The Hemagglutinin-Esterase of Mouse Hepatitis Virus Strain S Is a Sialate-4-\textit{O}-Acetylersterase

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Mouse hepatitis virus (MHV) is a positive-strand RNA virus belonging to the family \textit{Coronaviridae}. Several viruses have been classified as members of this family, which can be subdivided into three antigenic clusters (3, 28). MHV belongs to the same cluster as bovine coronavirus (BCV) and human coronavirus OC43. A major characteristic of this cluster is the presence of a hemagglutinin-esterase (HE) surface glycoprotein in addition to the viral spike protein. The latter is present in all coronaviruses, while the HE protein may be present or absent in viruses of the MHV cluster.

The HE protein of MHV is encoded by a gene located immediately upstream of the spike gene. It is expressed from mRNA 2-1; the molecular mass is approximately 60 to 69 kDa. In virions, it is found as a dimer anchored in the viral membrane by a C-terminal transmembrane region. Expression of the HE gene is highly variable between MHV strains. Functional HE proteins have been detected in MHV-JHM (27), MHV-S (38), and MHV-DVIM (31, 32). In MHV-S, large levels of the HE protein are found, while MHV-JHM expresses relatively low amounts (27, 38). Different MHV-JHM isolates express variable levels of HE, depending on the number of UCUAA repeats at the 3’ end of the leader RNA (18).

The presence of HE is not strictly required for MHV replication. MHV-A59 and several other MHV strains do not express HE (17, 27, 38). In MHV-A59, it is not expressed due to a missing initiation codon (17). In addition, the upstream promoter determinant of synthesis of mRNA 2-1 is destroyed in this strain. In other MHV strains, mutations and deletions at the 3’ end of the HE gene have been detected; as a result, HE proteins without a transmembrane anchor are encoded. Biochemical analysis of these truncated forms could be detected neither in lysates of infected cells nor in culture supernatants (38). Interaction of HE alone with target cells is apparently not sufficient for infectivity. MHV-DVIM replication is inhibited by a monoclonal antibody specific for the MHV receptor, indicating that interaction of the viral spike protein with cellular receptor molecules is mandatory for infection (6). The presence of HE may, however, modulate tissue tropism particularly within the central nervous system. MHV strains expressing an HE protein exhibit some preference for infecting neurons (for a review, see reference 1). Differences in neuropathogenicity of viruses with or without HE expression are at least partially derived from immune responses against HE. Passive immunization of mice with HE-specific monoclonal antibodies resulted in protection against a lethal infection, possibly by inhibition of virus spread through the central nervous system (39). MHV variants with mutations in the HE gene were isolated from such animals at late stages of infection (41). In a recent study, mice were infected with a chimeric MHV-A59 strain containing an HE protein derived from cells transfected with a defective interfering vector expressing the HE gene of MHV-JHM. Data obtained in this study indicated an enhanced early innate response caused by transient expression of HE (42).

In addition to MHV strains, several other viruses have been shown to express HE proteins. Among these, BCV and influenza C virus have been studied most extensively. HE proteins of these viruses are receptor-destroying enzymes, removing \textit{O}-acyl groups from sialic acid-containing cellular receptor glycoproteins (11, 23, 25, 34, 35). In contrast, data on substrate specificities of MHV esterases are limited. Enzymatic activity was mostly determined with \textit{p}-nitrophenylacetate (pNPA) as the substrate (6, 21, 40). Recently, the esterase of MHV-DVIM was found to remove acetyl groups from the natural...
substrate bovine mandibulary gland mucin (BSM) at very low levels (33).

We recently characterized the HE protein of puffinosis virus (PV), a coronavirus closely related to MHV (14). In that study, we compared substrate specificities of PV and influenza C virus. Results obtained from this comparison led us to propose that compounds different from 5-N-acetyl-9-O-acetyl sialic acid (Neu5,9Ac₂) may be natural substrates for the PV HE. Because of the high amino acid sequence similarity between the HE proteins of PV and MHV, we have now extended our investigation on the substrate specificity of the MHV esterase.

In this report, we provide evidence that 4-O-acetylated sialic acid (Neu4,5Ac₂), but not Neu5,9Ac₂, is a natural substrate for the HE protein of MHV-S.

MATERIALS AND METHODS

Viruses and cells. MHV-S was kindly supplied by M. Buchmeier (Scripps Research Institute, La Jolla, Calif.). MHV-A59 and MHV-S were grown in mouse L cells. Influenza C/JJ/50 virus was isolated from embryonated eggs as described elsewhere (34).

