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Substitutions of conserved amino acids in the receptor-binding domain of the spike glycoprotein affect utilization of murine CEACAM1a by the murine coronavirus MHV-A59

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Abstract

The host range of the murine coronavirus (MHV) is limited to susceptible mice and murine cell lines by interactions of the spike glycoprotein (S) with its receptor, mCEACAM1a. We identified five residues in S (S33, L79, T82, Y162 and K183) that are conserved in the receptor-binding domain of MHV strains, but not in related coronaviruses. We used targeted RNA recombination to generate isogenic viruses that differ from MHV-A59 by amino acid substitutions in S. Viruses with S33R and K183R substitutions had wild type growth, while L79A/T82A viruses formed small plaques. Viruses with S33G, L79M/T82M or K183G substitutions could only be recovered from cells that over-expressed a mutant mCEACAM1a. Viruses with Y162H or Y162Q substitutions were never recovered, while Y162A viruses formed minute plaques. However, viruses with Y162F substitutions had wild type growth, suggesting that Y162 may comprise part of a hydrophobic domain that contacts the MHV-binding site of mCEACAM1a.

Keywords: Murine coronavirus; Spike glycoprotein; Host range; Receptor specificity; Mutations; CEACAM1a; Receptor binding domain

Introduction

Viruses of the *Coronaviridae* family form three distinct antigenic and phylogenetic groups (Lai and Holmes, 2001). Coronaviruses in group II infect mice, rats, cattle or humans, as well as several other host species. Only one cellular glycoprotein has been identified as a receptor for a group II coronavirus. The murine coronavirus [murine hepatitis virus (MHV)] utilizes as receptors murine carcinoembryonic antigen cell adhesion molecule 1a (mCEACAM1a) and related murine glycoproteins in the CEA family of glycoproteins in the immunoglobulin (Ig) superfamily (Dveksler et al., 1993a, 1993b; Yokomori and Lai, 1992). The envelope of most group II coronaviruses contains a hemagglutinin esterase (HE) glycoprotein that binds to 9-*O*-acetylated neuraminic acid, but the A59 strain of MHV (MHV-A59) does not express HE (Yokomori et al., 1991). Thus, MHV-A59 infection of susceptible mice and murine cell lines depends solely on interactions of the viral spike glycoprotein (S) with mCEACAM1a and related murine glycoproteins (Hemmila et al., 2004).

Although the variety of hosts infected by coronaviruses illustrates that these viruses can emerge in a new host species, the mechanisms required for the introduction of a coronavirus into a new host are not well understood. The interaction of the viral spike glycoprotein with a specific cellular glycoprotein receptor is a major determinant of coronavirus host range. Cell lines from host species that are normally resistant to MHV, porcine coronavirus [transmissible gastroenteritis virus (TGEV)] or human coronavirus strain 229E (HCoV-229E) are rendered susceptible to infection by transfection with cDNA encoding the specific coronavirus receptors mCEACAM1a, porcine aminopeptidase N (pAPN) or human APN (hAPN), respectively

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(Delmas et al., 1992; Dveksler et al., 1991; Yeager et al., 1992). The 180-kDa S of MHV is a type I viral fusion protein that mediates both receptor binding and membrane fusion activities (Bosch et al., 2003; Gallagher and Buchmeier, 2001). S of MHV-A59 is post-translationally cleaved by a cellular protease into 90 kDa S1 and S2 proteins that remain non-covalently associated on the viral envelope. Variations in the amino acid (aa) sequence of S1 of MHV strains are associated with differences in tissue tropism and pathogenesis (Leparc-Goffart et al., 1998; Phillips et al., 2001). The membrane-anchored S2 contains a coiled-coil domain that is likely associated with membrane fusion and is more highly conserved among MHV strains than S1 (Bosch et al., 2003).

MHV and the blocking anti-mCEACAM1a monoclonal antibody, MAb-CC1, bind to the amino (N)-terminal Ig-like domain of mCEACAM1a (Dveksler et al., 1993a, 1993b). Binding of the N-terminal domain of soluble mCEACAM1a to S1 on MHV virions at 37 °C induces conformational changes in S1 and S2 proteins, and neutralizes virus (Gallagher, 1997; Lewicki and Gallagher, 2002; Matsuyama and Taguchi, 2002; Miura et al., 2004; Zelus et al., 1998). Incubation of purified MHV-A59 virions with soluble mCEACAM1a at neutral pH, or without receptor at pH 8.0, triggers major conformational changes in S2 (Zelus et al., 2003). These conformational changes may expose a hydrophobic domain in S that allows virus to bind liposomes and presumably initiates fusion of the viral envelope with host cell membranes, as well as cell-to-cell fusion. Mutational analyses of determinants in mCEACAM1a that alter MHV binding and entry implicated residues in the predicted CC′ loop and the C′ β sheet in the N-terminal domain of mCEACAM1a (Rao et al., 1997; Wessner et al., 1998). The predicted CC′ loop was also shown by mutational analyses to be critical for recognition of human CEACAM1 by bacterial pathogens such as *Neisseria*, as well as for homophilic cell adhesion (Bos et al., 1999; Virji et al., 1999; Watt et al., 2001). The crystal structure of mCEACAM1a[1,4] showed that the CC′ loop has a convoluted conformation unlike other Ig superfamily glycoproteins (Tan et al., 2002). The side chain of an isoleucine at residue 41 (I41) projects upwards away from the membrane and was predicted to play an important role in mCEACAM1a recognition by MHV S proteins.

The structural characterization of S is limited to fusion cores of S2 proteins of MHV and severe acute respiratory syndrome coronavirus (SARS-CoV) (Bosch et al., 2003; Liu et al., 2004; Tripet et al., 2004; Xu et al., 2004). Domains of S that are responsible for receptor binding have been identified for several coronaviruses. Aa 417–547 of S of HCoV-229E comprise a minimal receptor binding domain (RBD) for hAPN expressed on cell membranes (Bonavia et al., 2003; Breslin et al., 2003), while aa 1–330 of S of MHV (S330) comprise the minimal domain for binding to soluble mCEACAM1a in vitro and initiating infection via anchored mCEACAM1a (Kubo et al., 1994; Tsai et al., 2003). Aa 318–510 of S of SARS-CoV comprise a minimal domain for binding to angiotensin converting enzyme 2 (ACE2) in vitro (Babcock et al., 2004; Li et al., 2003; Wong et al., 2004; Xiao et al., 2003). Recently, we demonstrated that the N-terminal region of S1 containing 21 aa substitutions and a 7-aa insert derived from MHV/BHK, a virus variant generated during persistent MHV-A59 infection of murine cells, is sufficient to extend the host range of MHV-A59 in vitro (Sawicki et al., 1995; Schickli et al., 1997, 2004; Thackray and Holmes, 2004). Residues critical for receptor utilization and host specificity have not been identified for most coronaviruses, although several residues that play a role in the interactions of the MHV S protein with mCEACAM1a or of the SARS-CoV S protein with ACE2 have been identified (Saeki et al., 1997; Suzuki and Taguchi, 1996; Wong et al., 2004). With the emergence of SARS-CoV in humans and the search for effective drug-based interventions for coronavirus infection, the need to identify residues critical for the interactions of coronaviruses with their cellular receptors is greater than ever.

