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Identification of a Coronavirus Hemagglutinin-Esterase with a Substrate Specificity Different from Those of Influenza C Virus and Bovine Coronavirus

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We have characterized the hemagglutinin-esterase (HE) of puffinosis virus (PV), a coronavirus closely related to mouse hepatitis virus (MHV). Analysis of the cloned gene revealed approximately 85% sequence identity to HE proteins of MHV and approximately 60% identity to the corresponding esterase of bovine coronavirus. The HE protein exhibited acetylesterase activity with synthetic substrates *p*-nitrophenyl acetate, α-naphthyl acetate, and 4-methylumbelliferyl acetate. In contrast to other viral esterases, no activity was detectable with natural substrates containing 9-*O*-acetylated sialic acids. Furthermore, PV esterase was unable to remove influenza C virus receptors from human erythrocytes, indicating a substrate specificity different from HEs of influenza C virus and bovine coronavirus. Solid-phase binding assays revealed that purified PV was unable to bind to sialic acid-containing glycoconjugates like bovine submaxillary mucin, mouse α_1 macroglobulin or bovine brain extract. Because of the close relationship to MHV, possible implications on the substrate specificity of MHV esterases are suggested.

Members of several virus families possess surface glycoproteins with enzymatic activities. Well known are viral sialidases present in influenza A and B viruses, as well as in several paramyxoviruses. Sialidases remove sialic acids present on glycoproteins or glycolipids. Since viruses harboring sialidases bind to cellular receptors containing sialic acids, they are expressing an enzyme capable of removing virus receptors. Therefore, sialidases are also known as receptor-destroying enzymes (RDEs).

Besides sialidases, a second type of viral RDE is present in influenza C viruses (12, 33), several coronaviruses (for a review, see reference 1) and in bovine torovirus (2). RDEs of these viruses exhibit acetylesterase, as well as receptor-binding activity. Because of these two properties they are termed hemagglutinin-esterases (HEs).

HE proteins have been identified in several coronaviruses. An acetylesterase activity was first shown to be associated with bovine coronavirus (BCV), releasing acetate from bovine submaxillary mucin (BSM). BCV is able to remove its receptors from erythrocytes, as well as those for human coronavirus OC43 (HCV OC43) and for influenza C viruses (35). Since the HE protein of influenza C viruses was known to bind to cellular receptors containing 9-*O*-acetyl-5-*N*-acetyl sialic acids ($Neu5,9Ac_2$) as the major receptor determinant (12, 23, 33), it was concluded that the BCV esterase recognizes *O*-acetylated sialic-acid-containing receptors similar to those of influenza C viruses. It was further shown that the enzymatic activity is localized on a viral surface glycoprotein, which at that time was known as hemagglutinin or E3 protein (34). This protein was therefore renamed HE (14). Further studies confirmed the nature of the BCV receptor determinant as $Neu5,9Ac_2$ (26). Interestingly, the spike protein of BCV was found to be a stronger hemagglutinin than the HE protein and also bound to $Neu5,9Ac_2$ (25). In the case of BCV, it appears that the spike protein is the receptor binding entity, while the HE serves as the RDE. In contrast, for influenza C viruses the receptor-binding and receptor-destroying activities reside on a single surface glycoprotein (33, 9, 5).

More puzzling is the presence of an HE protein in mouse hepatitis virus (MHV), a virus belonging to the same antigenic cluster as BCV. Initiation of MHV infection is mediated by binding of the spike protein to a cellular receptor, which is a member of the murine carcinoembryonic antigens (38). The MHV receptor (MHVR, also known as Bgp1a or C-CAM) is a membrane-bound glycoprotein with four immunoglobulin-like domains. MHV binds via its spike protein to the N terminus of MHVR (3, 4). In contrast to BCV, interactions between the MHV spike protein and sialic acids, either on MHVR or on other glycoconjugates, have not been demonstrated. The HE protein is apparently not even required for virus replication. In MHV strain A59 no HE is present. Nevertheless, MHV-A59 replicates to high titers in tissue culture and causes infection in mice. Since this particular strain is very well adapted to tissue culture, it is widely used as a laboratory strain. For this reason, the gene encoding this protein was first found in MHV-A59 (16). It is, however, a pseudogene lacking the initiation codon. In addition, the mRNA is not expressed due to the lack of a consensus intergenic region upstream of the HE gene. Other MHV strains express this additional surface glycoprotein. For one strain, MHV-DVIM, hemagglutinating activity at a pH slightly below the optimum of the esterase activity was shown (31). Recombinant HE protein of MHV-JHM exhibits acetylesterase activity and is able to adsorb rat erythrocytes (21). On the other hand, hemagglutination of HE containing MHV strains is rather weak or undetectable (28, 32, 41).

One of the goals of this study was to determine whether additional coronaviruses express an HE protein. We have studied a coronavirus which was originally isolated during studies on puffinosis, a disease of birds (*Puffinus puffinus*) breeding on

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islands off the southwest coast of Wales (19). This coronavirus was later referred to as puffinosis virus (PV) (13). Tissue culture-adapted PV was found to express this protein. Moreover, determination of the substrate specificity revealed major differences to the esterases of BCV and influenza C virus. Because of the close relationship of the PV HE protein to those of different MHV strains, possible implications on substrate specificities of MHV HEs are suggested.

