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SHORT COMMUNICATION

A novel full-length isoform of murine *pregnancy-specific glycoprotein 16* (*psg16*) is expressed in the brain but does not mediate murine coronavirus (MHV) entry

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Abstract The mouse pregnancy-specific glycoprotein 16 (PSG16) has been reported to be an alternative receptor for mouse hepatitis virus (MHV), some strains of which cause encephalitis in mice lacking the canonical receptor CEACAM1a. The known isoforms of PSG16 are N-terminally truncated relative to other PSG family proteins and are expressed in neurons as well as in the placenta. We have cloned a novel full-length isoform of *psg16* that is also expressed in the brain, placenta, and retina but, like the truncated form, lacks MHV receptor activity when expressed on 293T cells, suggesting that PSG16 does not mediate CEACAM1a-independent spread of MHV.

Keywords Mouse hepatitis virus · Pregnancy-specific glycoprotein · Coronavirus entry · Receptor · CEACAM1a

Introduction

Spread of the JHM.SD strain of mouse hepatitis virus in CEACAM1a-deficient neurons is consistent with the use of an alternative receptor

A paradox of mouse hepatitis virus (MHV) pathogenesis is that while some strains of MHV are highly neurovirulent and neuronotropic, the canonical receptor CEACAM1a is poorly expressed in the mouse brain, especially in neurons; only trace amounts of *ceacam1a* mRNA are observed in pure neuronal cultures (Bender et al. 2010), and CEACAM1a protein has been detected in the central nervous system only in microglia (Ramakrishna et al. 2004) and endothelial cells (Godfraind et al. 1997). Furthermore, the spike protein of the extremely neurovirulent JHM.SD isolate, but not that of the moderately neurovirulent A59 strain, mediates spread among neurons from *ceacam1a*$^{-/-}$ mice (Bender et al. 2010), suggesting either an alternative receptor or a receptor-independent mechanism of spread. While JHM.SD performs "receptor-independent spread" (RIS) via syncytia formation in nonmurine cell monolayers (Gallagher et al. 1992), it is not clear that this phenomenon applies in vivo, especially as infected neuronal cell bodies are not observed to form syncytia (Bender et al. 2010). These results are consistent with the existence of an alternative receptor that allows JHM.SD to spread between neurons.

Pregnancy-specific glycoprotein-16, an unusual CEA family member, is a reported MHV receptor

The murine pregnancy-specific glycoprotein-16 (PSG16; previously brain carcinoembryonic antigen/bCEA) is expressed in the brain, especially in neurons, and has previously been reported to be an MHV receptor (Bender et al. 2010; Chen et al. 1995). The murine pregnancy-specific glycoproteins, which mediate placentation (Ha et al. 2010; Lisboa et al. 2011) and modulate interactions between the fetus and the maternal immune system (Bebo and Dveksler 2005), resemble the related carcinoembryonic antigen (CEA) proteins in their extracellular Ig-like domains but are secreted rather than membrane-anchored. To date, two full-length cDNA clones of *psg16* with different 3′ ends

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(designated C1 and C2) resulting from alternative splicing via an apparent cryptic splice donor site in exon 5 have been reported (Fig. 1a, 4N1*C1 and 4N1*C2; the truncated N1 domain is designated N1* by analogy to human CEACAM20; Zebhauser et al. 2005; Chen et al. 1995; Strausberg et al. 2002). Both cDNAs are truncated at the 5′ end relative to other *psg* family members, and the resulting proteins lack the signal sequence and the N-terminal portion of the Ig V-type N1 domain (Fig. 1b), suggesting that they may not enter the secretory pathway or reach the cell surface. RNA sequences corresponding to hypothetical upstream coding sequences of the *psg16* gene have been detected in a placental EST library (McLellan et al. 2005), but the full-length transcript has not been cloned. The combination of this alternative upstream sequence and the additional splice donor site within exon 5 could produce four possible isoforms of *psg16* (Fig. 1a, b). The region homologous to the MHV S protein-binding site on the N domain of CEACAM1a is absent from the truncated protein isoforms, although even the full-length N1 lacks critical virus-binding residues in the CC′ loop of CEACAM1a; the corresponding regions of the N2 and N3 domains, like those of the much weaker receptors CEACAM1b and CEACAM2, are dissimilar to the CEACAM1a sequence (Fig. 1c, boxed residues; Peng et al. 2011; Rao et al. 1997). The lack of a signal sequence, membrane anchor, or homology to the virus-binding site of CEACAM1a cast doubt on the MHV receptor activity of PSG16. We previously attempted to assess the potential MHV receptor activity of the commercially available 4N1*C2 isoform of *psg16* by constructing FLAG-tagged chimeric proteins expressing the extracellular domains of PSG16-4N1*C2 fused to the signal sequence and membrane anchors of the avian retrovirus receptor TVA in order to direct them to the plasma membrane; however, we were

