Glycoconjugate Journal 20, 551–561, 2004

Recombinant viral sialate-*O*-acetylesterases

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Viral *O*-acetylesterases were first identified in several viruses, including influenza C viruses and coronaviruses. These enzymes are capable of removing cellular receptors from the surface of target cells. Hence they are also known as "receptor destroying" enzymes. We have cloned and expressed several recombinant viral O-acetylesterases. These enzymes were secreted from Sf9 insect cells as chimeric proteins fused to eGFP. A purification scheme to isolate the recombinant *O*-acetylesterase of influenza C virus was developed. The recombinant enzymes derived from influenza C viruses specifically hydrolyze 9-*O*-acetylated sialic acids, while that of sialodacryoadenitis virus, a rat coronavirus related to mouse hepatitis virus, is specific for 4-*O*-acetylated sialic acid. The recombinant esterases were shown to specifically de-*O*-acetylate sialic acids on glycoconjugates. We have also expressed esterase knockout proteins of the influenza C virus hemagglutinin-esterase. The recombinant viral proteins can be used to unambiguously identify *O*-acetylated acids in a variety of assays. *Published in 2004.*

***Keywords:* *O*-acetylated sialic acids, virus receptor, influenza C virus, rat coronavirus, receptor-destroying enzyme**

Introduction

Sialic acids represent a family of acidic sugars that are found at terminal positions within the carbohydrate moieties of glycoproteins and glycolipids. One type of modification of sialic acids is *O*-acetylation which occurs at the glycerol side chain carbons 7, 8, and 9. In addition, *O*-acetylation of the ring carbon 4 is found regularly in glycoconjugates of horses and guinea pigs. The biochemical reaction pathways of the biosynthesis of *O*-acetylated sialic acids are not yet fully understood. *O*-acetylation of glycoconjugates may involve the formation of *O*-acetylated CMP-Neu5Ac and subsequent conjugation by sialyltransferases [1], or alternatively occur on the glycoconjugate itself [2].

O-acetylation is an important modification, which coincides with changes of cellular growth and differentiation, lymphocyte maturation, and diverse other biological phenomena. As an example, the 9-*O*-acetylated ganglioside GD3 is a differentiation marker also termed CD60b [3]. For more detailed overviews, the reader is referred to articles and reviews on sialic acid biochemistry and biology [4–13].

Glycoconjugates also represent receptors for the attachment of viruses. Several enveloped viruses utilize *O*-acetylated sialic acids for binding to target cells, the first step of viral infections. Viruses belonging to the families of the *Orthomyxoviridae* and *Coronaviridae* do not only bind to such sialic acids. In addition, they possess acetylesterase activities capable of de-*O*-acetylating cellular receptors. Due to this activity, the viral esterases have also been termed "receptor-destroying enzymes". Since several of these viral proteins also mediate agglutination of erythrocytes, they are now known as hemagglutinin-esterase (HE) proteins. Among the first viruses, which were shown to initiate infections via *O*-acetylated sialic acids were the influenza C viruses [14–16] as well as human and bovine coronaviruses [17]. All viruses mentioned so far specifically use 9-*O*-acetylated sialic acids (Neu5,9Ac_2) as receptor determinant. Another coronavirus, hemagglutinating encephalomyelitis virus of swine, exhibits a similar preference towards Neu5,9Ac_2 [18]. Other coronaviruses, like mouse hepatitis virus (MHV) strains S and JHM, and puffinosis coronavirus were recently found to hydrolyze *O*-acetylated sialic acids. In contrast to the above mentioned viruses, they specifically hydrolyze 4-*O*-acetylated sialic acids (Neu4,5Ac_2) [19]. In this report, the enzymatic properties of the HE protein of sialodacryoadenitis virus (SDAV), a rat coronavirus, will be described. Infectious salmon anemia virus (ISAV), a putative new member of the *Orthomyxoviridae*, was also shown to exhibit an

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Table 1. Viruses with O-acetylesterases