We compared the N-terminal 330 aa of S proteins of seven MHV strains with the N-termini of S proteins of the extended host range variant MHV/BHK, as well as related group II coronaviruses of rats, cattle and humans. We identified five residues that are highly conserved in S330 of all MHV strains and MHV/BHK, but are not found in S330 of rat, bovine or human coronaviruses of group II. To examine the roles of these residues in the receptor specificity of S, we used targeted RNA recombination (TRR) to generate isogenic viruses that differ from MHV-A59 at one or two aa in the RBD of S. We showed that Y162 is critical for the recovery of MHV-A59 from cells expressing mCEACAM1a. We also found that certain aa substitutions at S33, L79, T82 or K183 can inhibit the recovery of MHV-A59 from murine cells.

Results

Generation of recombinant viruses

To identify residues that are involved in the receptor specificity of the S glycoprotein of MHV, we compared the N-terminal 330 aa of S proteins of seven MHV strains with the N-termini of S proteins of related coronaviruses of rats, cattle and humans. We identified four residues, S33, T82, Y162 and K183, that are conserved in S330 of all MHV strains, but not in the corresponding domains of rat, bovine and human coronaviruses in group II (Fig. 1A). Another residue, L79, is conserved in S330 of all MHV strains, except MHV 2, but not in S330 of rat, bovine or human group II coronaviruses. These five residues are also conserved in the extended host range variant MHV/BHK that infects a wide range of non-murine cell lines while maintaining the ability to infect murine cells (Schickli et al., 2004; Thackray and Holmes, 2004). To examine the roles of

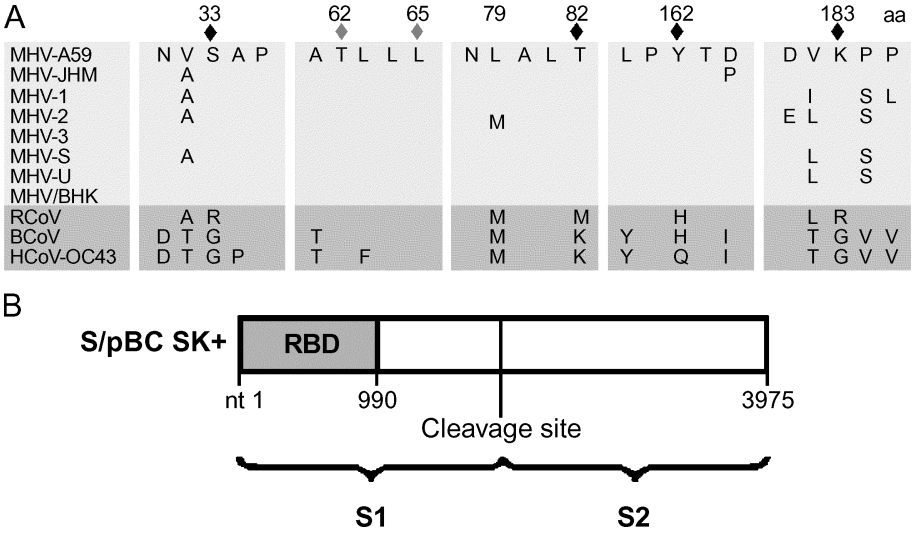

Fig. 1. (A) Residues conserved in spike glycoproteins of murine coronaviruses. Four residues (black diamonds) are conserved in the N-terminal 330 aa of S proteins of MHV strains (MHV-A59, MHV-JHM, MHV-1, MHV-2, MHV-3, MHV-S and MHV-U) and an extended host range variant of MHV-A59 (MHV/BHK), but not in the N-termini of S proteins of related rat (RCoV), bovine (BCoV) and human (HCoV-OC43) coronaviruses in group II. Two residues (grey diamonds) conserved in S proteins of murine, rat, bovine and human coronaviruses in group II are also indicated. (B) Composition of S construct used to introduce mutations into MHV-A59 using targeted RNA recombination. Mutations were engineered into the S/pBC SK+ plasmid and subcloned into the pMH54 plasmid. The minimal receptor binding domain (RBD) for murine CEACAM1a is shaded in gray.

these residues in the receptor specificity of S, we used TRR to introduce single aa substitutions at S33, Y162 or K183 and double aa substitutions at L79 and T82 into the genome of MHV-A59. The aa substitutions were chosen to reflect residues found in rat, bovine or human group II coronaviruses, a conservative Y to F substitution, or neutral alanine substitutions (Fig. 1A). Although aa substitutions at T62 or L65 in S330 of MHV-JHM were previously associated with reduced mCEACAM1a binding (Saeki et al., 1997; Suzuki and Taguchi, 1996), we observed that T62 and L65 were conserved in S330 of all MHV strains, as well as S330 of related rat, bovine and human coronaviruses (Fig. 1A). To examine the roles of these residues in the receptor specificity of S in the context of infectious isogenic viruses, we used targeted RNA recombination (TRR) to introduce aa substitutions at T62 or L65 into the genome of MHV-A59. The aa substitutions were chosen to reflect substitutions made previously, S and H for T62 and L65, respectively, (Saeki et al., 1997; Suzuki and Taguchi, 1996) or neutral alanine substitutions.

Donor RNAs, transcribed in vitro from pMH54 constructs, were transfected into feline (Fcwf) cells that had been inoculated with the chimeric helper virus fMHV (Kuo et al., 2000). RNA recombination occurs between fMHV, which contains a chimeric S gene with the ectodomain of feline infectious peritonitis virus and the rest of the MHV-A59 genome, and the pMH54 donor RNA containing the 3′-most 7.4 kb of the MHV-A59 genome (Kuo et al., 2000). The infected and transfected Fcwf 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. For each mutant pMH54 construct, three recombinant viruses (A, B and C) were independently recovered and plaque-purified to control for adventitious mutations that might arise during TRR. In addition, in every experiment, pMH54 RNA encoding wild-type MHV-A59 S protein was used to reconstitute wild-type MHV-A59 virus (SA59) in triplicate.

Extensive cytopathic effects (CPE) were seen at 48 h in 17 Cl 1 cell monolayers overlaid with fMHV-inoculated Fcwf cells transfected with pMH54 RNA or pMH54 RNAs encoding S33R, T62S, T62A, Y162F or K183R substitutions. In contrast, 17 Cl 1 cell monolayers overlaid with fMHV-inoculated Fcwf cells transfected with pMH54 RNAs encoding L65H, L65A or L79A/T82A substitutions exhibited less CPE at 48–72 h, while 17 Cl 1 cell monolayers overlaid with fMHV-inoculated Fcwf cells mock-transfected or transfected with pMH54 RNAs encoding S33G, L79M/T82M, Y162H, Y162Q, Y162A or K183G substitutions exhibited no detectable CPE even after 72 h.

Recovery of recombinant viruses from cells that express murine CEACAM1a

Most of the recombinant viruses formed uniform plaques on 17 Cl 1 monolayers, although recombinant viruses derived from the L65H or L65A constructs formed clear and opaque plaques (data not shown). Several mutant viruses were never recovered from 17 Cl 1 cell monolayers (Table 1), either because the mutant S proteins interacted inefficiently with mCEACAM1a or because the mutant S proteins were inefficiently incorporated into MHV virions. TRR using fMHV-inoculated Fcwf cells transfected with pMH54

Table 1
Recovery of viruses from cells expressing mCEACAM1a

Virus	Murine 17 Cl 1[a]	Hamster BHK + mCEACAM1a(I41R)[b]
MHV-A59	+	ND
SA59	+	+
S33R	+	ND
S33G	–	+
T62S	+	ND
T62A	+	ND
L65H	+	ND
L65A	+	ND
L79M/T82M	–	+
L79A/T82A	+	ND
Y162H	–	–
Y162Q	–	–
Y162A	–	–
Y162F	+	ND
K183R	+	ND
K183G	–	+

(ND) = not done.
[a] Determined by neutral red staining and immunoperoxidase labeling of viral N protein in 17 Cl 1 cell monolayers.
[b] Determined by neutral red staining and immunoperoxidase labeling of viral N protein in 17 Cl 1 and BHK + mCEACAM1a (I41R) cell monolayers.