MATERIALS AND METHODS

Viruses and cells. PV was obtained from P. A. Nuttall (NERC Oxford). MHV-S was kindly supplied by M. J. Buchmeier (Scripps Research Institute, La Jolla, Calif.). These viruses and MHV-A59 were grown in mouse L or DBT cells. Influenza C/JJ/50 virus was isolated from embryonated eggs as described earlier (33).

RNA isolation. For isolation of genomic RNA, PV was concentrated from tissue culture supernatants by precipitation with polyethylene glycol and purified on a sucrose gradient (35). Genomic and intracellular RNA from infected cells was isolated as described previously (30).

Protein labeling and radioimmunoprecipitation assays. For labeling viral proteins, L cells infected with PV (multiplicity of infection of 2) were incubated at 10 h postinfection (p.i.) with labeling medium containing 200 μCi of ^{35}S-Translabel (ARC, St. Louis, Mo.) per ml. After incubation for 3 h at 37°C, virus particles were purified from the supernatant by centrifugation on a 20 to 60% sucrose step gradient. Virus collected from the interphase was pelleted by ultracentrifugation and analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE). For radioimmunoprecipitation analysis, labeled cell lysates were collected 10 h p.i., incubated with rabbit antiserum specific for MHV-JHM (kindly provided by J. Thalhammer, University of Salzburg), and analyzed as described previously (33).

cDNA cloning and sequence analysis. Purified RNA was reverse transcribed (8) with Superscript II reverse transcriptase (Gibco) by using random hexanucleotides (Pharmacia). *Eco*RI linkers were ligated to double-stranded cDNA. Random-primed cDNA was ligated into the *Eco*RI site of pBluescript (Promega) and transformed into *Escherichia coli* DH5α by standard procedures (24). Nucleotide sequence analysis was performed with an automated sequencer (LICOR) on both strands of the cloned cDNA. The nucleotide sequence data presented here were submitted to the EBI/EMBL database and are available under accession number AJ005960.

Esterase assays. Acetylesterase activity with *p*-nitrophenylacetate (*p*NPA) as the substrate was determined as described previously (33). One unit was defined as the amount of enzymatic activity resulting in the cleavage of 1 μmol of *p*NPA per min.

Release of acetate from glycoconjugates was determined with a commercial test kit as described earlier (35). Acetate contents of sialoconjugates were determined by saponification in 0.2 M NaOH at room temperature and subsequent neutralization with 0.2 M HCl (44). BSM types I and I-S, porcine mucin type III, calf fetuin, and bovine brain extract type VI were obtained from Sigma-Aldrich. Mouse α_1 macroglobulin was purified from mouse serum similar to published procedures (10, 15) with the following modifications: purification involved precipitation with 12% polyethylene glycol 6000 and sequential chromatography on Blue Sepharose CL6B and HiTrap Q Sepharose connected to an ÄKTA purifier (Pharmacia). Hemagglutinin (HA) inhibition activity of fractions obtained was determined with influenza C/JJ/50 virus.

In situ staining of virus plaques was performed with α-naphthyl acetate (αNA) with a cytochemical esterase staining kit (Sigma). Approximately 48 h p.i., cells were fixed for 4 h by adding CAF solution (4.6 mM citric acid, 2.3 mM Na citrate, 3 mM NaCl, 66% acetone, 3% formaldehyde [pH 3.6]) on top of the agarose overlay. After removal of the agarose, cells were washed with H_2O. Esterase-expressing viral plaques were detected by incubation with αNA-Fast Blue BB solution for 15 to 30 min at 37°C according to the manufacturer's instructions. Reactions were stopped by washing the cells with H_2O.

HA and hemagglutinin-inhibition (HI) assays. HA and HI assays were performed as described previously (35) with 0.5% human erythrocytes obtained from the local blood bank. HA titers were expressed as the reciprocal of highest virus dilution resulting in full agglutination of erythrocytes.

Solid-phase binding assay. Virus binding assays were performed on coated 96-well microtiter plates as described elsewhere (44). Glycoproteins were dissolved in phosphate-buffered saline (PBS) and allowed to bind at 4°C overnight (50 μl/well). Bovine brain extract type VI was dissolved in methanol, added to microtiter wells (50 μl/well), and evaporated. Wells were then washed with PBS, and the remaining binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 2 h at room temperature. After removal of BSA, wells were washed with PBS and virus suspensions were added (50 μl/well) and incubated for 2 h at 4°C. After removal of virus, wells were washed three times with PBS. Bound virus was detected by incubation with the synthetic substrate 4-methylumbelliferyl acetate (4MUAc). A 5 mM stock solution (in acetone) was diluted 50-fold with PBS, and 100 μl was added to the microtiter wells and incubated at 37°C. Cleavage of substrate was monitored at an excitation wavelength of 365 nm.