Family	*Genus*	*Species*	*Substrate*
Orthomyxoviridae	Influenza C virus	Influenza C virus	Neu5,9Ac_2
	Isavirus	Infectious salmon anemia virus	Neu4,5Ac_2
Coronaviridae	Coronavirus	Human coronavirus OC43	Neu5,9Ac_2
		Bovine coronavirus	Neu5,9Ac_2
		Hemagglutinating encephalomyelitis virus	Neu5,9Ac_2
		Mouse hepatitis virus	Neu4,5Ac_2
		Puffinosis coronavirus	Neu4,5Ac_2
		Sialodacryoadenitis virus	Neu4,5Ac_2
	Torovirus	Bovine torovirus	n.d.
		Porcine torovirus	n.d.

n.d.: not determined.

acetylesterase activity [20,21]. In collaboration with Dr. Falk (Veterinary Institute, Oslo) a sialate-4-*O*-acetylesterase activity was identified recently [22,23]. Most recently we found, in collaboration with Dr. de Groot and Dr. Kamerling (University of Utrecht), that torovirus HE proteins, which also exhibit an acetylesterase activity [24], hydrolyze *O*-acetylated sialic acids (Smits *et al.*, in preparation). The viruses and their *O*-acetylesterases are summarized in Table 1.

In order to provide new tools for the analysis of *O*-acetylated sialic acids, we have expressed recombinant viral esterases derived from the cloned HE genes of influenza C/Cal/78 virus and SDAV.

Materials and methods

Cells and viruses

HeLa cells were grown in DMEM supplemented with 10% fetal calf serum (FCS), TK$^-$ 143B cells (ATCC # CRL 8303) were grown in MEM supplemented with 10% FCS, and Sf9 cells were grown at 27°C in IPL-41 medium containing 5% FCS. Chicken erythrocytes were kindly supplied by Dr. Norbert Nowotny (Veterinary University, Vienna). Influenza C/Cal/78 virus and influenza C/JJ/50 virus were grown in the amniotic cavity of embryonated chicken eggs as described previously [16]. SDAV was obtained from ATCC.

Construction of plasmids for the expression of the influenza C/Cal/78 virus HE gene

The cloned influenza C/Cal/78 virus HE gene was excised from plasmid pCHE4 [16] with restriction endonuclease *Sal*I and inserted into plasmid pATAgpt3Stop, resulting in pATAHE (Figure 1a). Details on the recombination vector pATAgpt3Stop were given earlier [25]. Recombinant Vaccinia virus (VV) was obtained by transfection of TK$^-$ 143B cells with recombination vectors and subsequent infection with wild type VV (VV-wt). Recombinant VV-HE was isolated by selection for a thymidine kinase (−) phenotype. Truncated forms lacking the cod-