RNAs encoding S33G, L79M/T82M, Y162H, Y162Q, Y162A or K183G substitutions was repeated several times, but none of these mutant viruses could be recovered from 17 Cl 1 cell monolayers.

In order to isolate these crippled viruses with mutant S proteins, we used hamster (BHK) cells stably expressing mCEACAM1a[1,4] containing an I41R substitution [BHK + mCEACAM1a(I41R)]. These cells expressed 10-fold more receptor than either 17 Cl 1 cells or BHK cells stably expressing wild type mCEACAM1a[1,4], as measured by flow cytometry using anti-mCEACAM1a MAb CC1 (data not shown). BHK + mCEACAM1a(I41R) cell monolayers overlaid with fMHV-inoculated Fcwf cells transfected with pMH54 or pMH54 RNAs encoding S33R, L79M/T82M or K183G substitutions exhibited extensive CPE at 48 h, while no CPE was detected even at 72 h in cells transfected with pMH54 RNAs encoding Y162H, Y162Q or Y162A substitutions. All three recombinant viruses derived from each of the SA59, S33G, L79M/T82M or K183G constructs formed uniform plaques on BHK + mCEACAM1a(I41R) cell monolayers, as well as on 17 Cl 1 cells (data not shown). We do not know whether the recovery of the S33G, L79M/T82M or K183R viruses from BHK + mCEACAM1a(I41R) cells was facilitated by the high level of receptor expression in these cells or the I41R substitution in the N-terminal domain of mCEACAM1a. However, the recovery phenotypes and plaque morphologies shared by the three replicates of each mutant virus suggest that the engineered mutations in S330 were responsible for the observed phenotypes of these viruses. We further characterized the S33G, L79M/T82M and K183G viruses that were subsequently plaque-purified and propagated in 17 Cl 1 cells.

Spread of EGFP recombinant viruses in cells expressing mCEACAM1a

Viruses derived from the Y162H, Y162Q and Y162A constructs were not recovered from either 17 Cl 1 or BHK + mCEACAM1a(I41R) cell monolayers (Table 1). TRR using fMHV-inoculated Fcwf cells transfected with pMH54 RNAs encoding Y162H, Y162Q or Y162A substitutions was repeated several times, but these mutant viruses were not recovered from BHK + mCEACAM1a(I41R) cell monolayers. To examine the infectivity and spread of these crippled viruses with mutant S proteins immediately after TRR, we used a pMH54-EGFP plasmid that contains the enhanced green fluorescent protein (EGFP) gene in place of gene 4 (Das Sarma et al., 2002). Gene 4 is not essential for replication of MHV-A59, since MHV-A59 EGFP has wild type growth in vitro and in vivo. Donor RNAs, transcribed in vitro from mutant pMH54-EGFP constructs, were transfected into Fcwf cells that had been inoculated with the chimeric helper virus fMHV (Kuo et al., 2000). The infected and transfected Fcwf cells were immediately overlaid in duplicate onto monolayers of 17 Cl 1 or BHK + mCEACAM1a(I41R) cells. For each mutant pMH54-EGFP construct, two recombinant viruses (A and B) were independently recovered. In addition, wild-type pMH54-EGFP RNA was used to reconstitute wild type MHV-A59 EGFP virus (SA59-EGFP) in duplicate.

Nearly all of the 17 Cl 1 or BHK + mCEACAM1a(I41R) cells overlaid with fMHV-inoculated Fcwf cells transfected with pMH54-EGFP or pMH54-EGFP encoding a Y162F substitution exhibited extensive CPE and strong EGFP expression at 48 h (Fig. 2). In contrast, less than 0.01% of the 17 Cl 1 or BHK + mCEACAM1a(I41R) cells overlaid with fMHV-inoculated Fcwf cells transfected with pMH54-EGFP encoding Y162H, Y162Q or Y162A substitutions exhibited CPE and EGFP expression at 48 h. Although Y162A-EGFP viruses induced several large syncytia in BHK + mCEACAM1a(I41R) cells, the number of cells infected by Y162A-EGFP viruses decreased between 24 and 48 h (Fig. 2). These results suggested that H, Q or A substitutions for Y162 that reflect residues found at corresponding residues in S330 of rat, bovine or human coronaviruses in group II altered the interaction of MHV-A59 virions with mCEACAM1a.

The detection of the mutant EGFP viruses in 17 Cl 1 cells prompted us to examine the plaque forming ability of the Y162H, Y162Q and Y162A-EGFP viruses on 17 Cl 1 cell monolayers. Both replicates of the SA59, Y162A and Y162F-EGFP viruses formed plaques that exhibited EGFP expression in 17 Cl 1 cells monolayers (data not shown). Both of the Y162F-EGFP viruses formed large, 4–5 mm plaques on 17 Cl 1 cell monolayers like MHV-A59 (Fig. 3). However, both of the Y162A-EGFP viruses formed minute, 0.5 mm plaques that were difficult to visualize with neutral red staining, but were readily apparent using immunoper-

