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Attachment of Mouse Hepatitis Virus to O-Acetylated Sialic Acid Is Mediated by Hemagglutinin-Esterase and Not by the Spike Protein▽

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The members of *Betacoronavirus* phylocluster A possess two types of surface projections, one comprised of the spike protein (S) and the other of hemagglutinin-esterase (HE). Purportedly, these viruses bind to O-acetylated sialic acids (O-Ac-Sias) primarily through S, with HE serving merely as receptor-destroying enzyme. Here, we show that, in apparent contrast to human and ungulate host range variants of *Betacoronavirus-1*, murine coronaviruses actually bind to O-Ac-Sias via HE exclusively. Apparently, expansion of group A betacoronaviruses into new hosts and niches was accompanied by changes in HE ligand and substrate preference and in the roles of HE and S in Sia receptor usage.

Receptor specificity is a major viral determinant of host preference, cell tropism, and pathogenesis. In coronaviruses (CoVs) (family *Coronaviridae*, order *Nidovirales*), which are enveloped, plus-strand RNA viruses of clinical and veterinary relevance, receptor binding is mediated by the class I fusion spike protein (S). Often, the receptors employed are glycoproteins and virion-receptor binding involves protein-protein interaction. Some CoVs, however, specifically bind to glycans (10, 19, 26, 30, 38). For example, members of phylocluster A in the genus *Betacoronavirus* (previously known as "coronavirus group 2A"; see http://talk.ictvonline.org/media/g/vertebrate-2008/default.aspx for official coronavirus taxonomy) use O-acetylated sialic acid (O-Ac-Sia) either as the primary receptor or as the initial attachment factor. Among them are human coronavirus OC43 (HCoV-OC43), bovine coronavirus (BCoV), and porcine hemagglutinating encephalomyelitis virus (PHEV), which are host range variants of the species *Betacoronavirus-1*, and mouse hepatitis virus (MHV) (species *Murine coronavirus* [MuCoV]) (22, 28, 34, 38). Interestingly, these viruses differ from other CoVs in that they code for an additional spike protein species, the hemagglutinin-esterase (HE) (1, 3, 9, 11, 17, 18). HE possesses sialate-*O*-acetylesterase receptor-destroying enzyme (RDE) activity (20, 29, 37, 42, 43), which allows virions to elute from sialylated surfaces (29, 34, 38). It may thus facilitate the release of viral progeny from infected cells and provide virions with a means of escape from irreversible attachment to non-cell-associated sialoglycoconjugates and off-target host cells.

Remarkably, group A betacoronaviruses differ from each other in their dependency on O-Ac-Sia cell surface expression. For the infection of cultured cells, HCoV-OC43, BCoV, and PHEV critically require 9-O-Ac-Sias as receptor determinants (13, 38), whereas MuCoVs rely exclusively on their primary receptor CAECAM1a (4–6, 39). As a likely reflection of this biological difference, HE expression is dispensable in MHV and rapidly lost during serial passage *in vitro* (17, 41). Indeed, many MHV laboratory strains, including the best-studied variant MHV-A59, carry a defective HE gene (18). As inferred from phylogenetic analyses, however, the HE gene is preserved in MHV field strains, implying that HE expression does offer a selective advantage during natural infection. These studies also revealed a relatively recent gene flow event during which the ancestor of one branch of MuCoVs exchanged its HE-coding sequence for that of an as-yet-unknown coronavirus (31). As a result, two MuCoV biotypes exist, exemplified by isolates MHV-DVIM and MHV-S, which differ primarily in their HEs, with the former possessing sialate-9- and the latter sialate-4-*O*-acetylesterase activity (22, 31, 34, 40).

Although CoV HEs also function as lectins (21, 34, 43) and even possess dedicated receptor-binding domains (43), their contribution to host cell attachment remains a subject of debate. Reportedly, HCoV-OC43, BCoV, and MHV bind to O-Ac-Sias primarily via their S proteins (14, 27, 29, 32, 40). Whereas there is strong evidence in support of this view for *Betacoronavirus-1* variants, the roles of the MuCoV HE and S proteins in O-Ac-Sia receptor usage has not been studied in great detail.