Recombinant vaccinia virus. RNA derived from L cells infected with MHV-S was isolated as described by Spaan et al. (29). Purified RNA was reverse transcribed using Superscript II reverse transcriptase (Gibco), using oligonucleotide C171 (5′-AGGGATACTTGTGATTTGGCAGTAATACAC-3′) as the primer. The underlined segment is complementary to the 3′ region of the HE gene in addition, an EcoRI site was added to allow cloning. Then the HE gene was amplified by PCR, using oligonucleotide C171 and upstream primer oligonucleotide C170 (5′-AGGCGATGCTGTTACATCCGTAAGTACAC-3′) as the primer. The resulting PCR product was digested with EcoRI and cloned into pUC21. Cloning of the authentic gene was verified by sequencing of the resultant recombinant plasmid. For expression in vaccinia virus, the cloned gene was amplified by PCR, using oligonucleotides MHV-S-forward (5′-CCGGAATTCTACGTAATTGGCAGTAATACAC-3′) and -reverse (5′-CCAGAATTCCTGCTTGCC-3′) and oligonucleotide MHV-S-reverse (5′-CCAATCTAAGCTAGTCTGATGATGCTG-3′). The PCR product was digested with EcoRI and SmaI and cloned into the EcoRI/SmaI fragment of pATA gpt stop3. This plasmid is a derivative of pATA-18 (30), modified by the addition of the Escherichia coli xanthine guanine phosphoribosyltransferase gene (gpt) under the control of the vaccinia virus early/immediate promoter I3 and insertion of stop codons in all three reading frames into the SalI/HindIII fragment of the polylinker. Homologous recombination was performed by infection of 100 ng of plasmid pATA-S-HE. Recombinant vaccinia viruses were isolated by TK selection (36), followed by threefold plaque purification in RK13 cells with gpt selection (4).

HA assay. Hemagglutination (HA) assays were performed as described previously (36) with 0.5% human type O erythrocytes obtained from the local blood bank or from infected HeLa cells. HA titers were calculated as the reciprocal of the highest virus dilution resulting in full agglutination of erythrocytes.

Esterase assays. Acetyl esterase activity was determined with pNPA as described previously (34). One unit of viral esterase was defined as the amount of enzymatic activity required to cleavage of 1 nmol of pNPA per mg of viral protein. After incubation with 4-methylumbelliferyl acetate (4-MUAc). Hydrolysis of substrate was monitored at an excitation wavelength of 365 nm.

RESULTS

Comparison of enzymatic activities of MHV-S and influenza C/JJ/50 virus esterases. In a recent study, we obtained data on major differences in substrate specificities between the esterases of PV and those of influenza C virus and BCV. Because of the high sequence similarities of the HE proteins of PV and MHV esterases (85% identical amino acid sequence), we assumed that MHV may exhibit substrate requirements similar to those of PV (14). Esterase activity of MHV strains with an expressed HE protein has been demonstrated in the past with a synthetic low-molecular-weight substrate, pNPA. Compared to assays determining acetate release from natural substrates as mucin, esterase activity can be more easily determined with pNPA (34). With this assay, esterase activities of MHV-JHM (40), MHV-DVIM (6), and HE derived from the cloned genome of MHV-JHM Wb1 expressed by vaccinia virus (21) have been determined. Although this type of assay allows rapid determination of esterase activity, it does not provide evidence for sialate-O-acetyltransferase activity of MHV. When we used a purified MHV-S preparation and compared its esterase activity with that of influenza C/JJ/50 virus, we found acetyltransferase activity associated with both viruses in a pNPA assay. For further experiments, we defined 1 U of viral esterase as the amount required to hydrolyze 1 μmol of pNPA/min. Other -p-nitrophenyl esters, like pN-propionate, -butyrate, and -valerate, were not hydrolyzed to a significant extent by the viruses tested, indicating a high specificity of both esterases towards acetyl esters (data not shown). In contrast, we used glycoconjugates as natural mimicking substrates, major differences between virus-S and C/JJ/50 virus were observed. The latter, as well as BCV, specifically removes acetyl groups at position 9 of sialic acids from BSM (10, 11, 34, 36, 37). When we used 30 μU of esterase of influenza C/JJ/50 virus, release of 3.7 and 3.6 μg of acetate/mg of substrate/h from two different BSM preparations was observed. In contrast, after incubation of these substrates with 30 μU of MHV-S, we detected no free acetate.