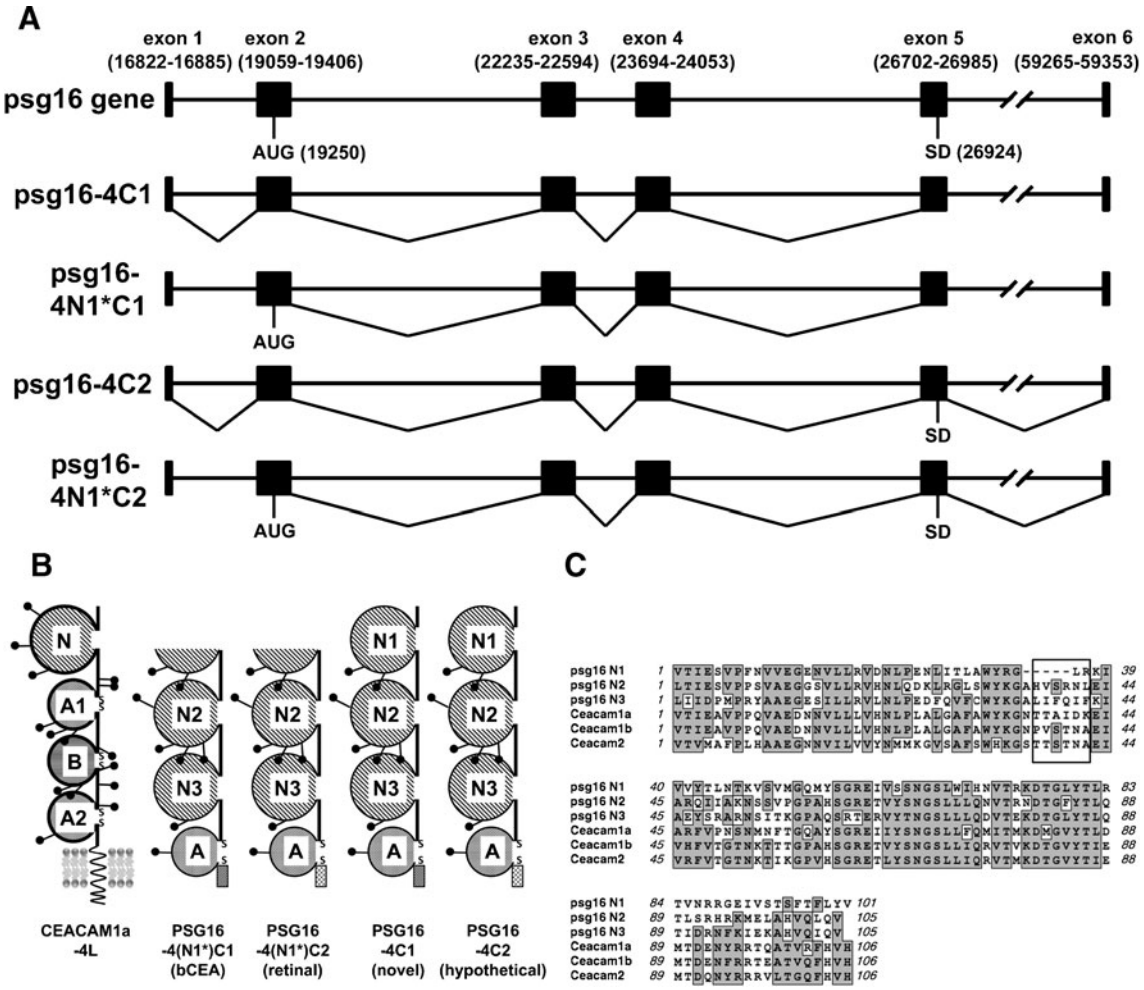


Fig. 1 Up to four isoforms of PSG16 are produced but lack homology to the virus-binding regions of CEACAM1a. **a** An alternative start codon (*AUG*) and a cryptic splice donor site (*SD*) in exon 5 allow synthesis of up to four distinct *psg16* mRNAs. **b** The previously published N1* isoforms of PSG16 lack the N-terminal portion of the N1 domain. CEACAM1a-4L is shown for reference. **c** Amino acid alignment of the N domains of PSG16, CEACAM1a, CEACAM1b, and CEACAM2. Note that all of the domains lack significant sequence similarity in the key virus-binding motif of CEACAM1a (*boxed*)

unable to detect either surface expression or receptor activity of the chimeric proteins (Bender et al. 2010) and therefore could not confirm the MHV receptor activity of PSG16.

Results and discussion

A novel full-length isoform of *psg16* is also expressed in the brain

While we could not detect receptor activity for the N-terminally truncated 4N1*C2 isoform of PSG16, we hypothesized that the theorized secreted full-length 4C1/2 isoforms with native signal sequences might be expressed in the brain and serve as MHV receptors. To obtain these isoforms, we prepared RNA from C57BL/6 mouse E15.5 placenta and successfully PCR-amplified full-length *psg16-4C1* from placental cDNA using primers based on the published hypothetical sequence. We next designed primers specific for each of the four possible isoforms of *psg16* (Table 