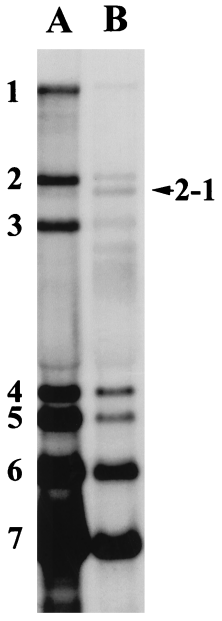

FIG. 1. Hybridization analysis of viral mRNA. Intracellular RNA was isolated from infected DBT cells at 8 h p.i. and subjected to electrophoresis on a denaturing agarose gel. The dried gel was hybridized to ^{32}P-labelled oligonucleotide O48 and autoradiographed. (A) MHV-A59-infected cells. (B) PV-infected cells. The numbers of viral mRNAs are indicated at the left, and mRNA 2-1 is indicated by an arrowhead.

RESULTS

During studies on puffinosis, a virus had been isolated by passage through suckling mouse brain and subsequent adaptation to mouse liver cell cultures. In this study, the virus was identified as coronavirus by electron microscopy. Serological assays revealed cross-reactions with MHV (19).

HE expression of PV. For further characterization of this virus, we first isolated RNA from infected cells. To determine the number of subgenomic RNAs transcribed from the genome of this virus, total RNA was subjected to electrophoresis on a denaturing agarose gel and hybridized with oligonucleotide O48 (5′-GTGATTCTTCCAATTGGCCATG-3′) complementary to a conserved region at the 3′ end of MHV and related viruses. Compared to MHV-A59 an additional RNA was found in cells infected with PV, migrating slightly faster than mRNA 2 (Fig. 1). A similar mRNA 2-1 encoding the HE protein is present in several MHV strains (28). The presence of this mRNA does not necessarily indicate expression of the HE protein. Due to point mutations or deletions in the coding region of the HE gene, several MHV strains do not express a functional HE (40). We therefore investigated whether PV does express this protein. Initial tests with the synthetic esterase substrate *p*NPA indicated relatively low levels of acetylesterase activity in virus preparations (data not shown). This might have been due to the low expression rates of the HE gene or to the presence of a mixture of viruses with or without a functional gene. We therefore plaque purified PV and screened individual isolates for acetylesterase activity. Among 24 preparations, isolates PV5 and PV14 were identified as expressing acetylesterase activity. To identify esterase-expressing virus plaques, we used an in situ staining procedure with αNA. This substrate has been used earlier to detect esterase

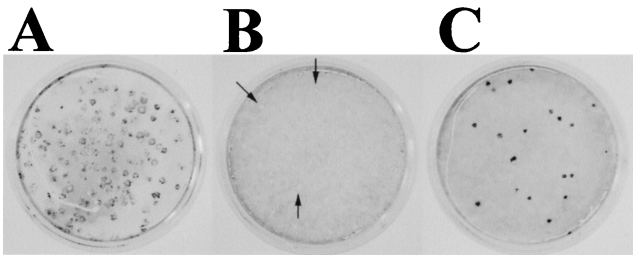

FIG. 2. In situ detection of acetylesterase activity in coronavirus plaques. L cells infected with coronavirus (20 to 100 PFU/dish) were fixed 36 h p.i. and stained with αNA for 15 to 30 min. (A) Plaque-purified isolate PV14. (B) MHV-A59. (C) MHV-S. Examples of unstained MHV-A59 plaques are marked with arrows.

activity of influenza C virus in infected MDCK cells (37) or immobilized on nitrocellulose filters and thin-layer plates (44). We have extended this method to detect coronavirus esterases in infected cells. Plaques of isolate PV14 were stained due to the esterase activity yielding insoluble α-naphthol–Fast Blue BB precipitate on infected cells (Fig. 2). As a negative control we used MHV-A59, which is devoid of HE protein (16). Unstained plaques were observed after incubation with αNA. For a positive control MHV-S, a strain expressing high levels of HE protein (39), was used in the assay. Other plaque-purified PV isolates exhibited no esterase activities. Thus, the initial low levels of esterase activity were caused by the presence of a mixed population. In the original PV preparation less than 10% of viruses expressed an active acetylesterase. PV14 exhibited acetylesterase activity comparable to that of MHV-S. By employing *p*NPA as a substrate, specific esterase activities of gradient-purified PV14 and MHV-S were found to be 5.9 and 6.7 mU/10^6 PFU, respectively.

Analysis of viral proteins. To determine expression rates and the apparent molecular weight of PV14 proteins, we prepared ^{35}S-labeled virus and subjected it to SDS-PAGE (Fig. 3A). The spike protein was found partially as uncleaved protein (S/gp180) and mainly as cleaved protein (S/gp90). In addition, the nucleoprotein (N) and at least three forms of the matrix protein (M) were clearly detectable. In contrast, the HE protein was found only in minor amounts after radioimmunoprecipitation with MHV-specific antiserum (Fig. 3B). Thus, expression rates of PV HE are comparable to those of MHV-JHM (29) and are lower than those described for MHV strains S and JHM(2) (39).

Cloning of the PV HE gene. For further characterization, we cloned the HE gene of PV, which was isolated from a set of clones reverse transcribed with random primers. Sequence analysis revealed that this gene is related to that of MHV. The PV HE gene encodes a protein with 439 amino acid residues, including a 24-amino-acid signal sequence. The putative active site serine residue of viral acetylesterases (36) within the conserved FGDS sequence is present at position 45 (Fig. 4). In the PV sequence, 85 to 87% of the amino acid sequences are identical, whereas 59 to 65 residues are different from the HE proteins of MHV strains. The mature PV HE protein contains 11 asparagine residues potentially serving as glycosylation sites. The corresponding proteins of MHV possess 9 (MHV-JHM) or 10 glycosylation sites (MHV-S and MHV-DVIM). All cysteine residues present in the mature PV protein are at the same positions as those of the MHV HE proteins (Fig. 5).

