced into the S gene of MHV-A59 by targeted RNA recombination (TRR) between pMH54-derived donor RNA containing engineered mutations and the chimeric helper virus fMHV essentially as previously described (Kuo et al. 2000). Briefly, confluent Fcwf cells were inoculated with fMHV (MOI 0.5). Capped transcripts were synthesized from *Pac*I-linearized pMH54 constructs with an mMessage mMachine T7 kit (Ambion, Austin, TX) as specified by the manufacturer. Donor RNA from each S construct was transfected into fMHV-inoculated Fcwf cells using a Gene Pulser II electroporation apparatus (BIO-RAD, Hercules, CA). Infected and transfected Fcwf cells were immediately overlaid onto 17 Cl 1 cells monolayers in triplicate. After 48 h, the culture media were collected,

clarified by centrifugation, and flash frozen. Three replicates (A, B, and C), independently derived from each S construct, were plaque-purified, and propagated in 17 Cl 1 cells.

Restriction enzyme digestion and sequence analysis of recombinant viruses

Total cellular RNA from 17 Cl 1 cells inoculated with MHV/BHK or the recombinant viruses was extracted with RNAzol B (Tel-Test, Inc., Friendswood, TX) and reverse transcribed with M-MLV reverse transcriptase (GIBCO) using primers A59.6 or 4a(IGS) (Schickli et al., 2004) as specified by the manufacturer. The cDNA was amplified with primers S(AvrII)+ and A59.C7 (Table 4 and Schickli et al., 2004). PCR amplifications were run for 32 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C using Taq DNA polymerase (Taq; Fisher Scientific, Pittsburgh, PA). The amplification products were incubated with *Hind*III and *Kpn*I. DNA fragments were separated on a 4% NuSieve agarose gel (BioWhittaker Molecular Applications, Rockland, ME) and visualized by ethidium bromide staining. *Kpn*I digestion of amplification products from all S genes generated a 225-bp fragment due to the presence of a ubiquitous *Kpn*I site at nt 1705 (Fig. 1).

To sequence the S genes of plaque-purified recombinant viruses, cDNA was amplified using four primer pairs: Sstart and A59.4, A59.5 and A59.6, A59.7 and A59.16, and A59.17 and 4a(IGS) (Table 4 and Schickli et al., 2004). PCR amplifications were run for 32 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C using Taq. Following purification with 30 000 NMWL filter units (Millipore Corp., Bedford, MA), the amplification products were sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility, as described above. Fifteen primers, Sstart, G133D-, A59.3, A59.4, A59.30, A59.22, A59.6, A59.7, A59.20, A59.19, A59.8, A59.9, A59.10, A59.11, and 4a(IGS) (Table 4 and Schickli et al., 2004), generated overlapping sequence for each S gene.

Detection of virus-infected cells

Virus inoculation of cells grown on coverslips and detection of newly synthesized viral nucleocapsid protein (N) were performed essentially as described previously (Dveksler et al., 1996; Schickli et al., 1997). Briefly, cells in 60-mm dishes were inoculated with 1.5 ml of virus for 3 h at 37 °C. At various time points post-inoculation (p.i.), cells were washed with phosphate-buffered saline (PBS) and fixed in methanol/acetic acid at –20 °C. Expression of N was detected with anti-N MAb followed by FITC-conjugated goat anti-mouse IgG. Controls that showed no immunolabeling were mock-inoculated cells incubated with anti-N MAb and virus-inoculated cells incubated with an isotype control MAb. Susceptibility of each cell line to each recombinant virus was tested in three independent experiments.

Determination of virus yields from murine or hamster cells

Murine cells grown in 60-mm dishes were inoculated with 1 ml of diluted virus for 1 h at 37 °C, whereas hamster cells were inoculated for 3 h at 37 °C. Cells were washed 3× with PBS and incubated with polyclonal goat anti-S AO4 serum for 1 h at 37 °C to neutralize unabsorbed virions. Cells were then washed 3× with PBS and 2× with media. The AO4 neutralization and wash were repeated twice. Cells were incubated in fresh media at 37 °C, and culture media were collected at various time points post-inoculation.