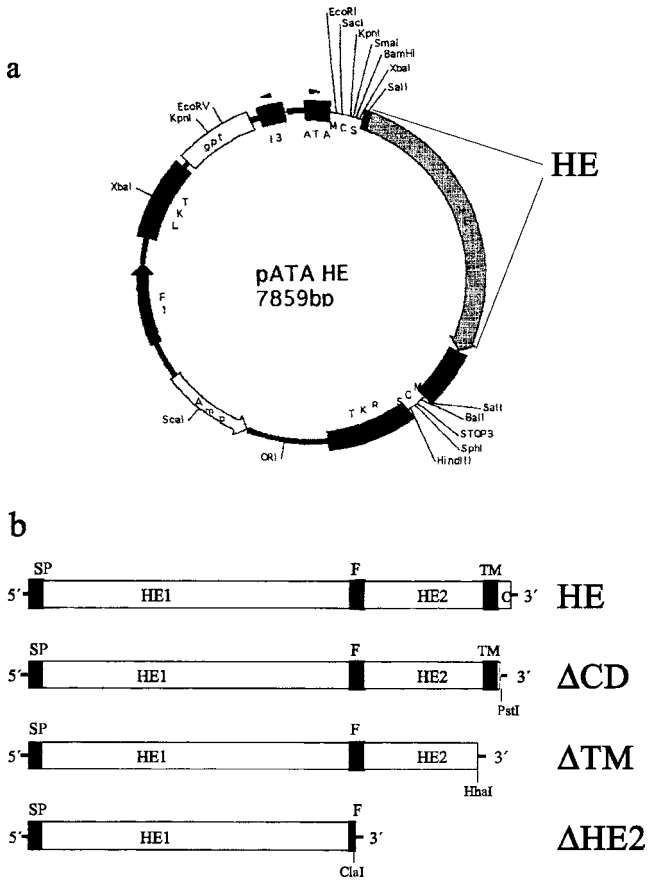

Figure 1. Schematic representation of vector constructs for expression by recombinant VV. (a) Structure of the recombination plasmid pATA HE. (b) schematic drawing of the influenza C virus HE gene and its encoded protein (HE), and of the truncated forms lacking the cytoplasmic domain (ΔCD), the transmembrane anchor (ΔTM) or the entire HE2 domain (ΔHE2). The positions of the signal peptide (SP), the HE domains (HE1, HE2), the fusion peptide (F), transmembrane anchor (TM), and the cytoplasmic domain (C) are indicated. Restriction sites used for truncation of the HE gene are indicated.

ing regions for the cytoplasmic domain, the transmembrane anchor, or the entire HE2 domain were constructed by restriction digests utilizing the *Pst*I site, the *Hha*I site, or the *Cla*I site of pATAHE, respectively. The restriction sites were blunt-ended and religated into the *Sma*I site of pATAgpt3Stop, resulting in pATAHEΔCD, pATAHEΔTM, and pATAHEΔHE2 (Figure 1b).

Construction of pBacHE1GFP

Plasmid pATAHE was cleaved with *Cla*I to obtain a fragment representing the HE1 subunit. This fragment was first blunt-ended by a Klenow fill-in reaction and subsequently digested with *Sac*I. The resulting HE1 fragment was ligated into vector pEGFP-N3 (CLONTECH Laboratories, Inc.) which had been linearized with *Bam*HI, followed by a Klenow fill-in reaction, and re-cleaved with *Sac*I. The ligation product encodes an in-frame fusion of HE1 and the green flourescent protein (GFP). The integrity of the fusion site was confirmed by DNA sequencing on both strands.

The new HE1-GFP fusion gene was excised from the plasmid by a *Sac*I/*Not*I restriction digest and ligated into the *Sac*I/*Not*I-cleaved baculovirus transfer vector pBacPAK8 (CLONTECH Laboratories, Inc.), resulting in the construct pBacHE1-GFP (Figure 2a).

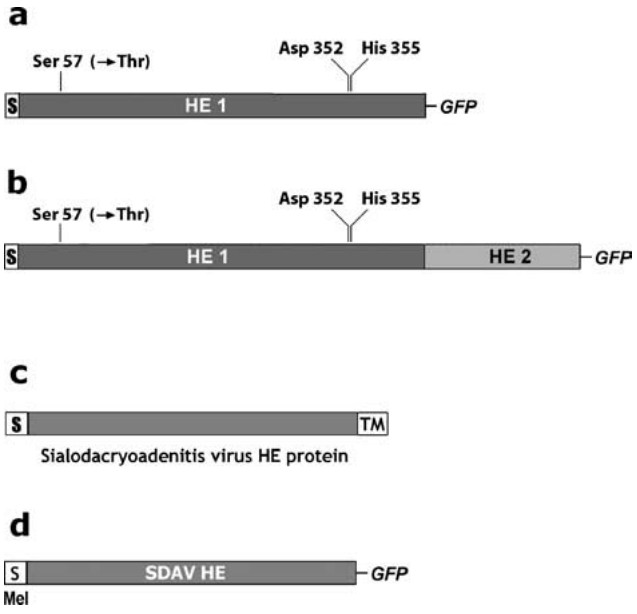

Figure 2. Schematic representation of recombinant proteins expressed by baculoviruses. The structures of the recombinant chimeric proteins HE1-GFP (a) and HE1/2-GFP (b) derived from influenza C/Cal/78 virus are shown. The position of the active site serine residue, which was replaced by a threonine, and the positions of the other active site residues (Asp, His) are indicated. (c) Structure of the SDAV HE protein, and (d) structure of the recombinant SDAV HE-GFP protein.