1) to determine their tissue expression profiles. We were able to detect *psg16* expression by isoform-specific PCR on cDNA from young adult (6–10-week-old) C57BL/6 mouse brain and retina, but not from the liver (Fig. 2), using the primers listed in Table 1. While the 4C1 isoform was detected in all three tissues, the alternative C2 3′ end, originally derived from a C57BL/6 mouse retinal cDNA library, was not detected in the retina or elsewhere despite generous amounts of template (each 25-μL PCR reaction contained 2 μL of cDNA reverse-transcribed from 0.75–1 μg RNA in a 20-μL reaction) and intensive amplification (50 cycles; note that the PCR should therefore be considered nonquantitative, as indicated by the relatively strong *ceacam1a* control signal in the brain). We do not question the authenticity of the C2 isoform(s) and suggest that this splicing event may be developmentally or otherwise regulated. Note that as the truncated 4N1* isoforms have no unique 5′ coding sequence, any primer in the coding region of 4N1* will also bind to the full-length transcript; therefore, while no tissue expressed the 4N1*-C1 isoform in the absence of 4C1, we did not attempt to determine whether 4N1*-C1 was expressed alongside 4C1. While the function of PSG16 in the mouse brain remains mysterious, we can now report that *psg16* mRNA with an intact N1 domain, encoding a potentially secreted protein, is expressed in mouse placenta, brain, and retina.