Neutralization of virus with soluble, murine CEACAM1a[1,4]

Virions were incubated with purified, anchorless mCEACAM1a[1,4] essentially as previously described (Zelus et al., 1998). Briefly, 30 µl of virus (5000 PFU) were pre-incubated with 180 µl of soluble mCEACAM1a[1,4] diluted in Tris-buffered saline with 5% glycerol (TBS-G) and 0.1 mg/ml bovine serum albumin Fraction V (BSA) or TBS-G with 0.1 mg/ml BSA alone as a control. After incubation for 1 h at 37 °C, virus survival was determined by plaque assay on 17 Cl 1 cell monolayers. Percentage of virus neutralization was calculated as: $100 - [(number\ of\ plaques\ from\ virus\ incubated\ with\ mCEACAM1a[1,4]) / (number\ of\ plaques\ from\ virus\ incubated\ with\ buffer\ alone)] \times 100$.

Receptor blockade with anti-murine CEACAM1a antibodies

Growth of virus in the presence of anti-CEACAM1a antibodies was performed essentially as described previously (Schickli et al., 1997). Briefly, 17 Cl 1 cells grown in 60-mm dishes were pretreated for 1 h with 1 ml of diluted anti-mCEACAM1a antibody or control antibody at 37 °C. MAb-CC1 and MAb-Ctrl were diluted to a final concentration of 0.05 or 0.5 mg/ml in growth medium, although polyclonal rabbit 649 serum and normal rabbit serum were diluted 1:10 in growth medium. 17 Cl 1 cells were inoculated with virus (MOI 10) in the presence of anti-mCEACAM1a or control antibody. After adsorption for 3 h at 37 °C, cells were washed 3× with PBS and 2× with media. Cells were incubated in fresh media at 37 °C in the presence of anti-mCEACAM1a or control antibody, and culture media were collected at various time points post-inoculation.

Expression and detection of recombinant S proteins

Full-length S proteins were produced from S constructs (Fig. 1) using a recombinant vaccinia virus, MVA-T7 pol, kindly provided by Dr. Bernard Moss (Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD). Hamster BHK cells grown in 100-mm dishes were inoculated with 900 µl of MVA-T7 pol (MOI 5). After adsorption for 1 h at 37 °C, BHK cells were incubated for 3 h at 37 °C in serum-free media. Fifteen micrograms of DNA encoding

the S constructs was transfected into MHV-T7 pol-infected BHK cells using 45 μ l of Lipofectamine 2000 (GIBCO), as specified by the manufacturer. An empty pBC SK+ vector was used to control for nonspecific binding of cell extracts. After 24 h at 37 °C, cell extracts were solubilized, clarified by centrifugation, and passed twice through a 0.22- μ m filter.

Cell extracts or purified virions were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp.), and blocked overnight at 4 °C in 25 mM Tris (pH 7.6), 150 mM NaCl and 0.05% Tween 20 (TBST) supplemented with 5% nonfat powdered milk (NFM) essentially as described previously (Zelus et al., 1998). S proteins were detected with polyclonal anti-S AO4 serum followed by HRP-conjugated rabbit anti-goat IgG. Bound horseradish peroxidase complexes were visualized using Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Inc., Boston, MA).

Binding of recombinant S proteins to soluble, murine CEACAM1a[1,4]

Ninety-six-well flat bottom microtiter plates (Immulon; Dynex Technologies, Inc., Chantilly, VA) were coated at 37 °C with 1 μ g/ml of purified, soluble mCEACAM1a[1,4] diluted in 0.1 M sodium carbonate buffer (pH 9.0) and blocked overnight at 4 °C with 150 mM NaCl, 50 mM Tris-HCl, 80 mM EDTA, 0.05% Tween 20, 0.1% BSA, pH 7.4, with 5% BSA essentially as previously described (Bonavia et al., 2003). Coated plates were incubated with 0.05 mg/ml MAb-CC1 or MAb-Ctrl, and incubated with vaccinia virus-infected cell extracts at room temperature. Binding of S proteins in cell extracts was detected using polyclonal AO4 serum followed by HRP-conjugated rabbit anti-goat IgG. Immobilized mCEACAM1a was detected using anti-mCEACAM1a 649 serum followed by HRP-conjugated donkey anti-rabbit IgG. Bound horseradish peroxidase complexes were detected using 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS Peroxidase Substrate System; KPL, Gaithersburg, MD). Binding is a measure of mean absorbance at 405 nm performed in duplicate. Absorbance at 405 nm was measured at 5 min for recombinant S proteins and 1 min for mCEACAM1a.