The MHV HE is unable to destroy influenza C virus receptors on erythrocytes. We then tried to remove influenza C virus receptors from erythrocytes by preincubation with MHV-S. If the MHV esterase cleaved these receptors, we expected a drop in HA titers of influenza C virus similar to data obtained with BCV esterase (36). We tested potential effects of the MHV esterase with human type O erythrocytes as well as murine erythrocytes from 6-week- and 6-month-old animals. In these assays, we used influenza C/JJ/50 virus, because it agglutinates human and murine erythrocytes. Mock-treated human erythrocytes were agglutinated by C/JJ/50 virus with the same titer as MHV-S-treated cells. As a control, we treated these cells with influenza C/JJ/50 virus, rendering them unagglutinable by C/JJ/50 virus (Table 1). From this control assay, we concluded that influenza C virus receptors can be removed from erythrocytes, provided that an enzyme with the correct substrate spec-
ificity is used. Similar data were obtained for murine erythro-
cytes. Since young mice are more susceptible to infection with
MHV than adult animals, we tested cells obtained from ap-
proximately 6-week-old as well as 6-month-old mice. Erythro-
cytes from young animals were agglutinated by influenza
C/JJ/50 virus with the same titers, regardless of whether cells
were preincubated with buffer or MHV-S. There was a slight
increase in HA titers with erythrocytes obtained from adult
animals after incubation with MHV-S. This may indicate that
the MHV esterase unmasks some additional influenza C virus
receptors. However, given the only twofold increase, it appears
more likely that this result can be explained by small experi-
mental variations. Unfortunately, we were unable to do the
reverse experiment using HA titration of MHV, because we
could not detect any HA activity of MHV-S with the erythro-
cytes used.

Identification of Neu4,5Ac2 as substrate for the MHV ester-
ase. First, to determine whether the differences observed were
attributable to the type of linkage of terminal sialic acids to
underlying sugars, we tested the esterase activity of MHV-S
with free sialic acid derivatives. We first incubated chemically
prepared Neu5,9Ac2 containing small amounts of Neu5Ac,
Neu5,7Ac2, and Neu5,8Ac2 with purified MHV-S. As a con-
trol, heat-inactivated virus was used. For a positive control, we
incubated sialic acids with influenza C/JJ/50 virus, which was
able to convert Neu5,9Ac2 to Neu5Ac (Fig. 1A). Analysis of
sialic acids after incubation with MHV-S revealed no detect-
able de-O-acetylation of Neu5,9Ac2 (Fig. 1B). These data
strongly indicate that 9-O-acetylated sialic acids are not hydro-
lyzed by the acetylesterase of MHV-S. Next, sialic acids iso-
lated from BSM were incubated with active or heat-inactivated
MHV-S. In addition to the above-mentioned sialic acid deriv-
atives, Neu5Gc, Neu5Gc9Ac, and Neu5,8,9Ac3 were present in
this preparation. Again, the esterase of MHV-S was unable to
cleave any of these O-acetylated sialic acids (Fig. 1C).

We then tested whether sialic acid with an O-acetyl group in
position 4 could serve as an alternative substrate for MHV-S.
When we incubated free Neu4,5Ac2 with MHV-S, we observed
an almost complete (99%) loss of the sialate-4-
O
-acetyl ester
and a corresponding increase of the Neu5Ac peak (Fig. 2A). In
control incubations with heat-inactivated virus, no cleavage
occurred, which indicates that this conversion was due to the
viral esterase. To date, glycoproteins containing this sialic acid

TABLE 1. Effects of erythrocyte pretreatments with viral esterase
on hemagglutination by influenza C/JJ/50 virus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HA titera</th>
<th>Human</th>
<th>Mouse</th>
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<tbody>
<tr>
<td>PBS</td>
<td>128</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>C/JJ/50b</td>
<td>&lt;2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MHV-Sb</td>
<td>128</td>
<td>256</td>
<td>512</td>
</tr>
</tbody>
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a Reciprocal of the highest virus dilution resulting in full agglutination. All
experiments were performed three times. ND, not determined.
b Erythrocytes were preincubated with 30 mU of viral esterase for 3 h at 37°C.