PSG16 isoforms do not support MHV expression when expressed at the cell surface

In order to investigate the MHV receptor activity of the novel PSG16 isoform, the previously published chimeric TVA-PSG16 and TVA-CEACAM constructs were transferred to the pCAGGs vector in order to increase protein expression. The extracellular domains of the *psg16-4C1* isoform cloned from placenta were then subcloned into the TVA chimeras to yield chimeric proteins with the native PSG16 signal sequence, a FLAG tag, and the transmembrane or GPI anchor from the TVA protein; an additional chimera with the PSG16 N1 domain in place of the CEACAM1a N domain (TVA-PSG16N1-CEACAM1a-TM) was also constructed. 293T cells were then transfected with these plasmids, analyzed for surface expression of chimeric protein by flow cytometry, infected in parallel with enhanced green fluorescent protein (EGFP)-expressing MHV, and analyzed for infection as previously described (Bender et al. 2010). All of the TVA-CEACAM1a and -CEACAM2 chimeras were expressed at the cell surface with the exception of TVA-CEACAM2-TM, which had been also been expressed at lower levels than the other chimeras from the pCB vector (Bender et al. 2010), suggesting that it may have been somewhat cytotoxic. The TVA-PSG16-4N1*C2 chimeras, which had not been detectable on the cell surface when expressed from the pCB vector (Bender et al. 2010), were detectable, albeit at low levels, when expressed from pCAGGS; we attribute the difference to stronger expression from the pCAGGS promoter. The TVA-PSG16-4C1 and -4C2 isoform chimeras were also readily detectable at the cell surface (Fig. 3a, b). We therefore infected TVA-CEA chimera-expressing cells 48 h postinfection with recombinant A59-EGFP [the virus for which PSG16 had receptor activity according to Chen et al. (1995)] and rA59/S_{JHM}-EGFP (a chimeric virus with the JHM spike in the A59 background, used because the JHM spike confers CEACAM1a independence). However, only the chimeras with CEACAM N domains supported MHV infection of transfected 293T cells (Fig. 3c; A59-EGFP data not shown because the results were identical); while occasional fluorescence was observed among the PSG16-transfected cells, these events were rare, were also present in the mock-transfected cells, and did not spread to adjacent cells (rA59/S_{JHM}-EGFP performs RIS) and are therefore best explained as fluorescent

Table 1 Primers used for specific amplification of *psg16* isoforms

Primer	F/R	Sequence (5′→3′)
4C1/4C2	F	CTTCTTCGTGTTGACAATCTGCCAGAGAATCTTATAACCTTAG
4N1*C1/C2	F	GATAGTGTCCAGCAACGGGTCC
4C1/4 N1*C1	R	CCAGGCTGACTGGGAGACTGGATTTC
4C2/4N1*C2	R	CCCCATGGTGTCAGTTGTTTCCTGC

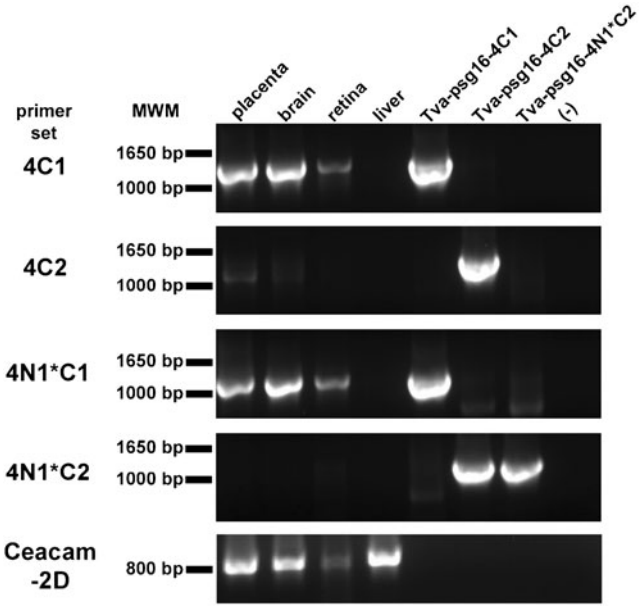


Fig. 2 *psg16-4C1* is expressed in the mouse placenta, brain, and retina. cDNA synthesized from the mouse placenta (positive control), brain, retina, and liver (negative control) was amplified with primers specific to exon 1, the unique portion of exon 5, and exon 6 to determine the mRNA tissue expression profiles of the four PSG16 isoforms. Plasmids encoding the 4C1, 4C2, and 4N1*C2 isoforms were used as controls. Primers specific to CEACAM1a were used as a positive control for the liver cDNA (the product shown represents the extracellular domains of the 2D isoforms)

debris from the unfiltered inoculum rather than infected 293T cells. We conclude that none of the isoforms of PSG16 is an MHV receptor when expressed on the surface of 293T cells.