To determine which MHV envelope protein (S, HE, or both) mediates attachment to Sia, we constructed by targeted RNA recombination (15) a set of recombinant MHV-A59 (rMHV-A59) derivatives in which the autologous genes for HE and S were replaced by those of MHV-S or MHV-DVIM (Fig. 1A). The construction of rMHV-A59-HE^{S} was described previously (17). Sequence analysis confirmed that all genetic modifications were as intended and that no inadvertent mutations were introduced (not shown). Synthesis of RNA2b (the HE mRNA), which is lost in MHV-A59, was restored in recombinant viruses rMHV-A59-HE^{S} (17) and rMHV-A59-HE^{DVIM} (Fig. 1B).

Virus stocks were prepared by pelleting virus particles from the supernatants of infected LR7 cells (15) through a 20% sucrose cushion (80,000 × *g*, 2 h, 4°C). For proper comparison of the viruses, it was crucial to confirm that the heterologous envelope proteins become incorporated into recombinant viri-

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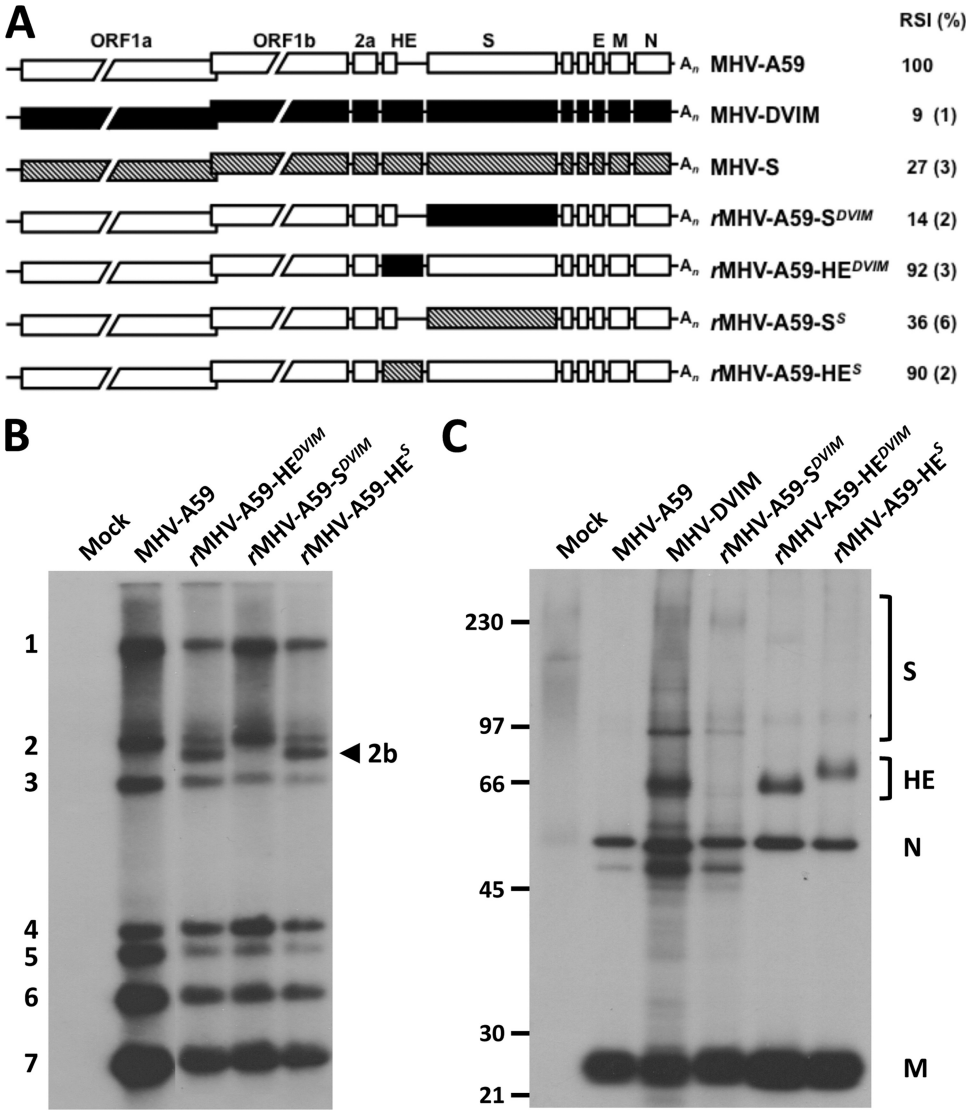