Construction of pBacHE1/2-GFP

A PCR fragment representing the HE2 subunit was amplified from plasmid pATAHE. This fragment starts at the unique *Xcm*I site in the HE1 subunit and extends to a *Hha*I site located downstream in the HE2 subunit. The sequences of the primers used were: the upstream primer: 5-CAATGCAATGTGACATGCTCCAGCTG-3 and the downstream primer: 5-GCCCTTGCTCACCATGGTGGCGATGGGCGCAGTTTCGTTGAACTTG-3. The *Hha*I site was changed into an *Xcm*I site (underlined) via the downstream primer. The resulting PCR fragment was digested with *Xcm*I and inserted into the *Xcm*I site of expression vector pBacHE1-GFP. By this means, the original *Xcm*I fragment in pBacHE1-GFP was replaced by the longer PCR fragment yielding vector pBacHE1/2-GFP. This new construct encodes both the HE1 subunit and almost the entire HE2 subunit lacking the last sixteen amino acids of the HE2 ectodomain, the transmembrane region, and the cytoplasmic tail, all of which are replaced by the in-frame fusion with GFP (Figure 2b).

Construction of pBacHE1-GFP (S → T) and pBacHE1/2-GFP (S → T)

Plasmid pATAHE ST encodes a mutant influenza C virus HE protein in which the active site serine at position 57 had been changed by site-directed mutagenesis to a threonine codon, thereby abolishing the esterase activity of the encoded protein. An 850bp *Eco*RI fragment containing this mutation was used to replace the respective wild type *Eco*RI fragment in both plasmids pBacHE1-GFP and pBacHE1/2-GFP, resulting in the new constructs pBacHE1-GFP (S → T) and pBacHE1/2-GFP (S → T), respectively (Figure 2a and b).

Construction of pBPAKMel8

To facilitate release of recombinant proteins from insect cells into the culture medium, a short DNA fragment encoding the melittin signal sequence [26] was inserted into pBacPAK8. The strategy employed was to create an artificial sequence by means of two complementary 74mer oligonucleotides: 5-GATCTGTCGACATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCG -3 and: 5-GATCCGCATAGATGTAAGAAATGTATACGACCATAAAAACAAGGGCAACGTTGACTAAGAATTTCATGTCGACA -3.

The purified oligonucleotides were mixed in equimolar amounts and phosphorylated with T4 polynucleotide kinase according to the manufacturer's recommendations. Then, the mixture was incubated at 96°C for 5 min in a waterbath and allowed to cool down slowly to room temperature to facilitate proper annealing of the oligonucleotides. The double-stranded product featured 5-protruding single-stranded GATC sequences on both ends, which were used for direct cloning into the *Bam*HI site of the pBacPAK8 polylinker. In the resulting plasmid, designated pBPAKMel8, the entire multiple

cloning site is located downstream of the melittin signal sequence. This construct was used for expression of the SDAV HE gene.

Cloning and expression of the SDAV HE gene

SDAV was obtained from ATCC and used directly for RNA isolation. RNA was extracted with RNAZol^{TM} (AMS Biotechnology Europe, UK), followed by reverse transcription utilizing an oligonucleotide that binds downstream of the SDAV HE coding region (primer SDAV HE2: 5-GTTTCTAGATCTAAAATCACCAATATACC -3). This cDNA was amplified by PCR with primer SDAV HE1: 5- GTATCTAGAATGAAGGGTTGTATGTGTTG -3, and primer SDAV HE2. Both primers contained *Xba*I restriction sites which were used for cloning the PCR fragment into plasmid pBluescript KS (Stratagene). A second PCR was performed on this recombinant vector with primers SDAV HE-SP fw: 5-GGAGATCTGTTCAATGAACCTATTAAC-3 and SDAV HE-TM rev: 5-AAGGTACCCGGTAGGGGGTCATAAAG -3. The PCR fragment was trimmed with *Bgl*II and *Kpn*I and ligated into the *Bgl*II/*Kpn*I-digested vector pEGFP-N3 (CLONTECH Laboratories, Inc.), resulting in a construct that encodes a truncated SDAV HE protein lacking its N-terminal signal peptide and, at the C-terminus, the transmembrane region and the cytoplasmic tail with the missing C-terminal sequences being replaced by an in-frame fusion with GFP. Finally, this new SDAV HE-GFP coding region was excised from the plasmid with *Bgl*II and *Not*I and ligated into the *Bgl*II/*Not*I window of pBPAKMel8, yielding construct pBPAKMelSDAVHE-GFP in which the SDAV HE-GFP fusion was placed downstream of the melittin signal sequence (Figure 2c and d).