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References

- Baric, R.S., Yount, B., Hensley, L., Peel, S.A., Chen, W., 1997. Episodic evolution mediates interspecies transfer of a murine coronavirus. *J. Virol.* 73 (1), 1946–1955.
- Baric, R.S., Sullivan, E., Hensley, L., Yount, B., Chen, W., 1999. Persistent infection promotes cross-species transmissibility of mouse hepatitis virus. *J. Virol.* 73 (1), 638–649.
- Beauchemin, N., Draber, P., Dveksler, G., Gold, P., Gray-Owen, S., Grunert, F., Hammarstrom, S., Holmes, K.V., Karlsson, A., Kuroki, M., Lin, S.H., Lucka, L., Najjar, S.M., Neumaier, M., Obrink, B., Shively, J.E., Skubitzi, K.M., Stanners, C.P., Thomas, P., Thompson, J.A., Virji, M., von Kleist, S., Wagener, C., Watt, S., Zimmermann, W., 1999. Redefined nomenclature for members of the carcinoembryonic antigen family. *Exp. Cell Res.* 252 (2), 243–249.
- Bonavia, A., Zelus, B.D., Wentworth, D.E., Talbot, P.J., Holmes, K.V., 2003. Identification of a receptor-binding domain of the spike glycoprotein of human coronavirus HCoV-229E. *J. Virol.* 77 (4), 2530–2538.
- Bos, E.C., Luytjes, W., Spaan, W.J., 1997. The function of the spike protein of mouse hepatitis virus strain A59 can be studied on virus-like particles: cleavage is not required for infectivity. *J. Virol.* 71 (12), 9427–9433.
- Bosch, B.J., van der Zee, R., de Haan, C.A., Rottier, P.J., 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J. Virol.* 77 (16), 8801–8811.
- Breslin, J.J., Mork, I., Smith, M.K., Vogel, L.K., Hemmila, E.M., Bonavia, A., Talbot, P.J., Sjostrom, H., Noren, O., Holmes, K.V., 2003. Human coronavirus 229E: receptor binding domain and neutralization by soluble receptor at 37 degrees C. *J. Virol.* 77 (7), 4435–4438.
- Chen, W., Baric, R.S., 1996. Molecular anatomy of mouse hepatitis virus persistence: coevolution of increased host cell resistance and virus virulence. *J. Virol.* 70 (6), 3947–3960.
- Compton, S.R., Stephensen, C.B., Snyder, S.W., Weismiller, D.G., Holmes, K.V., 1992. Coronavirus species specificity: murine coronavirus binds to a mouse-specific epitope on its carcinoembryonic antigen-related receptor glycoprotein. *J. Virol.* 66 (12), 7420–7428.
- Cyranoski, D., Abbott, A., 2003. Virus detectives seek source of SARS in China's wild animals. *Nature* 423 (6939), 467.
- Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjostrom, H., Noren, O., Laude, H., 1992. Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* 357, 417–420.
- Deregt, D., Gifford, G.A., Ijaz, M.K., Watts, T.C., Gilchrist, J.E., Haines, D.M., Babiuk, L.A., 1989. Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: demonstration of in vivo virus-neutralizing activity. *J. Gen. Virol.* 70, 993–998.
- Drosten, C., Gunther, S., Preiser, W., van der Werf, S., Brodt, H.R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R.A., Berger, A., Burguiere, A.M., Cinatl, J., Eickmann, M., Escouffier, N., Grywna, K., Kramme, S., Manuguerra, J.C., Muller, S., Rickerts, V., Sturmer, M., Vieth, S., Klenk, H.D., Osterhaus, A.D., Schmitz, H., Doerr, H.W., 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* 348 (20), 1967–1976.
- Dveksler, G.S., Pensiero, M.N., Cardellicchio, C.B., Williams, R.K., Jiang, G.S., Holmes, K.V., Dieffenbach, C.W., 1991. Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. *J. Virol.* 65 (12), 6881–6891.
- Dveksler, G.S., Pensiero, M.N., Dieffenbach, C.W., Cardellicchio, C.B., Basile, A.A., Elia, P.E., Holmes, K.V., 1993. Mouse hepatitis virus