FIG. 1. Construction of recombinant MHV viruses via targeted RNA recombination. (A) Genome organization of MHV-A59, MHV-DVIM, MHV-S, and recombinant MHV-A59 derivatives. White boxes, MHV-A59 genes; black boxes, MHV-DVIM genes; shaded boxes, MHV-S genes. Genes for the polymerase polyproteins (ORF1a and ORF1b) and the 2a, HE, S, small envelope (E), membrane (M), and nucleocapsid (N) proteins are indicated. A_n, poly(A) tail. Note that in MHV-A59, the HE gene is interrupted by a nonsense mutation at codon 15 (indicated by a shortened box) and that consequently MHV-A59 and derivates rMHV-A59-S^{DVIM} and rMHV-A59-S^S do not express the HE protein. Numbers on the right are the relative specific infectivity (RSI) values as calculated for the recombinant viruses from the PFU-to-genome copy ratio, with that of MHV-A59 set at 100%. Plaque assays in LR7 cells and TaqMan RT-PCR assays were performed in triplicate; standard deviations are given in parentheses. (B) mRNA profiles of MHV-A59 and recombinant viruses rMHV-A59-HE^{DVIM}, rMHV-A59-S^{DVIM}, and rMHV-A59-HE^S. Intracellular viral RNAs were [^{3}H]uridine labeled and separated in formaldehyde-0.8% agarose gels as previously described (17). mRNA species are numbered according to convention; mRNA2b is indicated by an arrowhead. (C) SDS-PAGE analysis of virus particles, metabolically labeled and immunopurified as described previously (17). Molecular masses (in kDa) are given at the left; bands corresponding to the structural proteins M, N, HE, and S are indicated at the right.

ons in amounts similar to those in the parental viruses and to perform Sia binding assays with equivalent numbers of virus particles. As there were consistent differences in specific infectivity between MHV-DVIM, MHV-S, and MHV-A59 (see below), uncorrected infectious titers are not a reliable indication of the amount of virions. Instead, the number of genome copies as determined by semiquantitative TaqMan reverse transcription-PCR (RT-PCR) was taken as a measure. RNA, isolated from purified virus using the QIAamp viral RNA minikit (Qiagen), was subjected to real-time RT-PCR using an ABI Prism 7700 sequence detector (Applied Biosystems) with primers MHV1b-F (5′-ACGGTGACGATGTTATCTTCAGC-3′) and MHV1b-R (5′-TTACCTTGTGGGCTCCGGTA-3′) and a fluorescent probe (5′-FAM-ATGGCTCGGTTCAAGGCTCCCTG TA-TAMRA-3′) to amplify and detect a conserved open reading frame 1b (ORF1b) region. The relative number of genome copies in each virus stock was calculated from a standard curve determined for purified MHV-A59 genomic RNA (not shown).

As shown for rMHV-A59-HE^{DVIM} in Fig. 1C and reported previously for rMHV-A59-HE^{S} (17), HE proteins were incorporated into the recombinant virions in quantities similar to those in the respective wild-type donor viruses. As an additional quantitative measure of HE incorporation in rMHV-A59-HE^{DVIM}, we determined virion-associated *O*-acetylesterase activity using *p*-nitrophenyl acetate as a substrate (22, 36). *O*-Acetylesterase titers for rMHV-A59-HE^{DVIM}, when corrected for virus particle concentration, corresponded to those for MHV-DVIM (relative enzyme activity of 1.0 ± 0.05 versus 1.5 ± 0.14 mU, respectively).