Analysis of substrate specificity. We then wanted to confirm that PV expresses sialate-9-*O*-acetylesterase activity. We first tested enzymatic activity with the synthetic substrate *p*NPA,

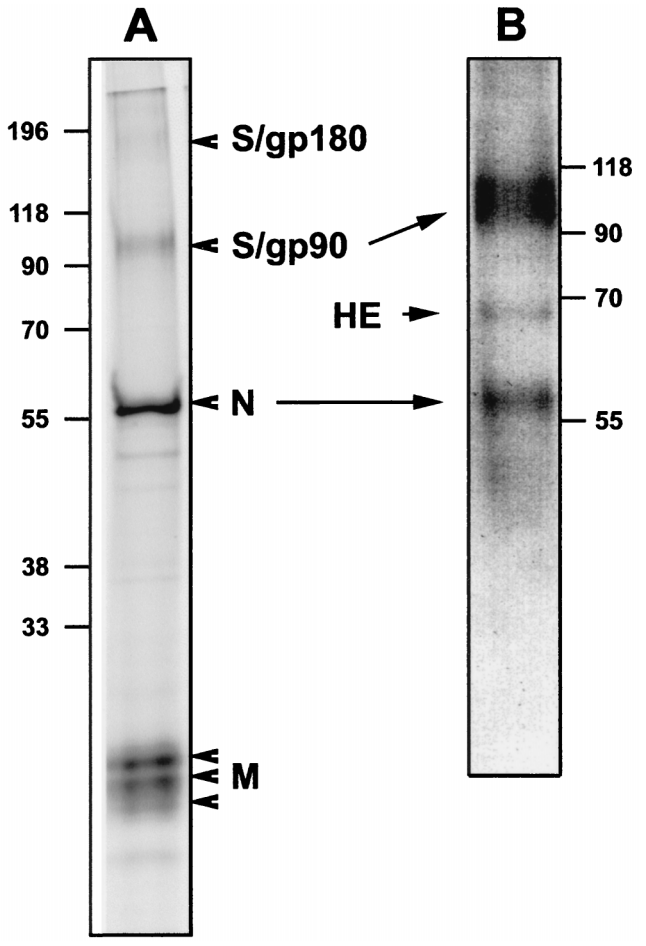

FIG. 3. Analysis of PV14 proteins. (A) L cells infected with PV14 were labelled 10 h p.i. with ^{35}S-Translabel for 3 h. Virus particles were purified from the supernatant by centrifugation on a 20 to 60% sucrose step gradient, pelleted, and analyzed by SDS–10% PAGE. (B) Radioimmunoprecipitation of PV14 proteins. Infected L cells were labelled 8 h p.i. with ^{35}S-Translabel and [^{3}H]leucine for 3 h. Then cells were lysed, and viral proteins were precipitated with rabbit anti-MHV-JHM and subjected to SDS-PAGE. Positions of the matrix protein (M), nucleoprotein (N), HE, and two forms of spike protein (S/gp90 and S/gp180) are indicated by arrows. Positions of the marker proteins (in kilodaltons) are also indicated.

and comparable rates of hydrolysis were observed for PV14 and influenza C/JJ/50 virus. To our surprise, we were unable to detect the release of acetate from BSM (Fig. 6). On the other hand, control assays indicated that this substrate was readily cleaved by the influenza C/JJ/50 virus esterase. In this assay, we used BSM with a sialic acid content of 12% and an acetate content of 1.6%. Similar results were obtained with another BSM preparation with 5% sialic acid and a 1.2% acetate content. Since other coronaviruses, including BCV (35, 20, 42), and hemagglutinating encephalomyelitis virus (27) are known to cleave *O*-acetylated sialic acids on mucin, these findings were unexpected. We then investigated a possible reason for observed differences in the enzymatic activities of influenza C/JJ/50 and PV. By using *p*NPA as a substrate, pH optima of PV and influenza C/JJ/50 esterase were determined and found to range between pH 7.4 and 7.8 (data not shown). Thus, the inability of PV esterase to release acetate from BSM due to a different pH optimum can be ruled out. This led us to speculate that the substrate specificity of PV esterase might be different from those of BCV and influenza C viruses.