The mechanism of CEACAM1a-independent spread by the JHM.SD strain of MHV remains unknown

We cannot at this time resolve the discrepancy between our results and the original report by Chen et al. (1995) of the MHV receptor activity of PSG16. One hypothesis is that PSG16 does have receptor activity but requires a coreceptor or other cofactor that is expressed on neurons and not on 293T cells. In theory, such a cofactor could both potentiate receptor activity and anchor native PSG16 to the cell surface; a soluble extracellular fragment of CEACAM1a has been shown to be surface expressed and to mediate MHV entry, presumably due to heterodimerization with native CEACAM proteins (Dveksler et al. 1996). However, the original demonstration of MHV receptor activity of PSG16 was performed in COS7 (African green monkey kidney) cells (Chen et al. 1995), and it seems improbable that COS7 cells and primary mouse neurons share a cofactor that is absent from 293T cells. Moreover, the receptor activity of PSG16 was originally measured by virus release from infected transfected cells. PSG16-transfected cells produced low levels of A59 and no JHM virus (Chen et al. 1995), whereas it is JHM that spreads in *ceacam1a*$^{-/-}$ neurons, suggesting that any PSG16 receptor activity observed in the bCEA/COS7 system is in any case unrelated to the CEACAM1a-independent interneuronal spread of JHM.SD. As no *psg16*$^{-/-}$ mouse is available and siRNA knockdown in primary neurons remains inefficient, we cannot exclude the possibility that neuronal PSG16 plays a role in MHV pathogenesis; however, it does not seem to be an alternative receptor for MHV. Whether JHM.SD spreads among *ceacam1a*$^{-/-}$ neurons by using an alternative receptor or by a receptor-independent mechanism remains to be seen.