The S protein is present in only small quantities in virus particles, is heterogeneously glycosylated, is subject to partial proteolytic cleavage, and is easily lost during virus purification (17). Consequently, the amount of S protein in virions cannot be assessed reliably by SDS-PAGE. As the S protein is the sole determinant of MHV entry *in vitro* (5, 6), the specific infectivity provides an indirect measure of the amount of functional S protein in the virion. The relative specific infectivity (RSI) of each virus was calculated from the ratio of the relative number of viral particles, determined by TaqMan RT-PCR, to PFU, with the RSI of parental virus MHV-A59 set at 100% (Fig. 1A). The RSIs of MHV-DVIM and MHV-S were consistently 11- and 4-fold lower, respectively, than that of MHV-A59, possibly reflecting differences in adaptation to propagation *in vitro*. Notably, rMHV-A59-S^{DVIM} and rMHV-A59-S^{S} had RSIs comparable to those of their respective donor viruses, MHV-DVIM and MHV-S, and thus apparently incorporate wild-type amounts of S protein into their virions.

Parental and HE-expressing recombinant viruses were tested in solid-phase whole-virion binding assays (22, 44) against bovine submaxillary mucin (BSM) and horse serum glycoproteins (HSG); these natural sialoglycoconjugates are rich in 9-O- and 4-O-Ac-Sias, respectively (7, 23). Virion binding was detected by HE-associated *O*-acetylesterase activity toward 4-methylumbelliferyl acetate (25). The HEs of MHV-S and MHV-DVIM cleave this substrate at comparable rates (Fig. 2B), allowing direct comparison of these viruses and their recombinant derivatives in the solid-phase assay.

As described previously (22), MHV-S bound to HSG but not to BSM. Conversely, MHV-DVIM bound exclusively to BSM. Importantly, recombinant virus rMHV-A59-HE^{DVIM} also tested positive in the binding assay but, like MHV-DVIM, bound only to BSM. Binding was abolished by pretreating BSM with bacterial neuraminidase or purified sialate-9-*O*-acetylesterase, while treatment with sialate-4-*O*-acetylesterase was ineffective. rMHV-A59-HE^{S}, like MHV-S, bound only to HSG. Its binding was sensitive to sialate-4-*O*-acetylesterase treatment but resistant to treatment with sialate-9-*O*-acetylesterase or neuraminidase (note that 4-O-Ac-Sias are resistant to all neuraminidases described to date [2]). These observations confirm and extend previous findings (22, 34) by showing that (i) both biotypes of MHV attach to Sia; (ii) binding is critically dependent on specific Sia modifications, i.e., 4-O- or 9-O-acetylation; (iii) MHV Sia receptor-binding specificity corresponds to the sialate-*O*-acetylesterase substrate preference of HE; and (iv) HE is sufficient to mediate virion binding to natural sialoglycoconjugates.

As can be inferred from the complementary binding profiles of rMHV-A59-HE^{S} and rMHV-A59-HE^{DVIM}, the S protein of