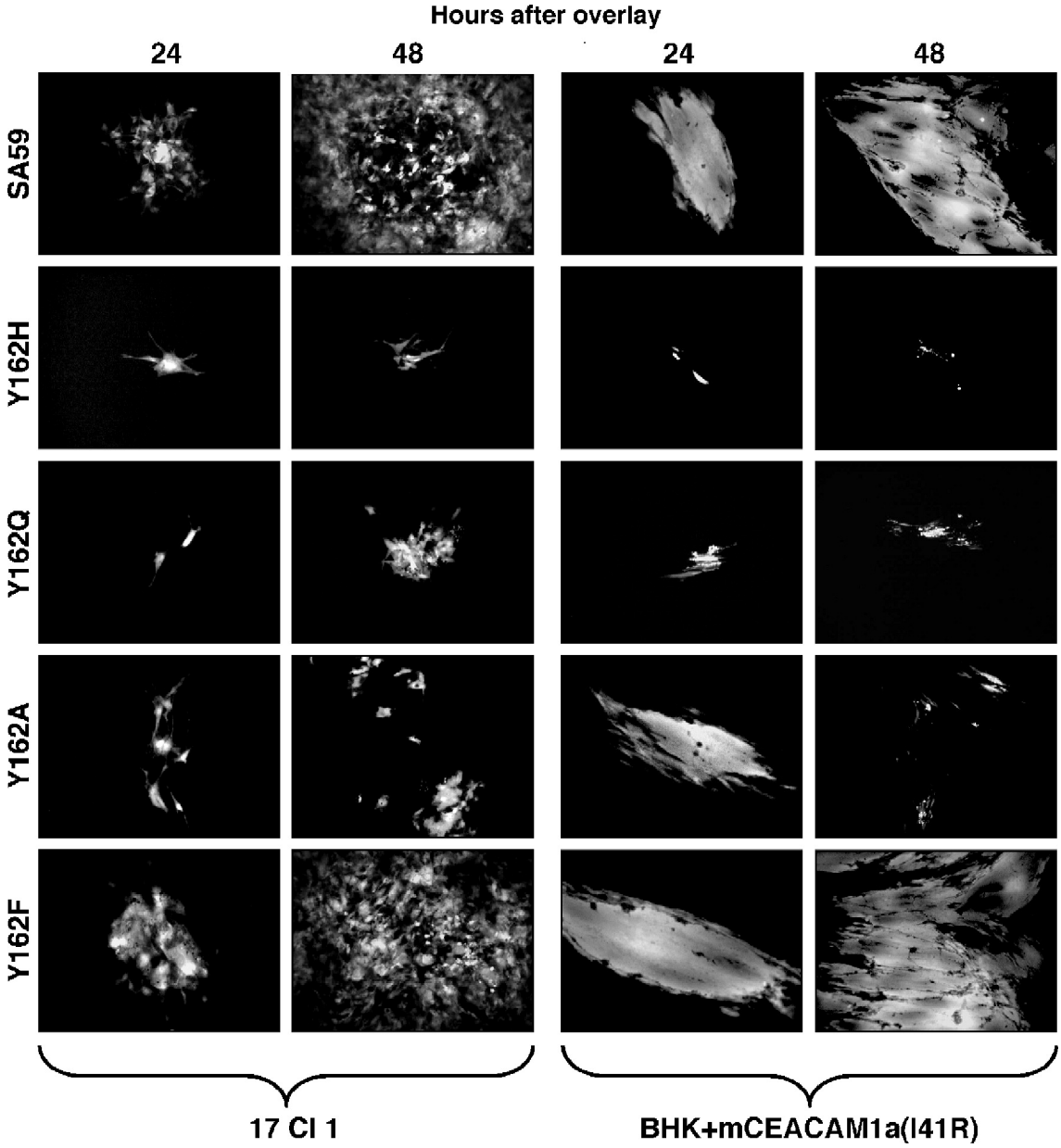

Fig. 2. Growth of EGFP viruses in murine 17 Cl 1 or hamster BHK + mCEACAM1a(I41R) cells. EGFP was visualized in cell monolayers overlaid with fMHV-inoculated Fcwf cells transfected with wild type (SA59) or mutant pMH54-EGFP RNAs. None of the 17 Cl 1 or BHK + mCEACAM1a(I41R) cells overlaid with mock-inoculated Fcwf cells transfected with wild type or mutant pMH54-EGFP RNAs exhibited EGFP expression. Magnification, 200×.

oxidase labeling of viral nucleocapsid (N) protein in 17 Cl 1 cell monolayers (Fig. 3). The yields of the Y162A-EGFP viruses from 17 Cl 1 cells were 10,000-fold lower than those of the SA59 and Y162F-EGFP viruses. Consequently, the EGFP viruses were not studied further.

Sequence analysis of recombinant viruses

To identify mutant viruses that were free of adventitious mutations in the S gene, we sequenced the S genes of plaque-purified recombinant viruses derived from mutant pMH54 constructs. Point mutations were found in the S genes of the SA59 A, S33G A and B, L65H A and B, L65A A and B and L79A/T82A A viruses (Table 2). The S genes of the SA59 B, S33R A, S33G C, T62S A, T62A A, L65H C, L65A C, L79M/T82M A, L79A/T82A B and K183G A viruses were free of adventitious mutations, while the S genes of the Y162F A and K183R A viruses had only non-coding adventitious mutations (Table 2). The lack of adventitious mutations in the S genes of the S33G C, L79M/T82M A, B, and C, and K183G A, B, and C viruses suggests that second-site mutations in the S gene were not responsible for the observed ability of these viruses to form plaques on 17 Cl 1 cells after their recovery from BHK + mCEACAM1a(I41R) cell monolayers.

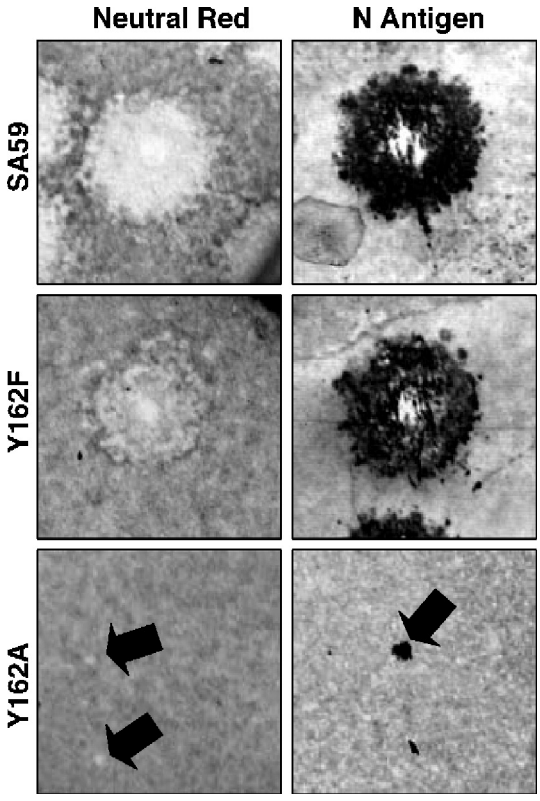

Fig. 3. Plaque morphologies of EGFP viruses on murine 17 Cl 1 cell monolayers. Plaques of the SA59, Y162F and Y162A-EGFP viruses were visualized at 72 h post-inoculation (p.i.) by neutral red staining (neutral red) or immunoperoxidase labeling of viral nucleocapsid protein (N antigen) in cell monolayers. Plaques formed by the Y162A-EGFP virus are indicated by black arrows.

Growth of recombinant viruses in murine cells

Most of the plaque-purified recombinant viruses released between 2×10^6 and 2×10^7 PFU/ml into the tissue culture medium by 48 h p.i., except for all three replicates of the L79A/T82A virus that released only 1×10^3 PFU/ml, as determined by plaque assay on 17 Cl 1 cell monolayers. To determine whether aa substitutions in the RBD of S altered the growth of MHV-A59 in murine cells, we examined the plaque morphologies of the plaque-purified recombinant viruses on 17 Cl 1 cell monolayers. When examined using neutral red stained cells, the SA59, S33R, L79M/T82M, Y162F and K183R viruses formed clear plaques like MHV-A59, whereas the S33G, T62S, T62A and K183G viruses formed turbid plaques (Fig. 4). The plaque-purified L65H and L65A viruses formed opaque plaques that were difficult to visualize with neutral red staining, but were readily apparent using immunoperoxidase labeling of viral N protein in 17 Cl 1 cell monolayers. Most of the recombinant viruses formed large, 4–5 mm plaques at 72 h like MHV-A59, whereas the three L79A/T82A viruses formed small, 2.9 mm plaques (Fig. 4). The plaque morphologies shared by the three replicates of each mutant virus indicate that the engineered mutations in S330 were responsible for the observed phenotypes of these viruses.

Interaction of recombinant viruses with murine CEACAM1a

Differences in plaque morphology could be caused by alterations in receptor binding or membrane fusion activities of mutant S proteins on virions. To investigate whether aa substitutions in the RBD of S altered the susceptibility of MHV-A59 virions to neutralization by soluble mCEACAM1a, we incubated the SA59 B, S33R A, S33G C, T62S A, T62A A, L65H C, L65A C, L79M/T82M A, Y162F A, K183R A and K183G A viruses at neutral pH and 37 °C with 70 nM of purified, anchorless mCEACAM1a[1,4]. This concentration of mCEACAM1a[1,4] was 100-fold higher than the ND_{50} for MHV-A59, but did not neutralize the extended host range variant MHV/BHK (Schickli et al., 1997; Zelus et al., 1998). Like MHV-A59, all of the recombinant viruses were neutralized by mCEACAM1a[1,4] at neutral pH and 37 °C (Fig. 5).The ability of soluble mCEACAM1a to neutralize the L79A/T82A B virus was not measured due to the low titer of this virus.