- strain A59 and blocking antireceptor monoclonal antibody bind to the N-terminal domain of cellular receptor. *Proc. Natl. Acad. Sci. USA* 90 (5), 1716–1720.
- Dveksler, G.S., Gagneten, S.E., Scanga, C.A., Cardellicchio, C.B., Holmes, K.V., 1996. Expression of the recombinant anchorless N-terminal domain of mouse hepatitis virus (MHV) receptor makes hamster or human cells susceptible to MHV infection. *J. Virol.* 70, 4142–4145.
- Frana, M.F., Behnke, J.N., Sturman, L.S., Holmes, K.V., 1985. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: host-dependent differences in proteolytic cleavage and cell fusion. *J. Virol.* 56 (3), 912–920.
- Gagneten, S., Gout, O., Dubois-Dalcq, M., Rottier, P., Rossen, J., Holmes, K.V., 1995. Interaction of mouse hepatitis virus (MHV) spike glycoprotein with receptor glycoprotein MHVR is required for infection with an MHV strain that expresses the hemagglutinin-esterase glycoprotein. *J. Virol.* 69 (2), 889–895.
- Gallagher, T.M., 1997. A role for naturally occurring variation of the murine coronavirus spike protein in stabilizing association with the cellular receptor. *J. Virol.* 71 (4), 3129–3137.
- Gallagher, T.M., Buchmeier, M.J., 2001. Coronavirus spike proteins in viral entry and pathogenesis. *Virology* 279 (2), 371–374.
- Guan, Y., Zheng, B.J., He, Y.Q., Liu, X.L., Zhuang, Z.X., Cheung, C.L., Luo, S.W., Li, P.H., Zhang, L.J., Guan, Y.J., Butt, K.M., Wong, K.L., Chan, K.W., Lim, W., Shortridge, K.F., Yuen, K.Y., Peiris, J.S., Poon, L.L., 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* 302 (5643), 276–278.
- Hajjema, B.J., Volders, H., Rottier, P.J., 2003. Switching species tropism: an effective way to manipulate the feline coronavirus genome. *J. Virol.* 77 (8), 4528–4538.
- Hensley, L.E., Baric, R.S., 1998. Human biliary glycoproteins function as receptors for interspecies transfer of mouse hepatitis virus. *Adv. Exp. Med. Biol.* 440, 43–52.
- Hensley, L.E., Holmes, K.V., Beauchemin, N., Baric, R.S., 1998. Virus–receptor interactions and interspecies transfer of a mouse hepatitis virus. *Adv. Exp. Med. Biol.* 440, 33–41.
- Holmes, K.V., 2003. SARS coronavirus: a new challenge for prevention and therapy. *J. Clin. Invest.* 111, 1605–1609.
- Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., Rollin, P.E., Dowell, S.F., Ling, A.E., Humphrey, C.D., Shieh, W.J., Guarnier, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.Y., Cox, N., Hughes, J.M., LeDuc, J.W., Bellini, W.J., Anderson, L.J., 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* 348 (20), 1953–1966.
- Kubo, H., Yamada, Y.K., Taguchi, F., 1994. Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino acids of the murine coronavirus spike protein. *J. Virol.* 68 (9), 5403–5410.
- Kuo, L., Godeke, G.J., Raamsman, M.J., Masters, P.S., Rottier, P.J., 2000. Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. *J. Virol.* 74 (3), 1393–1406.
- Lai, M.M.C., Holmes, K.V., 2001. Coronaviridae and their replication. In: Knipe, D., et al., (Ed.), *Fields' Virology*, 4th ed., vols. 1 and 2. Lipincott-Williams and Wilkins, Philadelphia, pp. 1163–1185.
- Ludwig, B., Kraus, F.B., Allwinn, R., Doerr, H.W., Preiser, W., 2003. Viral zoonoses—A threat under control? *Intervirology* 46 (2), 71–78.
- Luytjes, W., Sturman, L.S., Bredenbeek, P.J., Charite, J., van der Zeijst, B.A.M., Horzinek, M.C., Spaan, W.J., 1987. Primary structure of the glycoprotein E2 of coronavirus MHV-A59 and identification of the trypsin cleavage site. *Virology* 161, 479–487.
- Marra, M.A., Jones, S.J., Astell, C.R., Holt, R.A., Brooks-Wilson, A., Butterfield, Y.S., Khattri, J., Asano, J.K., Barber, S.A., Chan, S.Y., Cloutier, A., Coughlin, S.M., Freeman, D., Girm, N., Griffith, O.L., Leach, S.R., Mayo, M., McDonald, H., Montgomery, S.B., Pandoh, P.K., Petrescu, A.S., Robertson, A.G., Schein, J.E., Siddiqui, A., Smailus, D.E., Stott, J.M., Yang, G.S., Plummer, F., Andonov, A., Artsob, H., Bastien, N., Bernard, K., Booth, T.F., Bowness, D., Czub, M., Drebot, M., Fernando, L., Flick, R., Garbutt, M., Gray, M., Grolla, A., Jones, S., Feldmann, H., Meyers, A., Kabani, A., Li, Y., Normand, S., Stroher, U., Tipples, G.A., Tyler, S., Vogrig, R., Ward, D., Watson, B., Brunham, M., Krajden, M., Petric, M., Skowronski, D.M., Upton, C., Roper, R.L., 2003. The genome sequence of the SARS-associated coronavirus. *Science* 300 (5624), 1399–1404.