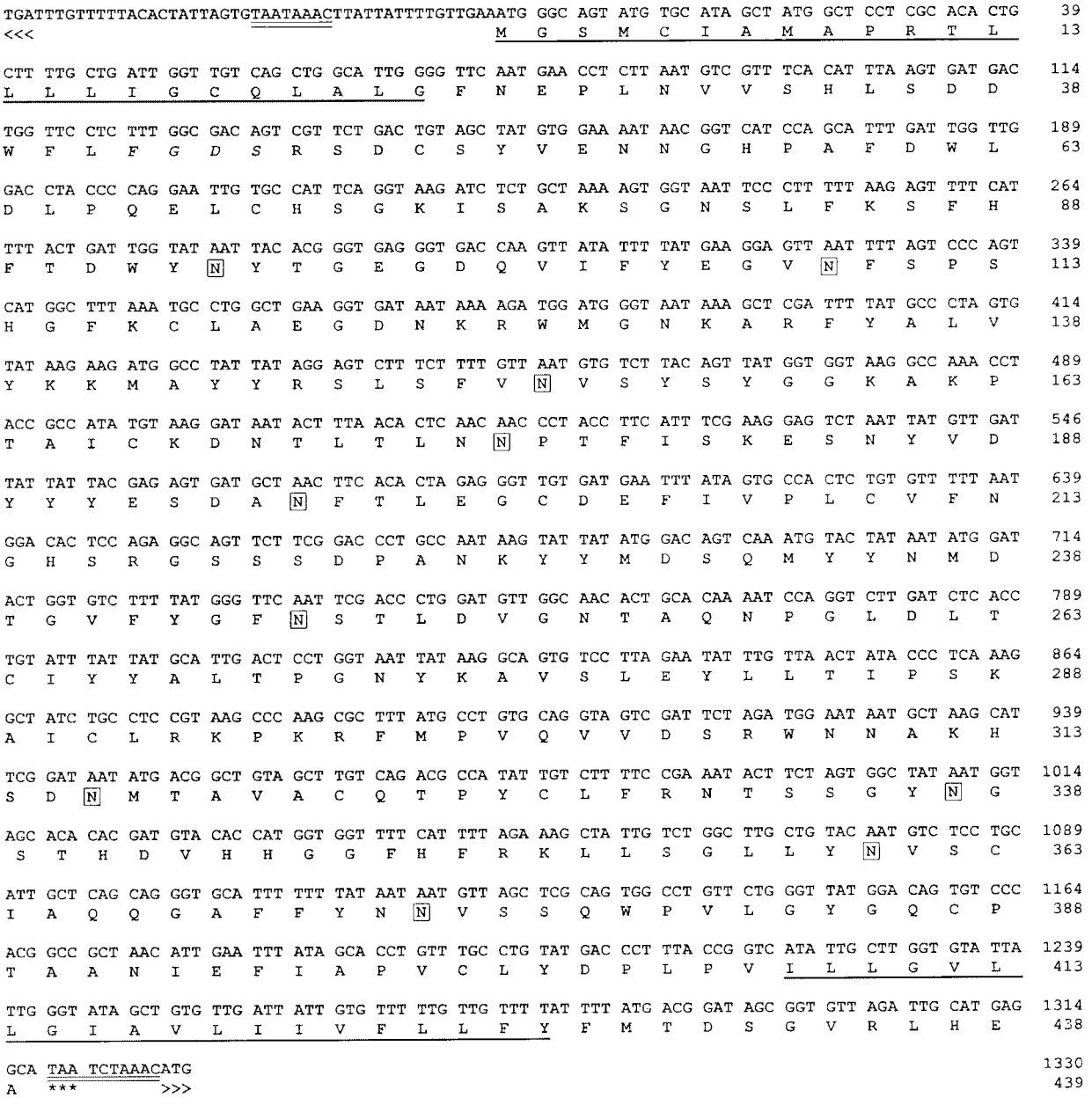

FIG. 4. Nucleotide sequence of the HE gene of PV. The stop codon of the upstream gene coding for the nonstructural protein 2a and the initiation codon of the downstream spike gene are indicated by <<< and >>>, respectively. Intergenic promoter sequences are double underlined, and the stop codon of the HE gene is indicated by asterisks. The deduced amino acid sequence is shown in the one-letter code. The predicted N-terminal signal sequence and the presumptive C-terminal transmembrane region are underlined. The conserved FGDS sequence with the putative active site serine residue is shown in italics. Potential *N*-glycosylation sites are boxed.

We then tested whether PV can remove receptors for influenza C virus from erythrocytes. Human erythrocytes were incubated with influenza C/JJ/50 virus, PV, or PBS. Then cells were washed three times with PBS and used for hemagglutination. Mock-treated or untreated erythrocytes were agglutinated by influenza C/JJ/50 virus (HA titer = 128). As expected (35), cells treated with influenza C virus were not agglutinated. In contrast, cells incubated with PV were agglutinated by C/JJ/50 with the same titer as the mock-treated cells. These data strongly indicate that Neu5,9Ac$_2$, a major receptor determinant for influenza C viruses (23), is not a substrate for PV acetylesterase.

PV exhibits no detectable affinity to sialic-acid-containing glycoconjugates. We then tested whether human erythrocytes or erythrocytes from mouse strains BALB/c and C57BL were agglutinated by PV at neutral pH and at pH 6.5. Under these conditions, MHV-DVIM was shown to agglutinate murine and rat erythrocytes (31). PV exhibited no agglutinin activity under the conditions tested. Due to this lack of HA activity, experiments to remove PV receptors from erythrocytes could not be performed. We therefore used a solid-phase assay on microtiter plates, which allows detection of virus binding to sialoconjugates (44). In this assay, bound virus is detected by its acetylesterase activity, which converts 4MUAc to 4-methyl

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PV       1 MGSMCIAMAPRTLLLLIGCQL.ALGF.NEPLNVVSHLSDDWFLFGDSRSDCSYVENNGHP  58
DVIM     1 --C-----------------.VF--.-----I----N-----------T--------  58
JHM      1 ---T----------------.VF--.-----I----N-----------T--------  58
S        1 --C-----------------.VF--.-----I----N-----------T--------  58
BCV      1       MFLLL-..FV-VS-IIGS---E-P-T------NG----------NH-V-TNPR  53