FIG. 1. Influenza C virus HE, but not MHV-S esterase, is able to hydrolyze acetate esters at the glycerol side chain of sialic acids. The reversed-phase C18 HPLC
chromatograms show fluorescent derivatives of free sialic acids. (A and B) Chemically O-acetylated sialic acids, free Neu5Ac (peak 1), Neu5,7Ac2 (peak 2), Neu5,8Ac2 (peak 3), and Neu5,9Ac2 (peak 4), were incubated with influenza C virus (A) or with MHV-S (B). (C) Sialic acids, Neu5Gc (peak 1), Neu5Ac (peak 2), Neu5,7Ac2 (peak 3), Neu5Gc9Ac (peak 4), Neu5,8Ac2 (peak 5), Neu5,9Ac2 (peak 6), and Neu5,8,9Ac3 (peak 7), released from BSM, were incubated with MHV-S. Samples in
the upper and lower chromatograms were treated with heat-inactivated and with active virus, respectively.
derivative have been identified in only a few animals (e.g., guinea pigs [for a review, see reference 24]). Therefore, to clarify whether the MHV-S HE protein also recognizes glycosidically linked Neu4,5Ac2 on glycoproteins, we used guinea pig serum proteins. These contain sialic acids, of which approximately 25 to 29% represent Neu4,5Ac2 (13). Again, an almost quantitative conversion of Neu4,5Ac2 to Neu5Ac was observed within 45 min at 37°C (Fig. 2B).

Expression of the HE protein by recombinant vaccinia virus.
To determine whether the observed sialate-4-O-acetylesterase activity is an intrinsic property of the viral HE protein, we cloned the corresponding gene and inserted it into the thymidine kinase gene of vaccinia virus by targeted recombination. Vaccinia virus expressing the MHV esterase, termed VV-S-HE, was used to infect HeLa cells. Expression of esterase activity was monitored by incubating infected cells with α-naphthyl acetate, which results in precipitation of an insoluble dye on cells expressing the recombinant enzyme (Fig. 3). Mock-infected cells were not stained by this procedure. We then isolated membranes from infected cells and incubated them with guinea pig serum. Degradation of Neu4,5Ac2 and a corresponding increase of the Neu5Ac peak were observed after incubation with membranes from cells infected with VV-S-HE. Control incubations with membranes of cells infected with wild-type vaccinia virus revealed no change in the Neu4,5Ac2 content of guinea pig serum (Fig. 4). These data confirm that specific O-acetylesterase activity is encoded by the HE gene of MHV-S. We additionally observed a small but reproducible decrease in the amount of Neu5Gc after incubation of guinea pig serum with membranes from HeLa cells infected with recombinant VV-S-HE.

MHV-S exhibits binding activity toward glycoproteins with Neu4,5Ac2.
Next we examined if MHV-S is able to bind to Neu4,5Ac2 on glycoproteins. We used a solid-phase binding assay with immobilized proteins coated in microtiter plates. Since viral esterases commonly can cleave fluorogenic sub-
strates (7, 25), we first investigated if the MHV esterase exhibits enzymatic activity with fluorescein diacetate or 4-MUAc. Incubation of MHV-S with these substrates clearly resulted in cleavage of substrates (data not shown), comparable to rates observed with influenza C/JJ/50 and PV (14). In the assay, we used 4-MUAc as the substrate to detect virus binding. Since guinea pig serum glycoproteins were a substrate for the MHV esterase, we also used them in the assay. MHV-S exhibited binding activity with these immobilized glycoproteins in a concentration-dependent manner (Fig. 5). Binding was abolished by saponification of acetyl esters, indicating that this binding activity is specific for 4-O-acetyl esters present on guinea pig serum proteins. Similar results were obtained when we used horse serum, which is also a source of Neu4,5Ac2 (8). Again, MHV-S was able to bind to these immobilized proteins, and no binding was observed after removal of O-acetyl groups by mild alkali treatment. In contrast, no reactivity of MHV-S was observed when we used plates coated with BSM. These data suggest that the HE protein of MHV-S binds to Neu4,5Ac2 but not to other O-acetylated sialic acids.

**DISCUSSION**

In this study, we investigated the substrate specificity of the HE protein of MHV-S. Other viruses known to express evolutionarily related proteins are influenza C viruses (5, 11, 34), BCV (35, 36), human coronavirus OC43 (12, 43), hemagglutinating encephalomyelitis virus (26), and bovine torovirus (2). Several of these viral esterases have been characterized in terms of their substrate specificities and in all instances tested have been shown to recognize 9-O-acetylated sialic acids. Enzymatic activities of HE proteins in MCV strains have been described, but few data on their substrates and binding activities have been published (for a review, see reference 1). Recently, more data became available. First, Sugiyama et al. (33) reported significant differences on cleavage of a natural substrate known to contain high amounts of O-acetylated sialic acids. They found that MHV-DVIM, the only MHV strain exhibiting hemagglutinating activity, can hydrolyze O-acetylated sialic acids present on the natural substrate BSM, but at limited rates compared to other viral esterases. Furthermore, data published in this work indicated that MHV-S was essentially unable to liberate acetic acid from BSM (33). We have recently investigated another coronavirus, PV, and found similar differences regarding acetate release from Neu5,9Ac2. Particularly, BSM was found to be no substrate for PV, a virus closely related to MHV (14). These data had prompted us to hypothesize that other, unidentified O-acetylated compounds may be substrates for PV and closely related coronaviruses.