The neutralization of the mutant viruses by soluble mCEACAM1a[1,4] at neutral pH and 37 °C suggested that the soluble receptor bound to mutant S1 proteins on the recombinant virions and induced a conformational change in S2 like that observed for MHV-A59 (Gallagher, 1997;

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

Virus	Mutations[b] (nt)	Substitutions (aa)
SA59 A	A254G	N85S
SA59 B[c]	–	–
S33R A	–	–
S33G A	A2350T	T784S
S33G B	A2511G	S837S
S33G C	–	–
T62S A	–	–
T62A A	–	–
L65H A	T1626C	–
	C3074A	A1025D
L65H B	G857T	S286I
L65H C	–	–
L65A A	G2500T	D834Y
L65A B	C3116T	A1039V
L65A C	–	–
L79M/T82M A	–	–
L79M/T82M B	–	–
L79M/T82M C	–	–
L79A/T82A A	C1010A	A337D
L79A/T82A B	–	–
Y162F A	C939A	–
K183R A	T2874A	–
K183G A	–	–
K183G B	–	–
K183G C	–	–

(–) indicates no adventitious mutations.
[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.
[c] Recombinant viruses indicated in bold were characterized further in infection, neutralization and antibody blockade experiments.

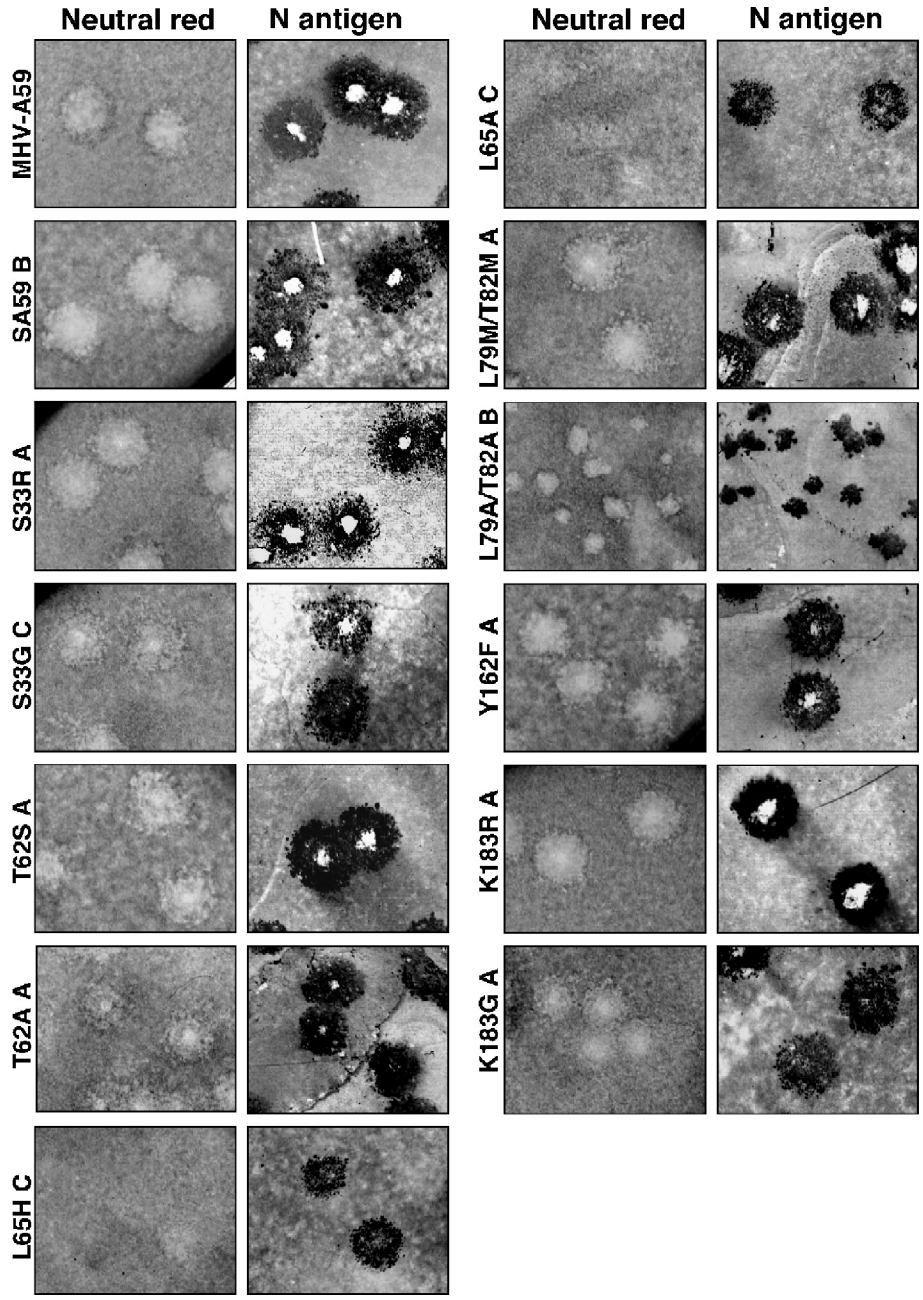

Fig. 4. Plaque morphologies of recombinant viruses on murine 17 Cl 1 cell monolayers. Plaques of MHV-A59 or the plaque-purified recombinant viruses were visualized at 72 h p.i. by neutral red staining (neutral red) or immunoperoxidase labeling of viral nucleocapsid protein (N antigen) in cell monolayers.

Matsuyama and Taguchi, 2002; Zelus et al., 1998, 2003). To further characterize the mCEACAM1a utilization of the mutant viruses, we measured the yields of the SA59 B, S33R A, S33G C, T62S A, T62A A, L65H C, L65A C, L79M/T82M A, L79A/T82A B, Y162F A, K183R A and K183G A viruses from 17 Cl 1 cells treated with anti-mCEACAM1a MAb-CC1. MAb-CC1 binds to the N-terminal domain of mCEACAM1a using an epitope that overlaps, but is not identical to, the virus-binding site (Dveksler et al., 1993a, 1993b; Wessner et al., 1998). Like MHV-A59, none of the recombinant viruses infected 17 Cl 1 cells in the presence of MAb-CC1 (Fig. 6). Thus, aa substitutions at S33, T62, L65, L79/T82, or K183 in S did not prevent binding of MHV-A59 virions to soluble or anchored mCEACAM1a.

Growth of recombinant viruses in non-murine cells

Since some of the aa substitutions in the RBD of S were chosen to reflect residues found in S330 of related rat, bovine or human coronaviruses in group II (Fig. 1A), we examined the ability of the SA59 B, S33R A, S33G C, T62S A, T62A A, L65H C, L65A C, L79M/T82M A, L79A/T82A B, Y162F A, K183R A and K183G A viruses to infect

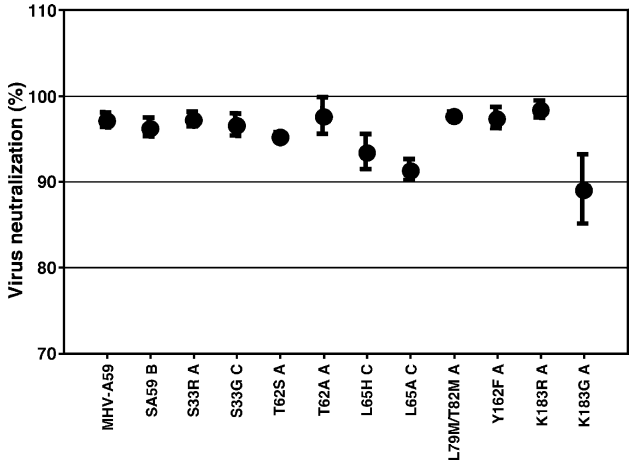

Fig. 5. Neutralization of recombinant viruses with soluble, murine CEACAM1a. MHV-A59 or the recombinant viruses (5000 PFU) were pre-incubated with 70 nM of soluble mCEACAM1a[1,4]. Virus survival was determined and percent neutralization calculated as described in Materials and methods. Averages of percent neutralization ± SEM of 2 independent experiments are shown.