PV      59 AFDWLDLPQELCHSGKISAKSGNSLFKSFHFTDWYNYTGEGDQVIFYEGVNFSPSHGFKC 118
DVIM    59 KL-----DPK--N--R----------R----I-F---S---------------------- 118
JHM     59 KL-----DPK--N-------------R------F---------IV---------N----- 118
S       59 KL-----DPK--N--R-Y--------R----I-F---S---------------------- 118
BCV     54 NYSYM--NPA--D-----S-A---I-R------F-------Q-I--------T-Y-A--- 113

PV     119 LAEGDNKRWMGNKARFYALVYKKMAYYRSLSFVNVSYSYGGKAKPTAICKDNTLTLNNPT 178
DVIM   119 --Y---------------R--E---Q-----------A---N----S----K-------- 178
JHM    119 --Y---------------R--E---Q---------P-A--------S---HK-------- 178
S      119 --Y---------------R--E---Q-----------A---N----S----K-------- 178
BCV    114 TTS-S-DI--Q--GL--TQ---N--V----T----P-V-N-S-QS--L--SGS-V----A 173

PV     179 FISKESNYVDYYYESDANFTLEGCDEFIVPLCVFNGHSRGSSSDPANKYYMDSQMYYNMD 238
DVIM   179 ---------------E-----Q---------------K----------T---S----- 238
JHM    179 ---------------E-----A---------------K--------------S----- 238
S      179 ---------------E-----Q-------T---S----KS---------T-A-S---- 238
BCV    174 Y-AR-A-FG----KVE-D-Y-S----Y-----I---KFLSNT.....---D---Y-F-K- 228

PV     238 TGVFYGFNSTLDVGNTAQNPGLDLTCIYYALTPGNYKAVSLEYLLTIPSKAICLRKPKRF 298
DVIM   239 ---L------------V---------R-L---------------SL----------S- 298
JHM    239 ---L-------------KD--------R-L---------------SL---------- 298
S      239 ---L------------V---------R-L---------------SL----------S- 298
BCV    229 ---I--L---ETI.....TT-F-FN-H-LV-PS---L-I-N-L---V-T-----N-R-D- 283

PV     298 MPVQVVDSRWNNAKHSDNMTAVACQTPYCLFRNTSSGYNGSTHDVHHGGFHFRKLLSGLL 358
DVIM   299 ----------STRQ----------L---F-----AD-S-G-------D----Q------ 358
JHM    299 ----------STRQ----------L---F-----AD-S-G-------D----Q------ 358
S      299 ----------STRQ----------L---F-----AD-S-G-------D----Q------ 358
BCV    284 T------------RQ----------P---Y---STTN-V-.VY-IN--DAG-TSI----- 342

PV     358 YNVSCIAQQGAFFYNNVSSQWPVLGYGQCPTAANIEP.IAPVCLYDPLPVILLGVLLGIA 417
DVIM   359 ------------V------S--AY---H-------GY.M----I---------------- 417
JHM    359 L----------L------S--AY----------GY.M----I------V---------- 417
S      359 ------------V------S--AY---H-------GY.M----I-E------------- 417
BCV    343 -DSP-FS---V-R------V--LYS--R-----D-NTPDV-I-V-----L----I---V- 402

PV     417 VLIIVFLLFYFMTDSGVRLHEA                                      439
DVIM   418 -------M--------------                                      439
JHM    418 -------IL-------------                                      439
S      418 -------M---------                                          434
BCV    403 -I---V--L---V-N-T---D-                                     424
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FIG. 5. Sequence alignment of the HE protein of PV with the corresponding proteins of MHV-DVIM (accession number PID g2662175), MHV-JHM (PID g543553), MHV-S (PID g555242), and BCV-Mebus (PID g122851). Amino acid residues identical to the PV sequence are shown as dashes; gaps introduced to allow optimal alignment are shown as dots. The putative catalytic site is underlined, potential glycosylation sites are shown double underlined. Cysteine residues are marked with an asterisk.

umbelliferone, a fluorescent dye. First, we tested whether PV was able to cleave 4MUAc. Serial dilutions of PV or influenza C/JJ/50 virus were incubated in microtiter wells with 0.1 mM 4MUAc at 37°C, and cleavage of the substrate was monitored at 365 nm. Both viruses were able to cleave this substrate at

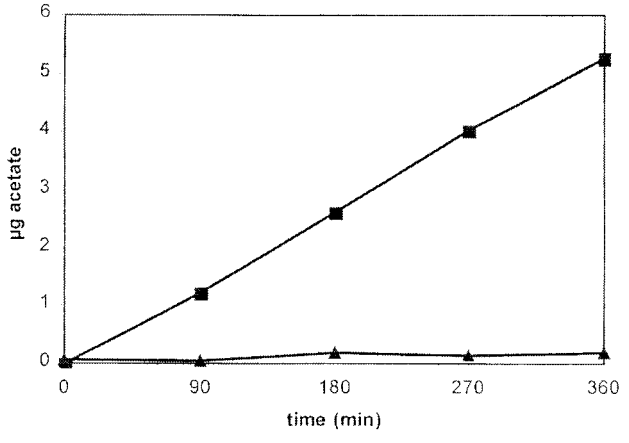

FIG. 6. Acetate release from BSM. A total of 2.5 mU of purified PV or influenza C/JJ/50 virus was incubated with BSM (12.5 mg/ml) at 37°C. At the times indicated, the incubation was stopped by heating at 95°C, and the free acetate content was determined with a commercial test kit (Boehringer Mannheim). Triangles indicate the incubation of BSM with PV; squares indicate the incubation with influenza C virus.