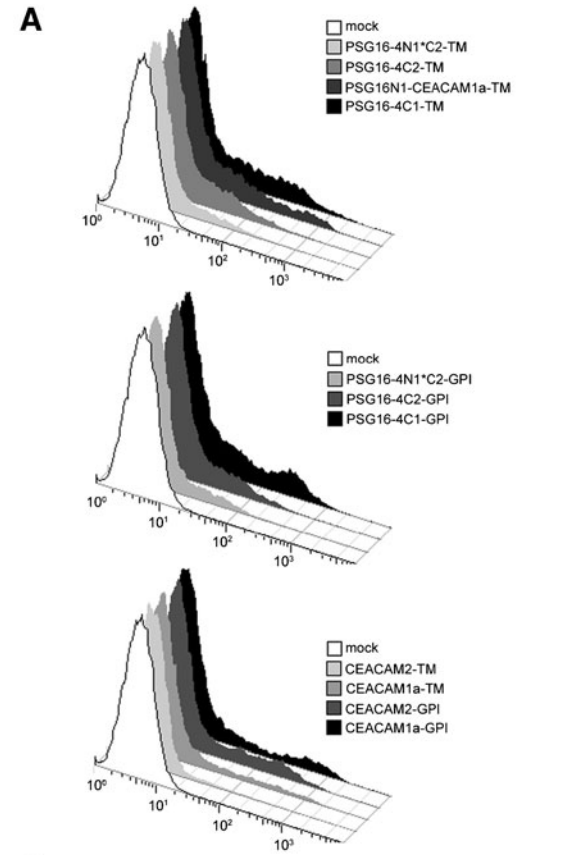
Materials and methods

PCR amplification of *psg16* and plasmid construction

RNA was prepared from E15.5 mouse placenta and young adult (6–10-week-old) mouse brain, liver, and retina using the Qiagen RNeasy Mini-Kit (Qiagen) according to the manufacturer's instructions with the optional β-mercaptoethanol added to the lysis buffer. cDNA was synthesized using Superscript III (purchased from Invitrogen and the primers from Integrated DNA Technologies). Each reverse transcription reaction contained 350 ng (for initial PSG16-4C1 amplification from placenta) or 800–1000 ng (for isoform-specific amplification) RNA and 50 ng random hexamer primers in a final volume of 20 μL containing 0.5 mM mixed dNTPs and 5 mM DTT in 1× First Strand Buffer. The initial PSG16-4C1 amplification from placental cDNA was performed using 1× ThermoPol Buffer (New England Biolabs), 200 μM dNTP mix (Invitrogen), 1 μM each forward primer 5′-CTAGAACCGGTACCATGGAGGTGTTCTCTGTGCTTCC-3′ and reverse primer 5′-GGCCGACGCGTTTATTTTCCTTTGGACCCCAAGTCTCACTTAAAATGATGG-3′, 2 μL cDNA template, and 1 μL Vent Polymerase (New England Biolabs) in a 100-μL reaction and amplified with a program of 5 min at 95°C, 10 cycles of 30 s at 95°C, 30 s at 58.0°C, and 1 min 30 s at 72°C, followed by 20 cycles of 30 s at 95°C, 30 s at 65°C, and 2 min at 72°C, followed by a final extension of 10 min at 72°C. The isoform-specific PCR amplification was performed using 1× PCR Rxn buffer (–$MgCl_2$; Invitrogen), 2 μM $MgCl_2$, 200 μM dNTP mix (Invitrogen), 1 μM each of the forward and reverse primers shown in Table 1, 2 μL cDNA template or 1 ng plasmid template, and 0.25 μL Taq polymerase (Invitrogen) in a 25-μL reaction and amplified with a program of 5 min at 95°C, 50 cycles of 30 s at 95°C, 30 s at 60°C, and 2 min at 62.5°C, and a final extension of 10 min at 72°C.

Plasmid construction

The PSG16-4C1 amplified from placental cDNA was inserted into the TOPO TA Cloning® for Sequencing kit



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Plasmid	Percent positive	Median fluorescence intensity
CEACAM1a-TM	12.3	59.2
CEACAM1a-GPI	19.1	77.9
CEACAM2-TM	3.9	24.5
CEACAM2-GPI	21.1	84.8
PSG16-4(N1*)-TM	10.9	32.1
PSG16-4(N1*)-GPI	10.1	31.8
PSG16-4C1-TM	30.3	68.7
PSG16-4C1-GPI	34.5	52.6
PSG16-4C2-TM	22.2	36.7
PSG16-4C2-GPI	21.4	33.6
PSG16-N1-CEACAM1a-TM	32.2	48.5