non-murine cells lines that are normally resistant to MHV-A59 infection. As determined by immunofluorescence labeling of viral N protein and plaque formation, none of the mutant viruses infected rat (RIE), human (HRT-18G) or hamster (BHK) cells. RIE cells were susceptible to infection by rat coronavirus (RCoV), HRT-18G cells were susceptible to infection by bovine coronavirus and human coronavirus strain OC43, while all three cell lines were susceptible to infection by the extended host range variant MHV/BHK (Schickli et al., 1997). In addition, viruses derived from the Y162H, Y162Q and Y162A constructs did not infect RIE, HRT-18 or BHK cell lines. Thus, aa substitutions at S33, T62, L65, L79/T82, Y162 or K183 did not extend the host range of MHV-A59.

Discussion

We used comparative analysis of S proteins from coronaviruses in group II to identify residues in the receptor binding domain, S330, that may be important for the mCEACAM1a specificity of MHV virions. To study the biological significance of aa substitutions in the RBD of S, we used targeted RNA recombination (TRR) to generate isogenic recombinant viruses that differ from MHV-A59 at one or two residues in S330. Aa substitutions were selected by identification of five residues, S33, L79, T82, Y162 and K183, that are highly conserved in S330 of seven MHV strains and the extended host range variant MHV/BHK, but not in S330 of related group II coronaviruses of rats, cattle and humans. Since MHV-A59 does not bind or infect rat, bovine or human cell lines and RCoV does not bind to or utilize mCEACAM1a (Compton et al., 1992; Gagneten et al., 1996), we reasoned that these residues might be important for the interactions of MHV-A59 with mCEACAM1a.

In this study, we found that Y162 in the RBD of S is critical for the recovery of MHV-A59 from cells expressing mCEACAM1a. Fcwf cells inoculated with fMHV and transfected with pMH54-EGFP or pMH54-EGFP RNAs encoding Y162H, Y162Q, Y162A or Y162F substitutions showed limited EGFP expression when plated alone (data not shown). However, fMHV-inoculated Fcwf cells transfected with pMH54-EGFP or pMH54-EGFP RNAs encoding Y162H, Y162Q, Y162A or Y162F substitutions that were overlaid onto 17 Cl 1 or BHK + mCEACAM1a(I41R) cell monolayers showed increased CPE and EGFP expression (Fig. 2). These data strongly suggested that the majority of CPE and EGFP expression observed was due to infection of 17 Cl 1 or BHK + mCEACAM1a cells. Recombinant viruses with Y162H and Y162Q substitutions inefficiently infected 17 Cl 1 and BHK + mCEACAM1a(I41R) cell monolayers (Fig. 2) and these viruses were not recovered from either 17 Cl 1 or BHK + mCEACAM1a(I41R) cells (Table 1). Although viruses with Y162A substitutions were recovered from 17 Cl 1 and BHK + mCEACAM1a(I41R) cells, the Y162A viruses formed minute plaques on 17 Cl 1 cell monolayers (Fig. 3) and had markedly lower yields than MHV-A59. In contrast, F substitutions for Y162 were well tolerated by the S proteins of MHV-A59, since the Y162F viruses infected both 17 Cl 1 and mCEACAM1a(I41R) cells as well as MHV-A59 (Figs. 2–4 and 6). These data suggest that the phenyl ring of Y162 may form an essential hydrophobic contact with the MHV-binding domain of mCEACAM1a, and that this hydrophobic contact is disrupted in S proteins with H, Q or A substitutions at Y162. Alternatively, it is possible that H, Q or A substitutions at Y162 may alter the local conformation or stability of S proteins, since a S310G substitution in the S of MHV-JHM is associated with decreased S1–S2 stability

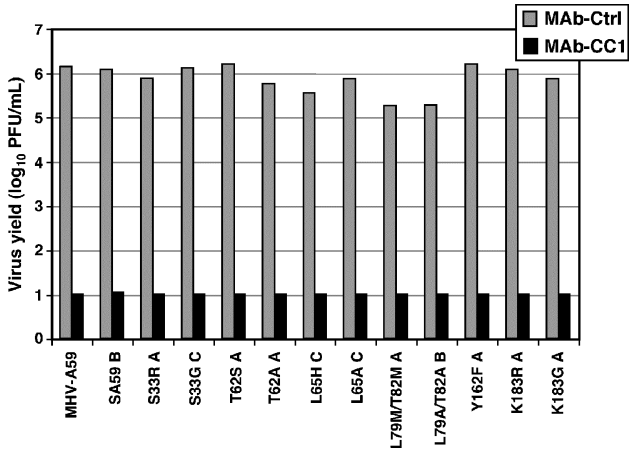

Fig. 6. Growth of recombinant viruses during anti-receptor antibody blockade of murine 17 Cl 1 cells. Yields of MHV-A59 or the recombinant viruses at 24 h p.i. from cells treated with 0.05 mg/ml of anti-CEACAM1a MAb-CC1 or isotype matched MAb-Ctrl before, during and after inoculation. The yield of the L79A/T82A B virus was assayed at 72 h p.i. due the low titer of this virus. Virus yields shown are representative of two independent experiments.

(Ontiveros et al., 2003) and a Q294L substitution in the S of infectious bronchitis virus has been shown to inhibit maturation and incorporation of S proteins into virions (Shen et al., 2004). Additional studies will be needed to determine whether Y162 participates directly in mCEACAM1a binding, triggers the fusion activity of S and/or maintains the conformation of the RBD of the MHV S protein.

In this study, we found that viruses with S33R, L79A/T82A or K183R substitutions in the RBD of S were readily recovered from 17 Cl 1 cells. The S33R and K183R viruses had wild-type growth in 17 Cl 1 cells, while the L79A/T82A viruses formed small plaques on murine cell monolayers (Fig. 4) and had reduced viral yields compared to MHV-A59. Viruses with S33G, L79M/T82M or K183G substitutions in S330 were never recovered from 17 Cl 1 cells. However, these viruses could initially be recovered from BHK + mCEACAM1a(I41R) cells that express 10-fold more receptor than 17 Cl 1 cells or BHK cells stably expressing wild type mCEACAM1a. The S33R, S33G, L79M/T82M, K183R and K183G viruses were neutralized like MHV-A59 by soluble mCEACAM1a at neutral pH and 37 °C (Fig. 5). In addition, just like MHV-A59, the S33R, S33G, L79M/T82M, L79A/T82A, K183R and K183G viruses bound mCEACAM1a using an epitope that overlaps that of the blocking anti-mCEACAM1a MAb-CC1 (Fig. 6). The recovery, growth and plaque phenotypes shared by each independently generated replicate of each mutant virus strongly suggests that the engineered mutations in S330, rather than adventitious mutations outside of the S gene, were responsible for the observed phenotypes of these viruses.