- Matsuyama, S., Taguchi, F., 2002. Receptor-induced conformational changes of murine coronavirus spike protein. *J. Virol.* 76 (23), 11819–11826.
- Miura, H.S., Nakagaki, K., Taguchi, F., 2004. N-terminal domain of the murine coronavirus receptor CEACAM1 is responsible for fusogenic activation and conformational changes of the spike protein. *J. Virol.* 78 (1), 216–223.
- Navas, S., Weiss, S.R., 2003. Murine coronavirus-induced hepatitis: JHM genetic background eliminates A59 spike-determined hepatotropism. *J. Virol.* 77 (8), 4972–4978.
- Peiris, J.S., Lai, S.T., Poon, L.L., Guan, Y., Yam, L.Y., Lim, W., Nicholls, J., Yee, W.K., Yan, W.W., Cheung, M.T., Cheng, V.C., Chan, K.H., Tsang, D.N., Yung, R.W., Ng, T.K., Yuen, K.Y., 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 361 (9366), 1319–1325.
- Popova, R., Zhang, X., 2002. The spike but not the hemagglutinin/esterase protein of bovine coronavirus is necessary and sufficient for viral infection. *Virology* 294 (1), 222–236.
- Rao, P.V., Gallagher, T.M., 1998. Intracellular complexes of viral spike and cellular receptor accumulate during cytopathic murine coronavirus infections. *J. Virol.* 72 (4), 3278–3288.
- Rao, P.V., Kumari, S., Gallagher, T.M., 1997. Identification of a contiguous 6-residue determinant in the MHV receptor that controls the level of virion binding to cells. *Virology* 229 (2), 336–348.
- Rest, J.S., Mindell, D.P., 2003. SARS associated coronavirus has a recombinant polymerase and coronaviruses have a history of host-shifting. *Infect. Genet. Evol.* 3, 219–255.
- Rota, P.A., Oberste, M.S., Monroe, S.S., Nix, W.A., Campagnoli, R., Icenogle, J.P., Penaranda, S., Bankamp, B., Maher, K., Chen, M.H., Tong, S., Tamim, A., Lowe, L., Frace, M., DeRisi, J.L., Chen, Q., Wang, D., Erdman, D.D., Peret, T.C., Burns, C., Ksiazek, T.G., Rollin, P.E., Sanchez, A., Liffick, S., Holloway, B., Limor, J., McCaustland, K., Olsen-Rasmussen, M., Fouchier, R., Gunther, S., Osterhaus, A.D., Drosten, C., Pallansch, M.A., Anderson, L.J., Bellini, W.J., 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 300 (5624), 1394–1399.
- Sawicki, S.G., Lu, J.H., Holmes, K.V., 1995. Persistent infection of cultured cells with mouse hepatitis virus (MHV) results from the epigenetic expression of the MHV receptor. *J. Virol.* 69 (9), 5535–5543.
- Schickli, J.H., Zelus, B.D., Wentworth, D.E., Sawicki, S.G., Holmes, K.V., 1997. The murine coronavirus mouse hepatitis virus strain A59 from persistently infected murine cells exhibits an extended host range. *J. Virol.* 71 (12), 9499–9507.
- Schickli, J.H., Thackray, L.B., Sawicki, S.G., Holmes, K.V., 2004. The N-terminal region of the murine coronavirus spike glycoprotein is associated with the extended host range of viruses from persistently infected murine cells. *J. Virol.*, in press.
- Schultze, B., Herrler, G., 1992. Bovine coronavirus uses *N*-acetyl-9-*O*-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells. *J. Gen. Virol.* 73, 901–906.
- Siddell, S.G., Snijder, E.J., 1998. Coronaviruses, toroviruses and arteriviruses. In: Mahy, B.W.J., Collier, L. (Eds.), *Topley and Wilson's Microbiology and Microbial Infections*, 5th ed. Edward Arnold, London, pp. 463–484.
- Smith, A.L., Cardellicchio, C.B., Winograd, D.F., de Souza, M.S., Barthold, S.W., Holmes, K.V., 1991. Monoclonal antibody to the receptor for murine coronavirus MHV-A59 inhibits viral replication in vivo. *J. Infect. Dis.* 163, 879–882.
- Stanhope, M.J., Brown, J.R., Amrine-Madsen, H., 2004. Evidence from the