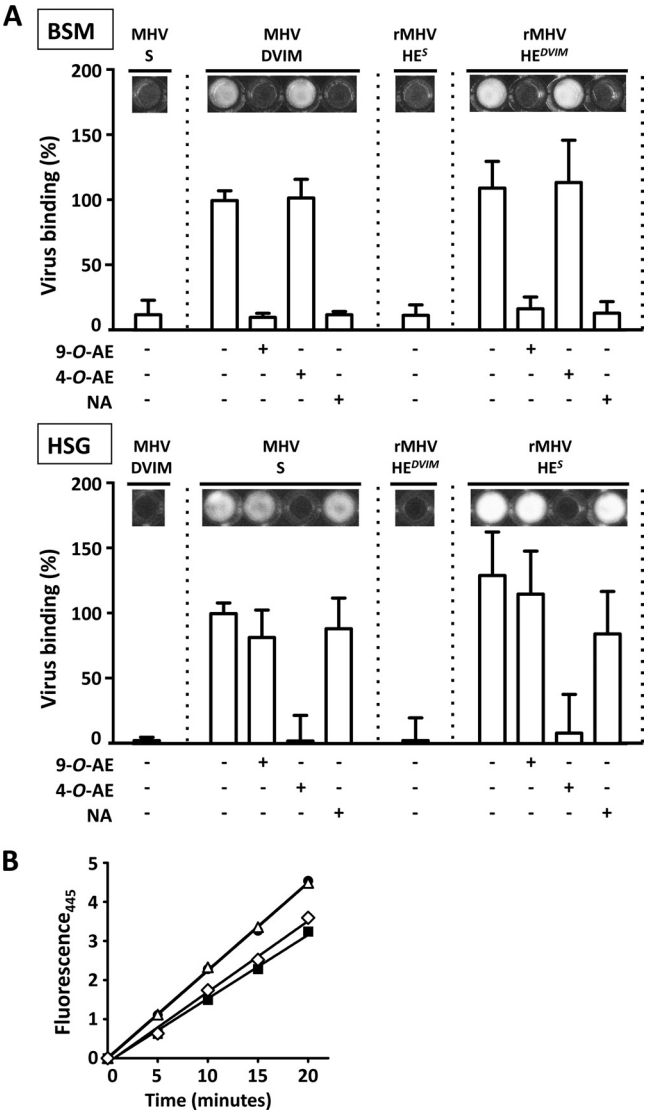

FIG. 2. Attachment of wild-type and recombinant MHVs to O-Ac-Sias as measured by solid-phase whole-virion binding assay. (A) HE is sufficient to mediate MHV virion binding to natural sialoglycoconjugates. BSM and HSG, applied to 96-well Maxisorp plates (Nunc), were either mock treated or treated with purified 9-*O*-acetylesterase of porcine torovirus strain Markelo HE (9-*O*-AE) (16, 31), purified 4-*O*-acetylesterase of MHV strain S HE (4-*O*-AE) (22), or *Arthrobacter ureafaciens* neuraminidase (NA) (Roche Applied Science). The solid-phase assay was performed as described previously (12, 44) with equal amounts of virus particles (equivalent to 1.0×10^7 PFU of MHV-A59). Binding of viruses was measured by fluorophotometric detection (excitation and emission wavelengths of 330 nm and 445 nm, respectively) of 4-methylumbelliferone released from the synthetic substrate 4-methylumbelliferyl acetate (4-MUAc) by virion-associated HE *O*-acetylesterase activity. The data are presented as bar charts, with the fluorescence measured for MHV-DVIM bound to BSM and for MHV-S bound to HSG set at 100%. (B) HEs of MHV-S and MHV-DVIM hydrolyze 4-MUAc at comparable rates. Equal amounts of virus particles (equivalent to 2.0×10^6 PFU of MHV-A59) in 100 μl phosphate-buffered saline (PBS) with 0.2 mM 4-MUAc were incubated at ambient temperature, and hydrolysis of the substrate was followed over time. Black squares, MHV-DVIM; black dots, MHV-S; open diamonds, rMHV-A59-HE^{DVIM}; open triangles, rMHV-A59-HE^{S}.