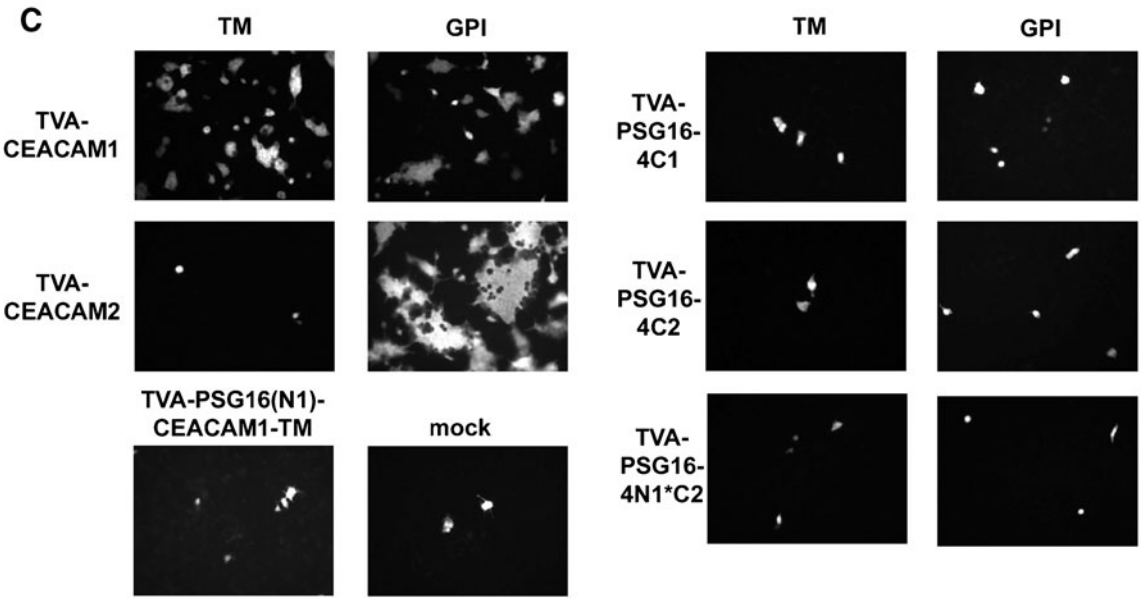

Fig. 3 No known isoform of PSG16 is an MHV receptor when expressed at the surface of 293T cells. **a** Anti-FLAG cell surface staining and flow cytometry 40 h posttransfection showed surface expression of all PSG16 constructs. TVA-CEACAM2-TM was not expressed. **b** Percentages and median fluorescence intensities of FLAG-positive cells. **c** 293T cells were transfected with the indicated TVA-CEA constructs, infected 48 h later with rA59-EGFP (not shown) or rA59/S_{JHM}-EGFP (a chimeric virus with the JHM.SD spike on the rA59 background), and assessed 16 h later for EGFP expression by fluorescence microscopy. Note that fluorescence of PSG16-transfected cells is similar to that of the mock-transfected background

(Invitrogen) vector by postamplification addition of 3′A overhangs and ligation according to the manufacturer's protocol. The PSG16-4C1 sequence was confirmed by sequencing the resulting plasmid. Plasmids pCB-PSG16-FLAG-4C1-FLAG-Tm and -GPI were generated by replacing the KpnI-SacII fragments of pCB-TVA-CEACAM1a-

FLAG-Tm and -GPI with a PCR product in which primers were used to add a KpnI site and a Kozak consensus sequence to the 5′ end and to remove the stop codon and add a SacII site to the 3′ end of the PSG16-4C1 sequence; this insert replaced the chimeric TVA signal peptide of the pCB-TVA-CEACAM1a plasmids with the authentic PSG16 sequence. Plasmids pCB-PSG16-4C2-FLAG-Tm and -GPI were made by replacing the KpnI-PmlI fragments of pCB-TVA-PSG16-FLAG-Tm and -GPI (Bender et al. 2010); pCB-TVA-PSG16-FLAG-Tm and -GPI were subsequently renamed pCB-TVA-PSG16-4N1*C2-FLAG-Tm and -GPI to incorporate the new designation for the N-terminally truncated isoforms. All of the PSG16 and CEACAM constructs were then transferred from pCB into pCAGGS-MCS by replacing the EcoRI-XhoI fragment of pCAGGS-MCS with MfeI-XhoI fragments amplified from the pCB plasmids with primers that added a 5′ MfeI site and a 3′ XhoI site. The chimeric pCAGGS-PSG16N1-CEACAM1a-Tm construct was made by replacing the EcoRI-SacII fragment of pCAGGS-CEACAM1a-Tm with a two-step PCR product in which the 5′ end was amplified from pCAGGS-PSG16-4C1-FLAG-Tm and the 3′ end from pCAGGS-CEACAM1a-FLAG-Tm. The PCR-amplified portions of all plasmids were confirmed by sequencing analysis.

CEA protein expression and MHV infection

The expression and analysis of the pCAGGS-TVA-CEA constructs and the MHV infections were performed as previously described (Bender et al. 2010). Briefly, 293T cells in six-well plates were transfected with 400 ng of each plasmid and 600 ng of empty pCAGGS-MCS using FuGene 6. Forty hours posttransfection, cells were surface stained with a mouse M2 anti-FLAG primary antibody (Amersham) and a goat antimouse AlexaFluor 488-conjugated secondary antibody and analyzed by flow cytometry. The threshold for considering cells positive for expression was set at the level at which 99% of mock-transfected cells were negative. In parallel, cells were infected with rA59-EGFP or rA59/S_{JHM}-EGFP 48 h posttransfection and fixed and analyzed for EGFP expression 16 h postinfection.

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