In this study we used MHV-S, a strain expressing high levels of HE protein (38). In contrast to BCV and influenza C virus, MHV-S exhibited no esterase activity with BSM and was in addition unable to remove influenza C virus receptors from
Sialate-9-O-acetylesteserase: Influenza C Virus
Bovine Coronavirus

AcO

Sialidase (Neuraminidase): Influenza A, B, and Parainfluenza Virus

AcO

Sialate-4-O-acetylesteserase: Mouse Repeptidase Virus S

COO-

erythrocytes. To clarify the reasons for these differences, we wanted to gain further information on the enzymatic activity of the MHV-S HE protein. We used either chemically synthesized sialic acid derivatives or sialic acids prepared from BSM, ESM, or guinea pig serum glycoproteins to characterize substrate specificities of the MHV-S esterase. We identified Neu4,5Ac2 as the only sialic acid derivative hydrolized by MHV-S. Other sialic acids with O-acetylation on the glycerol side chain were not de-O-acetylated at detectable amounts. MHV-S was able to hydrolize acetyl esters from free as well as glycosidically linked Neu4,5Ac2. In addition, we have demonstrated that this novel substrate specificity of MHV-S is a property of the viral HE protein. HE expressed by recombinant vaccinia virus exhibited the same reactivity with Neu4,5Ac2 as observed with MHV-S.

Recently, Sugiyama et al. reported acetae release by MHV-DVIM from isolated murine brush border membranes (33). However, in the case of MHV-DVIM, it remains to be determined whether this MHV strain also exhibits 4-O-acetylesteserase or the more classical 9-O-acetylesteserase activity. Taking into consideration the close relationship between amino acid sequences of MHV esterases, it appears likely that all MHV HE proteins are specific for Neu4,5Ac2. On the other hand, the exclusive specificity observed for the HE protein of MHV-S may be the result of subtle changes in the three-dimensional configuration of the viral enzyme during evolution. Possibly there exist viral esterases that recognize O-acetyl esters on sialic acids in positions 4 and 9. The possibility arises that in addition to MHV, other viruses with an esterase specific for Neu4,5Ac2 exist, infecting particularly animals which are known to possess such sialic acid derivatives, e.g., horses or guinea pigs (13). However, to our knowledge there is no evidence that MHV-S itself causes infections in these animals. It was concluded from experiments designed to infect cells expressing influenza C virus receptors containing Neu5,9Ac2. Such cells were not infected by MHV-DVIM unless the MHV receptor was expressed from the transfected gene. Since we now provide evidence that influenza C virus receptors are not bound by the HE protein of MHV-S, it remains to be determined if this also applies to MHV-DVIM. In the future, we will test whether MHV-S can infect cells expressing Neu4,5Ac2 but lacking the MHV receptor. Such experiments may shed light on whether the presence of the MHV receptor is a prerequisite for infection by MHV-S. Neu4,5Ac2 may either serve as secondary receptor modulating tissue tropism of HE-expressing MHV strains or represent an alternative receptor facilitating infection of cells devoid of the MHV receptor.

Since Neu4,5Ac2 has not yet been found in mice (13), the question arises about potential substrates for the HE protein in this host. In further experiments, it may be rewarding to explore the binding activity of recombinant, soluble MHV-S HE with 4-O-acetylated sialic acid-bearing glycoconjugates. This may be a useful tool for the histochemical detection of Neu4,5Ac2 in mice. Similar approaches to detect Neu5,9Ac2 have been described for recombinant, soluble influenza C virus HE (15, 16) as well as for purified influenza C virus HE (10, 44, 45).

In summary, we have identified a viral enzyme exhibiting a previously unidentified specificity. In addition to the sialidases of influenza A and B viruses and paramyxoviruses and the sialate-9-O-acetylesteserases of influenza C and BCV, a third type of receptor-destroying enzyme specifically cleaving 4-O-acetyl groups, has now been identified (Fig. 6).

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