Preparation of recombinant baculoviruses

Sf9 cells were seeded into a 25 cm^2 flask at a density of 1×10^5 cells/cm^2 in serum-free growth medium and cotransfected with 100 ng of predigested baculovirus DNA (BaculoGold Baculovirus DNA; PharMingen) and 500 ng of recombinant baculovirus transfer vector using LipofectAce transfection reagent (Life Technologies) according to the manufacturer's recommendations. Four hours post transfection, the volume of the culture medium was increased five-fold by addition of IPL-41 including 10% FCS, and the cells were incubated at 27°C for five days. Subsequently, recombinant virus was harvested from the culture supernatant and isolated by three consecutive rounds of plaque-purification. After each round, recombinant virus could be easily detected by infecting Sf9 cultures with lysates from individual plaques and monitoring GFP expression within the cells by flourescence microscopy. Viral working stocks with titers ranging from 5×10^7 to 1×10^8 per ml were prepared from supernatants of infected cell cultures and stored at 4°C.

Esterase assays

The acetylesterase activity of the recombinant proteins was determined with p-nitrophenyl acetate (*p*NPA), as described previously [16,27]. One unit of viral esterase activity was defined as the amount of enzymatic activity resulting in the hydrolysis of 1 μmol pNPA per minute. For assays involving glycosidically bound sialic acids, recombinant viral esterases or influenza C/JJ/50 virus were incubated at 37°C either with BSM (Sigma type I-S) or horse serum glycoproteins. Reactions were stopped by heating for 10 min at 96°C and analyzed either with a commercial acetate test kit as described previously [16] or by fluorimetric HPLC. Reverse-phase HPLC analysis of sialic acids was performed essentially as described previously [28]. Esterase-staining in non-denaturing SDS-PAGE with α-naphtyl acetate was performed essentially as described previously [19].

Radioimmunoprecipitation assays

Recombinant viral proteins were labeled with ^{35}S-Translabel^{TM}(American Radiolabeled Chemicals, Inc; St. Louis) and immunoprecipitated by rabbit anti-C/JJ/virus antiserum as described previously [16,29].

Results

Expression of the influenza C/Cal/78 virus hemagglutinin-esterase by recombinant vaccinia virus

Viral HE proteins are located at the viral surface, anchored via their carboxy-terminal ends to the viral membrane. While the HE protein of influenza C virus is a homotrimer [30], the corresponding proteins of group 2 coronaviruses are dimers. In order to express a soluble recombinant influenza C virus HE, we first investigated whether the deletion of the membrane anchor results in the expression of a secreted esterase. For these experiments, we used recombinant vaccinia viruses (VV). The influenza C/Cal/78 HE gene was inserted into the thymidine kinase gene by targeted recombination as described in Materials and Methods. It was placed under the control of the VV 11 K late promoter. The recombinant vaccinia virus (VV-HE) was then tested for its potential to express the influenza HE protein. By labeling of HeLa cells infected with recombinant VV-HE and radioimmunoprecipitation two types of HE were detected. Besides the uncleaved HE_0 with approximately 88 kDa a second form of 75 kDa was precipitated by the influenza C virus specific antiserum (Figure 3). Cleaved HE1 and HE2 were not detected, indicating that HeLa cells do not cleave the precursor. During a chase for 3 h with unlabeled medium the 75 kDa protein gradually disappeared. We therefore assume that this protein is the unglycosylated precursor of the HE_0 protein.