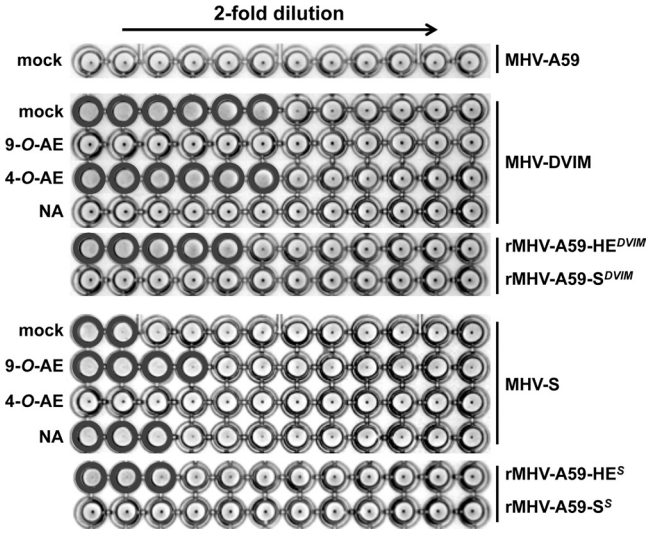

FIG. 3. Attachment of wild-type and recombinant MHVs to O-Ac-Sias as measured by hemagglutination assay. Rat erythrocytes (*Rattus norvegicus* strain Wistar) were mock treated or treated with 9-*O*-acetylesterase (9-*O*-AE), 4-*O*-acetylesterase (4-*O*-AE), or *A. ureafaciens* neuraminidase (NA) prior to hemagglutination assay with twofold serial dilutions of virus. Assays were performed in PBS with final dilution of erythrocytes to 0.2% and with equal amounts of virus particles (starting amounts equivalent to 1.0×10^7 PFU of MHV-A59). The arrow indicates the direction from low to high virus dilutions. Gray circles indicate wells displaying hemagglutination.

MHV-A59 does not mediate virion binding to Sia. However, the results do not allow conclusions about the Sia-binding properties of the S proteins of MHV-DVIM and MHV-S, strains that are less adapted to tissue culture conditions and, presumably, more representative of naturally occurring MuCoVs. As an alternative to the solid-phase binding assay, viruses were tested for hemagglutination. In accordance with the literature (33–35), MHV-DVIM readily agglutinated rat erythrocytes (Fig. 3). Surprisingly, and in contrast to earlier reports (35), we also observed hemagglutination by MHV-S. Agglutination was observed only when fresh erythrocytes were used and rapidly decreased during storage of the red blood cells at 4°C. Assays performed with neuraminidase- or sialate-*O*-acetylesterase-treated erythrocytes confirmed that MHV-DVIM and MHV-S specifically bound to cell surface 9-O- and 4-O-Ac-Sias, respectively. In fact, removal of 9-*O*-acetyl groups even led reproducibly to an ~4-fold increase in MHV-S hemagglutination titers, suggesting that MHV-S prefers 4-mono-O- over 4,9-di-O-acetylated Sia species (Fig. 3). MHV-DVIM and rMHV-A59-HE^{DVIM}, when tested at equal particle concentrations, agglutinated rat erythrocytes to identical titers. No agglutination was observed for rMHV-A59-S^{DVIM}. Likewise, MHV-S and rMHV-A59-HE^{S} hemagglutinated to similar extents, while rMHV-A59-S^{S} tested negative. The combined findings conclusively show that MHV-DVIM and MHV-S bind to O-Ac-Sias via their HE and not via their S proteins. As these isolates have a limited passage history in cultured cells and are representative of the two MuCoV biotypes, we submit that our observations can be extrapolated to all naturally occurring MuCoV variants.

HE appears to be a recent addition to the betacoronavirus proteome and, as it originated from an influenza virus C-like hemagglutinin-esterase fusion protein, would have come with 9-O-Ac-Sia-binding activity (8, 24, 36, 43). It may thus be envisaged that the acquisition of the HE by the betacoronavirus group A ancestor at once led to a two-protein strategy for virion attachment, with S mediating binding to the main receptor (a specific glycoprotein) through protein-protein interactions and HE mediating binding to O-Ac-Sias. While some group A betacoronaviruses, including MHV, apparently continued to use this strategy, others seem to have taken the next step. In contrast to MHV, BCoV and related viruses that are united in the species *Betacoronavirus-1* critically require O-Ac-Sias for infection and bind to these receptor determinants also via their S proteins (13, 38). It is tempting to speculate that in the course of *Betacoronavirus-1* evolution, the S protein extended or maybe even completely shifted receptor specificity from protein to glycan moieties to recognize a new receptor determinant, 9-O-Ac-Sia, originally used by HE alone.

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