- evolutionary analysis of nucleotide sequences for a recombinant history of SARS-CoV. *Infect. Genet. Evol.* 4, 15–19.
- Stavriniades, J., Guttman, D.S., 2004. Mosaic evolution of the severe acute respiratory syndrome coronavirus. *J. Virol.* 78 (1), 76–82.
- Taguchi, F., 1993. Fusion formation by the uncleaved spike protein of murine coronavirus JHMV variant cl-2. *J. Virol.* 67, 1195–1202.
- Tan, K., Zelus, B.D., Meijers, R., Liu, J.H., Bergelson, J.M., Duke, N., Zhang, R., Joachimiak, A., Holmes, K.V., Wang, J.H., 2002. Crystal structure of murine sCEACAM1a[1,4]: a coronavirus receptor in the CEA family. *EMBO J.* 21 (9), 2076–2086.
- Tresnan, D.B., Levis, R., Holmes, K.V., 1996. Feline aminopeptidase N serves as a receptor for feline, canine, porcine, and human coronaviruses in serogroup I. *J. Virol.* 70 (12), 8669–8674.
- Tsai, J.C., Zelus, B.D., Holmes, K.V., Weiss, S.R., 2003. The N-terminal domain of the murine coronavirus spike glycoprotein determines the CEACAM1 receptor specificity of the virus strain. *J. Virol.* 77 (2), 841–850.
- Wentworth, D.E., Holmes, K.V., 2001. Molecular determinants of species specificity in coronavirus receptor aminopeptidase N (CD13): influence of N-linked glycosylation. *J. Virol.* 75, 9741–9752.
- Wessner, D.R., Shick, P.C., Lu, J.H., Cardellicchio, C.B., Gagneten, S.E., Beauchemin, N., Holmes, K.V., Dveksler, G.S., 1998. Mutational analysis of the virus and monoclonal antibody binding sites in MHVR, the cellular receptor of the murine coronavirus mouse hepatitis virus strain A59. *J. Virol.* 72 (3), 1941–1948.
- Williams, R.K., Jiang, G.S., Snyder, S.W., Frana, M.F., Holmes, K.V., 1990. Purification of the 110-kilodalton glycoprotein receptor for mouse hepatitis virus (MHV)-A59 from mouse liver and identification of a nonfunctional, homologous protein in MHV-resistant SJL/J mice. *J. Virol.* 64 (8), 3817–3823.
- Woolhouse, M.E., 2002. Population biology of emerging and re-emerging pathogens. *Trends Microbiol.* 10 (Suppl. 10), S3–S7.
- Yeager, C.L., Ashmun, R.A., Williams, R.K., Cardellicchio, C.B., Shapiro, L.H., Look, A.T., Holmes, K.V., 1992. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* 357, 420–422.
- Yokomori, K., Lai, M.M.C., 1992. Mouse hepatitis virus utilizes two carcinoembryonic antigens as alternative receptors. *J. Virol.* 66, 6194–6199.
- Yokomori, K., Banner, L.R., Lai, M.M., 1991. Heterogeneity of gene expression of the hemagglutinin-esterase (HE) protein of murine coronaviruses. *Virology* 183 (2), 647–657.
- Zelus, B.D., Wessner, D.R., Williams, R.K., Pensiero, M.N., Phibbs, F.T., deSouza, M., Dveksler, G.S., Holmes, K.V., 1998. Purified, soluble recombinant mouse hepatitis virus receptor, Bgp1(b), and Bgp2 murine coronavirus receptors differ in mouse hepatitis virus binding and neutralizing activities. *J. Virol.* 72 (9), 7237–7244.
- Zelus, B.D., Schickli, J.H., Blau, D.M., Weiss, S.R., Holmes, K.V., 2003. Conformational changes in the spike glycoprotein of murine coronavirus are induced at 37 degrees C either by soluble murine CEACAM1 receptors or by pH 8. *J. Virol.* 77 (2), 830–840.