To demonstrate correct folding and cell surface expression, we analyzed the cellular distribution of acetylesterase activity. HeLa cells were infected with VV-HE virus and fractionated by standard methods. Subcellular fractions were then tested for acetylesterase activity with the *p*NPA assay (Table 2). In

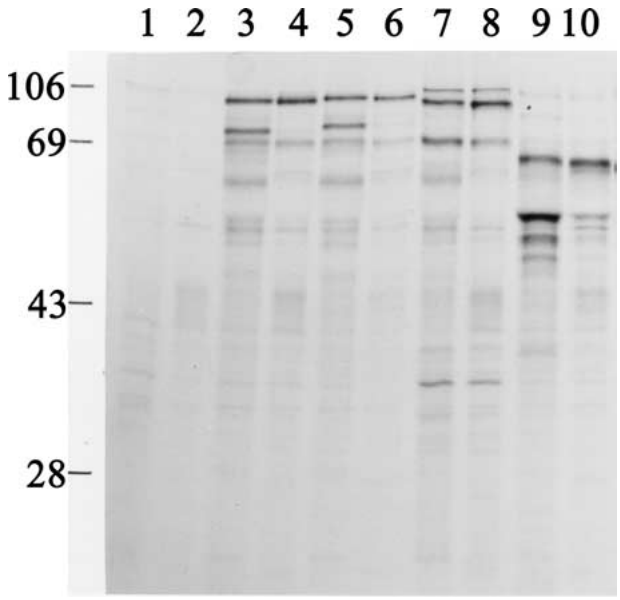

Figure 3. Pulse-chase labeling of truncated HE proteins. HeLa cells were infected with recombinant vaccinia viruses indicated (m.o.i. = 5) and incubated for 16 h. Then labeling media were added for 60 min. Cells were lysed immediately (lanes 1, 3, 5, 7, and 9) or further incubated with chase media for 3 h (lanes 2, 4, 6, 8, and 10). Recombinant HE protein was precipitated with rabbit anti-influenza C/JJ/50 antiserum and analyzed by 10% SDS-PAGE und autoradiography. Positions and molecular weight (kDa) of marker proteins are indicated at the left. Cells were infected with VV-wt (lanes 1, 2), VV-HE (lanes 3, 4), VV-HEΔCD (lanes 5, 6), VV-HEΔTM (lanes 7, 8), or VV-HEΔHE2 (lanes 9, 10).

VV-HE infected cells, esterase activity in the plasma membrane fraction was five- to six-fold higher than in the soluble cytoplasmic fraction, indicating insertion of the recombinant HE protein into the membrane. Since *p*NPA is also hydrolyzed by cellular esterases, some background activity was detected in VV-wt infected cells. The influenza C virus esterase is characterized as a serine esterase highly susceptible to inhibition by di-isopropyl fluorophosphate (DFP) and low reactivity with phenylmethylsulfonyl fluorophosphate (PMSF) [31,32]. To confirm that enzymatic activity found in the plasma membrane fraction of VV-HE-infected cells was derived from expression of the influenza C virus esterase, we determined activities in the presence of these inhibitors. DFP treatment of membranes reduced esterase activity to background level, while PMSF treatment resulted in no significant reduction of activity in membranes of VV-HE-infected cells (Table 2). In addition, we also determined the correct substrate specificity of the recombinant esterase using BSM as substrate. HeLa cells (2×10^6 cells/dish) infected with recombinant VV were washed with PBS and incubated in the presence of 500 μl BSM (25 mg/ml) for 2 h at 37°C. In the supernatant of VV-HE-infected cells approximately 12.8 μg free acetate was detected, while concentrations in control assays with VV-wt were below detection limits (Table 3). Thus, the recombinant influenza C/Cal/78 HE protein is expressed at the cell surface at high levels. We therefore concluded that expression of the influenza C/Cal/78 virus HE by recombinant VV yields a biologically active glycoprotein, which is in agreement with earlier expression studies [16].