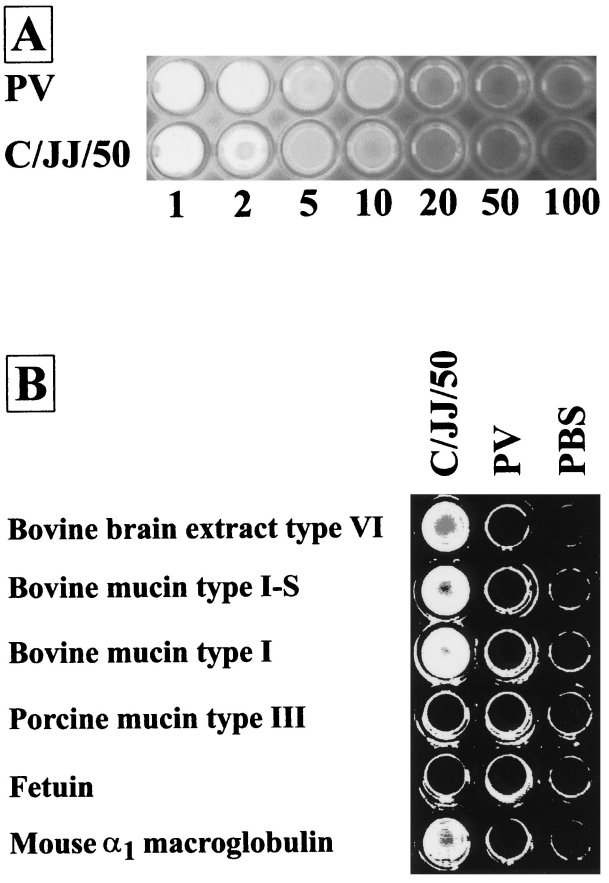

FIG. 7. (A) Cleavage of 4MUAc. PV and influenza C virus (C/JJ/50) were diluted with PBS and incubated with 0.1 mM 4MUAc for 30 min. Cleavage of substrate was monitored at a 365-nm excitation wavelength. In the first wells, 1.7 mU of PV or 0.9 mU of influenza C virus esterase was present. Reciprocals of the virus dilutions are indicated. (B) Solid-phase binding assays in microtiter plates. Microtiter wells were coated with glycoproteins (125 µg/well) or bovine brain extract (12.5 µg/well) and blocked with 3% BSA. Then 1.7 mU of PV or 0.5 mU of influenza C/JJ/50 virus was added to each well. For a control, PBS was used. After incubation for 2 h at 4°C, wells were washed three times with ice-cold PBS. Bound virus was detected by determination of the acetylesterase activity with 0.1 mM 4MUAc. The glycoconjugates used for coating are indicated on the left.

comparable rates (Fig. 7A). We then coated microtiter wells with glycoconjugates and tested the binding of viruses. Of several glycoproteins tested in this assay, influenza C/JJ/50 virus exhibited strong binding activity towards bovine mucin, as expected from the esterase assays described above. In addition, murine α_1 macroglobulin exhibited binding activity towards C/JJ/50. No binding of influenza C virus was observed in wells coated with porcine mucin and, as expected (44), with calf fetuin. In contrast, PV did not bind to any of these glycoproteins under the conditions tested. It should be mentioned that compared to C/JJ/50 virus, a 3.4-fold-higher concentration of PV, as calculated from esterase activities, was applied to coated microtiter wells. When we used even higher concentrations of PV, some binding activity with porcine mucin type III was detectable (data not shown). Since porcine mucin is believed to be devoid of *O*-acetylated sialic acids (18), we are currently investigating whether this observation is due to nonspecific interactions. In any case, we were unable to detect bound *O*-acetyl groups in porcine mucin after saponification. In addition to 9-*O*-acetylated glycoproteins, other glycoconjugates, such as gangliosides, can serve as influenza C virus

receptors. Bovine brain gangliosides have been used to restore susceptibility to infection by influenza C virus of sialidase-treated MDCK cells (11). For this reason, we also tested the binding of viruses to bovine brain extract containing a mixture of phospholipids and glycolipids, including gangliosides. Again, the binding of influenza C/JJ/50 virus was observed but no reaction of PV with bovine brain extract was detectable (Fig. 7B).

DISCUSSION

In this report, we describe cloning of an HE gene from a coronavirus, which was isolated during an investigation on a zoonosis affecting seabirds (19) and later referred to as PV (13). Due to the passage history in mouse brain it could not be strictly ruled out that it may be an MHV variant (19). Final proof clearly will require identification of the virus or viral genes in infected birds. Sequence data obtained in this study may allow design of specific primers to clarify the exact origin of PV. Regardless of whether PV is a coronavirus isolated from infected seabirds or an MHV variant, we wanted to determine whether an HE protein is expressed. In addition, we wanted to compare the enzymatic properties of the PV HE protein with those of other viral esterases. Data indicate that we have detected and characterized a coronavirus HE with a substrate and binding specificity different from those of other viral HE proteins.