The failure of the S33G, L79M/T82M and K183G viruses to be recovered from 17 Cl 1 cells may have been due to impaired interactions between mutant S proteins and mCEACAM1a. During TRR, feline Fcwf cells infected with fMHV and transfected with mutant MHV-A59 RNAs may generate virions whose envelopes contain both chimeric fMHV S proteins and mutant MHV-A59 S proteins due to phenotypic mixing. The phenotypically heterogeneous S33G, L79M/T82M and K183G virions may have impaired interactions with mCEACAM1a. However, phenotypically homogenous S33G, L79M/T82M and K183G virions, generated in BHK + mCEACAM1a cells, may regain the ability to interact with mCEACAM1a on 17 Cl 1 cells. Alternatively, the high level of receptor expression on BHK + mCEACAM1a(I41R) cells may facilitate the recovery of these crippled viruses by increasing the number of receptors available on cell membranes. In a similar manner, increased expression of its cellular receptor partially restores the infectivity of a Friend murine leukemia virus with aa substitutions in its receptor binding pocket (Davey et al., 1999). In addition, the I41R substitution in the N-terminal domain of mCEACAM1a may facilitate the recovery of crippled viruses with mutant S proteins.

Several residues in S330 that may be important for binding to mCEACAM1a have previously been identified. A single T62S substitution or a triple T212S, Y214S, Y216S substitution in soluble S330 of MHV-JHM reduced binding to soluble mCEACAM1a in vitro (Suzuki and Taguchi, 1996). Soluble receptor resistant (srr) mutants of MHV-JHM with L65H substitutions in S330 also had reduced binding to soluble mCEACAM1a in vitro (Saeki et al., 1997). However, these srr mutants had wild-type interactions with anchored mCEACAM1a expressed in hamster cells (Matsuyama and Taguchi, 2000). In this study, we found that isogenic recombinant MHV-A59 viruses with T62S, T62A, L65H or L65A substitutions had wild-type growth in 17 Cl 1 cells and were neutralized, like MHV-A59, by soluble mCEACAM1a at neutral pH and 37 °C (Figs. 5 and 6). The T62S and T62A viruses formed turbid plaques on 17 Cl 1 cell monolayers, while the L65H and L65A viruses formed opaque plaques (Fig. 4). Thus, although T62 and L65 are not essential for mCEACAM1a utilization by MHV-A59, these residues may nevertheless influence spike-receptor interactions (Rao and Gallagher, 1998). We noted that T62 and L65 are conserved in S330 of murine, rat, bovine and human coronaviruses in group II (Fig. 1A). Since MHV-A59 does not bind rat, bovine or human tissues or infect rat, bovine or human cell lines (Compton et al., 1992), T62 and L65, individually, most likely do not determine the receptor specificity of MHV.

In this study, we demonstrated that single aa substitutions at S33, T62, L65, Y162 or K183 and double aa substitutions at L79 and T82 in the RBD of S of MHV-A59 did not allow the virus to interact with alternative receptors on murine or non-murine cell lines. Like wild type MHV-A59, infection of murine cells by viruses with substitutions at S33, T62, L65, L79/T82, Y162 or K183 was blocked by anti-mCEACAM1a MAb-CC1 (Fig. 6). None of the viruses with substitutions at S33, T62, L65, L79/T82, Y162 or K183 infected rat, hamster or human cell lines. Perhaps multiple aa substitutions are needed to change the host range of MHV-A59. We recently showed that 21 aa substitutions and a 7-aa insert in the N-terminal region of S1 of MHV-A59 are sufficient to extend the viral host range (Schickli et al., 2004; Thackray and Holmes, 2004). Isogenic recombinant viruses that differ from MHV-A59 by the 21 aa substitutions and the 7-aa insert in S not only utilize mCEACAM1a, but also as yet unidentified receptors on murine and non-murine cells (Thackray and Holmes, 2004). Alternatively, residues in the S proteins of rat, bovine and human coronaviruses in group II critical for receptor binding may not be found in S330. An aspartic acid at residue 454 in the RBD of the distantly related SARS-CoV S protein is essential for binding to ACE2 in vitro (Wong et al., 2004).

The mCEACAM1a-binding site in the MHV S glycoprotein has previously been proposed to be conformation-dependent based on the inhibition of MHV infection by conformation-dependent anti-S MAbs (Suzuki and Taguchi, 1996). We postulate that Y162 may comprise part of a hydrophobic pocket or patch in the RBD of S that interacts with hydrophobic residues in the MHV-binding site of

mCEACAM1a. Hydrophobic residues such as Y, F and W are known to play important roles in spike-receptor interactions of other enveloped viruses such as herpes simplex virus, human immunodeficiency virus and murine retroviruses (Carfi et al., 2001; Connolly et al., 2003; Kwong et al., 1998; Wu et al., 1996; Zhang et al., 2003). Residues S33, T62, L65, L79, T82 and K183 are most likely located peripheral to this hydrophobic binding site, since binding hot spots in spike-receptor interactions are often surrounded by energetically less critical residues, predominantly charged in nature (Bogan and Thorn, 1998; Wang, 2002). Residues that extend the host range of MHV-A59 may also be located peripheral to the hydrophobic binding site. During persistent MHV infection in murine cells, the important mCEACAM1a binding residues in S are retained (Baric et al., 1999; Schickli et al., 1997), while additional aa substitutions may be selected that enhance the affinity or avidity of S for mCEACAM1a and allow MHV to utilize as yet unidentified alternative receptors on murine and non-murine cells.

Materials and methods

Antibodies

A mouse monoclonal antibody (MAb) to the MHV nucleocapsid protein (N) (anti-N MAb) was provided by Julian Leibowitz (Department of Pathology and Laboratory Medicine, Texas A and M University, College Station, TX). Mouse anti-mCEACAM1a MAb-CC1 blocks binding of MHV to mCEACAM1a and infection of murine cells that express mCEACAM1a, such as 17 Cl 1 cells (Dveksler et al., 1993a, 1993b; 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.

Cell lines

The 17 Cl 1 cell line of spontaneously transformed BALB/c 3T3 fibroblasts, rat intestinal epithelial (RIE) cells, baby hamster kidney BHK-21 (BHK) cells, *Felis catus* whole fetus (Fcwf) cells, *Felis catus* lung epithelial (AK-D) cells and African green monkey kidney (Vero 76) cells were propagated as previously described (Schickli et al., 1997; Thackray and Holmes, 2004). Human rectal tumor clone G cells (HRT-18G) were kindly provided by Johannes Storz (Department of Veterinary Microbiology and Parasitology, Louisiana State University School of Veterinary Medicine, Baton Rouge, LO) and propagated in Dulbecco's modified Eagle medium (DMEM; GIBCO, Invitrogen Corporation, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT), 2% antibiotic-antimycotic (PSF; GIBCO) and 1.5 g/L sodium bicarbonate.

To generate BHK cells stably transfected with a murine CEACAM1a cDNA encoding an I41R substitution [BHK + mCEACAM1a(I41R)], site-directed mutagenesis of mCEACAM1a[1,4] in pCI-Neo (Invitrogen) was performed with the mutagenic forward primer 5′CTACGGCTagaGACAAAGAAATTG, and reverse primer 5′CAATTTCTTTGTCtctAGCCGTAG. BHK cells were transfected with cDNA encoding the mCEACAM1a(I41R) or wild type mCEACAM1a construct using Lipofectamine 2000 (Invitrogen), as specified by the manufacturer, and selected using 500 μg/ml of Geneticin (GIBCO). Stably transfected BHK cells were sorted twice on a Cytomation MoFlo cell sorter (Ft. Collins, CO) for high levels of mCEACAM1a expression using anti-CEACAM1a MAb-CC1 followed by phycoerythrin (PE)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Viruses

MHV-A59 and fMHV were propagated in 17 Cl 1 and Fcwf cells, respectively, as previously described (Kuo et al., 2000; Schickli et al., 1997). Virus titers were measured by plaque assay on 17 Cl 1, Fcwf, AK-D, RIE or BHK cells as previously described (Gagneten et al., 1995; Kuo et al., 2000). Virus-inoculated HRT-18G cells were incubated under 0.8% SeaKem agarose (BioWhittaker Molecular Applications, Rockland, ME) and MEM (GIBCO) with 8% FBS and 2% PSF.