Table 2. Intracellular distribution of recombinant influenza C/Cal/78 virus esterase expressed in HeLa cells

Virus	*Plasma membrane*[a]	*Cytoplasm*[a]
VV-HE	30.6 (±4.6)	5.6 (±0.1)
VV-HE + 1 mM DFP	4.1 (±0.5)	n.d.[b]
VV-HE + 1 mM PMSF	31.4 (±0.4)	n.d.[b]
VV wt	7.2 (±0.4)	6.8 (±0.1)
VV wt + 1 mM DFP	3.3 (±0)	n.d.[b]
VV wt + 1 mM PMSF	5.8 (±0.1)	n.d.[b]

[a] acetylesterase activity (mU/10^6 cells) of subcellular fractions was determined with 1mM *p*NPA.
[b] n.d.: not determined.

Table 3. Release of acetate from bovine submaxillary mucin by truncated influenza C/Cal/78 HE proteins[a]

Virus	*Cell lysate*	*Plasma membrane*	*Supernatant*
VV-HEΔCD	++	++	−
VV-HEΔTM	++	−	−
VV-HEΔHE2	++	−	−
VV-HE	++	++	−
VV wt	−	−	−

[a] Total cell lysates, plasma membrane fractions, and culture supernatants were incubated with BSM (25mg/ml) for two hours at 37°C. Release of acetate was determined with a test kit. ++, >20 μg acetate/2 × 10^6 cells; −, below detection limit.

Compared to the influenza C virus HE protein, those of corona- and toroviruses are smaller in size. The latter ones resemble the HE-1 subunit and the hydrophobic N-terminus of the HE-2 subunit of the influenza C virus esterase. This prompted us to test whether expression of truncated forms of the influenza C virus HE protein results in recombinant proteins with acetylesterase activity. Constructs were prepared to test the requirement of different regions of the HE protein (Figure 1b). We deleted the cytoplasmic domain (HE-ΔCD), the transmembrane anchor (HE-ΔTM), and the entire HE2 subunit except for the five amino-acid residues of the putative fusion peptide (HE-ΔHE2). Recombinant VVs were prepared, and protein expression was determined in a pulse-chase experiment. As shown in Figure 3, the truncated proteins were precipitated from lysates of infected HeLa cells by polyclonal antiserum. Similar to the authentic HE, additional faster migrating bands were detected in the modified proteins. During a 3 h chase these additional bands gradually disappeared. In presence of monensin which blocks the intracellular transport and glycosylation [33], only

the faster migrating forms were detected (data not shown), indicating that these bands represent unglycosylated precursors.

None of the truncated HE proteins could be precipitated from tissue culture supernatants (data not shown). We therefore investigated acetylesterase activity in supernatant, in plasma membrane fraction, and in total cell lysates. As shown in Table 3, esterase activity was found in lysates of infected cells expressing HE-ΔCD, HE-ΔTM, and HE-ΔHE2. In cell culture supernatants no esterase activity was detected. In plasma membranes of cells infected with VV-HE-ΔCD, acetylesterase activity was detectable, indicating that this modified glycoprotein was transported to the plasma membrane. Other constructs exhibited no membrane-associated activity, indicating that anchorless and ΔHE2 were retained intracellularly.

Expression of chimeric HE-GFP proteins by recombinant baculoviruses

In order to facilitate the purification of the recombinant esterases, it was desirable to express the protein in a way to allow the purification from the cell culture supernatant. In the laboratory of Dr. Varki it had been shown that expression of a chimeric influenza C virus HE fused to an immunoglobulin resulted in the constitutive expression of a secreted protein from a stable cell line [5]. In order to obtain large amounts of the recombinant esterase, we decided to use a viral vector. Due to biosafety considerations, we decided to switch to a baculovirus expression system considered biosafety level 1 while the expression by VV requires biosafety level 2 facilities. In order to further facilitate monitoring of expression, we selected a strategy yielding a chimeric protein consisting of the viral esterase and the green fluorescent protein (Figure 2). Recombinant baculoviruses were designed, which directed the expression of two soluble chimeric esterases (HE1/2-GFP and HE1-GFP). It had been shown earlier that inhibition of of the esterase activity of recombinant HE-Fcγ by DFP resulted in a protein which specifically binds to Neu5,9Ac_2 [5]. To avoid handling of the highly toxic DFP, we were aiming to express a recombinant protein devoid of the esterase activity. To express such a protein, we changed the codon of the active site serine residue [30,32] to a threonine codon. By these means we constructed recombinant baculoviruses expressing HE1/2-GFP(S → T) and HE1-GFP(S → T) (Figure 2).