PV expressed an mRNA 2-1 encoding the HE protein. From a mixed population of viruses with or without an active esterase, we have plaque purified an esterase-expressing isolate termed PV14. A mixture of different viruses may have already existed in the animals from which PV was originally isolated. Infection of mouse brain with MHV can rapidly result in the formation of viruses defective in HE expression (40). It is tempting to speculate that isolation procedures, including the passage of coronaviruses in suckling mouse brain, or tissue culture adaptation is at least one reason for the existence of viruses without a functional esterase. In contrast to influenza C virus, HE expression is not required for replication of PV or MHV in tissue culture. On the other hand, the presence of HE has consequences on the tissue tropism of MHV. Passive immunization of mice with HE-specific antibodies alters the neurotropism of MHV-JHM (39). Using a defective interfering (DI) vector, it has recently been shown that even transient expression of the HE protein in chimeric virus particles has pronounced effects on the outcome of central nervous system infection (43).

Sequence analysis of the cloned gene revealed a relationship to the HE proteins of MHV strains. Approximately 85 or 60% of the amino acid sequence is identical between PV and either MHV or BCV HE proteins, respectively.

It has been shown that the esterases of influenza C virus and bovine coronavirus remove 9-*O*-acetyl groups from sialic acids (12, 34). In addition, several synthetic compounds, e.g., *p*NPA, 4MUAc, or αNA, are cleaved by these esterases (7, 33, 44). These low-molecular-weight substrates were also cleaved by PV esterase. In contrast, acetyl esters on BSM, containing high concentrations of Neu5,9Ac_2, were not cleaved at detectable levels by this enzyme. Due to the detection limits of our assay system, we cannot strictly exclude that 9-*O*-acetylated sialic acids may be cleaved at very low rates. The kinetic parameters of cleavage of this substrate are at least different from those of influenza C virus and BCV, while several nonspecific substrates such as *p*NPA or 4MUAc are cleaved by the PV enzyme at rates comparable to the influenza C virus esterase. Furthermore, the treatment of erythrocytes with PV had no influence on the HA titers of influenza C virus, strongly suggesting that receptors for influenza C virus and BCV are not recognized by the PV esterase.

In solid-phase binding assays, influenza C virus was found to bind to BSM, mouse α_1 macroglobulin, and glycoconjugates from bovine brain extract. In contrast, PV exhibited no affinity for BSM. In addition to Neu5,9Ac_2, other substituted forms of sialic acids are present. Modifications include *O*-acetylation at position 7 or 8. In addition, di- or tri-*O*-acetylated forms of sialic acids are present in BSM (22). Considering the high sensitivity of the solid-phase binding assay, with a detection limit of approximately 65 fmol of 9-*O*-acetylated sialic acid (44), the possibility arises that sialic acids with *O*-acetyl groups at position 7, 8, or 9 are not involved in the binding of PV to target cells. Alternatively, structural requirements, e.g., the type of linkage of sialic acids to other sugars, may be different for the binding of PV.

Taken together, we have demonstrated that the HE protein of PV exhibited an acetylesterase activity towards synthetic *O*-acetyl esters that was similar to that of other viral esterases. Natural substrates for influenza C virus and BCV were not cleaved by this enzyme. Furthermore, no binding activity towards a series of sialic-acid-containing glycoconjugates was detectable. Although we cannot strictly exclude the possibility that *O*-acetylated sialic acids may serve as substrates, our data suggest that other unidentified natural substrates exist. Clearly, further studies involving pure *O*-acetylated sialic acids are required to define the specificity of this enzyme. Expression of the cloned gene with a recombinant vaccinia virus may further clarify the binding specificities of the PV HE protein. Because of the high degree of similarity of the PV HE protein with those of MHV strains, substrate specificities of the latter HEs may be different from those of BCV and influenza C viruses as well. In fact, publications showing the acetylesterase activity of MHV do not necessarily exclude this possibility. In several instances, enzymatic activity was determined with *p*NPA but not with BSM or other glycoconjugates (6, 21, 41). In case MHV esterases exhibit substrate specificities similar to those of the PV HE, new models on the role of this enzyme during infection would be required. It is interesting to speculate that unidentified *O*-acetylated cellular proteins may be involved in the neurotropism of HE-containing MHV-like viruses. Alternatively, the presence of an acetylesterase may modify acetylated proteins or peptides involved in cell signalling or the regulation of the immune system. In particular, it has been suggested that HE gene expression in MHV may modify a function of nonspecific innate immunity (43). Removal of negatively charged *O*-acetyl groups from cellular or viral surfaces may very well modify binding sites for complement factors, e.g., factor C3 or complement regulatory H protein. In the future, it will be interesting to test whether HE proteins of MHV-S or MHV-JHM indeed have substrate specificities similar to that of the PV esterase. It should be noted that results do not necessarily suggest that PV is a separate coronavirus species. In addition, it would be interesting to test the possible influences on complement activation by viral HE proteins.

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