Detection of virus-infected cells

Virus inoculation of cells grown on coverslips and detection of newly synthesized viral nucleocapsid (N) protein by immunofluorescence were performed as previously described (Thackray and Holmes, 2004). Virus-inoculated cell monolayers were incubated under agar, and plaques were visualized by neutral red staining or immunolabeling of viral N protein in cell monolayers. Cell monolayers were washed with isotonic phosphate buffered saline and fixed in methanol/acetic acid at −20 °C for 10 min. Expression of N protein was detected using anti-N MAb followed by biotinylated anti-mouse IgG and avidin DH/biotinylated horseradish peroxidase (HRP) H complexes (VECTASTAIN Elite ABC kit; Vector Laboratories Inc., Burlingame, CA). Avidin–HRP complexes were visualized by deposition of 3,3′-diaminobenzidine (DAB; Vector Laboratories Inc., Burlingame, CA).

Alignment of S330 of Group II coronaviruses

S330 sequences of MHV strains and the corresponding domains of other group II coronaviruses were obtained using the following GenBank accession numbers: MHV-A59 (AY497328), MHV-JHM (X04797), MHV-1 (D83333), MHV-2 (D83334), MHV-3 (D83335), MHV-S

(D83337), MHV-U (D83336), MHV/BHK (AY497331), RCoV (AAF97738), BCoV strain L9 (P25191) and HCoV-OC43 (Z21849). Deduced aa sequences were aligned and five residues that were highly conserved in S330 of all MHV strains, but not in S330 of other group II coronaviruses were identified (Fig. 1A).

Generation of S constructs

The S constructs used in this paper were assembled in pBC SK+ (Stratagene, La Jolla, CA) (Fig. 1B) and used to replace the S gene of pMH54 (Kuo et al., 2000) or pMH54-EGFP (Das Sarma et al., 2002). The transcription vector pMH54-EGFP, containing the 3′-most 7.4 kb of the MHV-A59 genome and the gene for enhanced green fluorescent protein (EGFP) in place of gene 4, was kindly provided by Susan Weiss (Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA). S gene sequences in this paper were numbered according to Genbank accession number AY497328 (Schickli et al., 2004).

Site-directed mutagenesis was used to introduce point mutations into a cDNA encoding the S protein, the S/pBC SK+ construct containing an *Avr*II site at nt 37 (Thackray and Holmes, 2004). Mutant S constructs were amplified with cloned Pfu DNA polymerase (PFU; Stratagene, La Jolla, CA) using various primer pairs (Table 3) essentially as previously described (Wentworth and Holmes, 2001). Mutant S constructs were screened by restriction enzyme digestion and/or sequence analysis. All 14 S constructs were subcloned into the S gene of pMH54 using *Avr*II and a *Dra*III site at nt 1271. Y162H, Q, A and F mutant S constructs were also subcloned into pMH54-EGFP. The 5′ ends of each construct were 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). Six primers: Sstart, G133D-, A59.3, A59.4, A59.30 and A59.22 (Schickli et al., 2004; Thackray and Holmes, 2004) were used to generate overlapping sequences for each construct.

Table 3
Oligonucleotides used for site directed mutagenesis and sequencing

Primer	Sequence (5′–3′)[a]
Forward	
K183G+	GCACACGGATGTAggACCCCCAATTTGTG
K183R+	GCACACGGATGTAAggCCCCCAATTTGTG
L65A+	GCCACATTATTGgcTACTGGTTACTACCCG
L65H+	GCCACATTATTGCaaACTGGTTACTACCCG
L79A/T82A+	GTTTAGAAACgcCGCTCTTgCGGGAACTAACTC
L79M/T82M+	GTTTAGAAACaTgGCTCTTAaGGGAACTAACTC
S33G+	GGTGCTAATGTTgGTGCTCCAAGCATTAGC
S33R+	GGTGCTAATGTTcGTGCTCCAAGCATTAGC
S(*Not*I)+	GCTGTTATAGcGGCCGcGTTTCTGCTGC
S(*Xba*I)+	GCTTAGGTGTTcTAGAAGATAATTGTGG
T62A+	CGAGTTTATTTAAATGCCgCtTTATTGCTTACTGG
T62S+	CGAGTTTATTTAAATGCCtCtTTATTGCTTACTGG
Y162A+	CCATTTGTCAGTTACCTgcCACTGATTGTAAGC
Y162F+	CCATTTGTCAGTTACCTTcaACTGATTGTAAGC
Y162H+	CCATTTGTCAGTTACCTcACACTGATTGTAAGC
Y162Q+	CCATTTGTCAGTTACCTcAaACTGATTGTAAGC
Reverse	
572	TCGCTTTAACACACAAATTGG
BZ25	CCGTCTAGATATGTGGATCTTTTCAACGTC

[a] Nucleotides that differ from the S gene sequence of MHV-A59 (GenBank accession no. AY497328) are shown in lower case. Nucleotides that encode amino acid substitutions are underlined.

Targeted RNA recombination

Mutations were introduced into the genome of MHV-A59 by targeted RNA recombination (TRR) as previously described (Thackray and Holmes, 2004). Briefly, Fcwf cells were inoculated with the chimeric helper virus fMHV, transfected with pMH54 or pMH54-EGFP derived donor RNAs containing engineered mutations in the S gene, and immediately overlaid onto 17 Cl 1 or BHK + mCEACAM1a(I41R) cell monolayers in individual 25-cm^2 flasks. After 48 h, culture media were collected, clarified by centrifugation, and flash frozen. Independently recovered replicates were plaque-purified and propagated in 17 Cl 1 cells.

Sequence analysis of recombinant viruses

Total cellular RNA from 17 Cl 1 cells inoculated with each of the recombinant viruses was extracted and reverse transcribed as previously described (Thackray and Holmes, 2004). The cDNA was amplified with primers Sstart and A59.6 or A59.7 and 4a(IGS) as previously described (Thackray and Holmes, 2004). Amplification products were sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility as described above. Eleven primers: 572, A59.3, A59.30, A59.C7, S(*Xba*I)+, A59.20, S(*Not*I)+, A59.19, A59.10, A59.11, and BZ25 [Table 3 and (Schickli et al., 2004; Thackray and Holmes, 2004)] generated overlapping sequences for each S gene.

Neutralization of viruses with purified, soluble murine CEACAM1a protein

Virions were incubated with purified, anchorless mCEACAM1a[1,4] essentially as described previously (Zelus et al., 1998). Briefly, 30 μl of virus (5000 PFU) were incubated with 180 μl of soluble mCEACAM1a 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. After incubation for 1 h at 37 °C, virus survival was determined by plaque assay on 17 Cl 1 cell monolayers. Percent virus neutralization was calculated as: 100 − [(number of plaques from virus incubated with mCEACAM1a/number of plaques from virus incubated with buffer alone) × 100].

Receptor blockade with an anti-murine CEACAM1a monoclonal antibody

Virus inoculation of murine cells in the presence of 0.05 mg/ml anti-CEACAM1a MAb-CC1 or isotype matched MAb-Ctrl was performed as previously described (Schickli et al., 1997; Thackray and Holmes, 2004).

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