De-*O*-acetylation of BSM by the recombinant HE1-GFP - and HE1/2-GFP esterases

For monitoring the esterase activities of the recombinant HE proteins, cell culture supernatants of baculovirus-infected cells containing either HE1-GFP or HE1/2-GFP were incubated with *p*NPA. Esterase activity was detected in the supernatants of cells expressing HE1-GFP and HE1/2-GFP. Typical yields were in the range of 10–30 Units /100 ml (data not shown). No specific activity was found in the supernatants of cells expressing the esterase knockout mutants HE1/2-GFP(S → T) and HE1-GFP(S → T). All recombinant proteins were clearly detectable in a Western blot (data not shown). To test whether the recombinant proteins exhibited the correct substrate specificity, we incubated culture supernatants with BSM. Free acetate was determined in each sample with a commercially available kit. Figure 4 shows a time course of acetate release from BSM by HE1-GFP, HE1/2-GFP, and influenza C/JJ/50 virus. The total amount of acetate bound to BSM was determined by saponification with 200 mM NaOH for 30 min at room temperature. Both recombinant esterases were found to de-*O*-acetylate BSM over time, indicating they exhibited the correct substrate specificity.

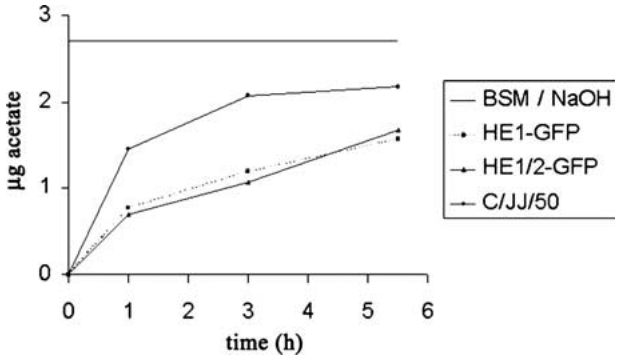

Figure 4. Acetate release from BSM. 90 μg of BSM were incubated with cell culture supernatants containing HE1-GFP (closed rectangles), HE1/2-GFP (closed triangles), or influenza C virus (closed circles). The solid line represents the amount of acetate released from BSM by saponification.

Receptor-destroying activity of the recombinant HE1-GFP and HE1/2-GFP fusion proteins

In order to determine the ability of the recombinant fusion proteins to recognize cell surface receptors as substrates, chicken erythrocytes were incubated with the different culture supernatants, or with influenza C/JJ/50 virus (Figure 5). The pre-incubated erythrocytes were washed twice with PBS and subjected to a hemagglutinin (HA) titer assay using an influenza C/JJ/50 virus preparation with an HA titer of 6. Agglutination was observed on erythrocytes that had been pre-incubated with PBS, wild-type baculovirus supernatant, or the two supernatants containing the inactive S → T mutants. Pre-incubation with HE1-GFP and HE1/2-GFP abolished the agglutinability of the erythrocytes, indicating the de-*O*-acetylation of cell surface receptors by the esterase activity of the recombinant proteins.

Expression of recombinant SDAV sialate-4-*O*-acetylesterase

Next, we wanted to express a recombinant esterase specific for Neu4,5Ac_2. Earlier, we had found that coronaviruses related to MHV expressed an HE which specifically hydrolyzed glycosidically bound Neu4,5Ac_2, but not Neu5,9Ac_2 [19,27,28]. We had hypothesized that other MHV-related coronavirus esterases are also specific for Neu4,5Ac_2 [19]. To test this hypothesis, we cloned the HE gene of rat coronavirus SDAV. The amino acid

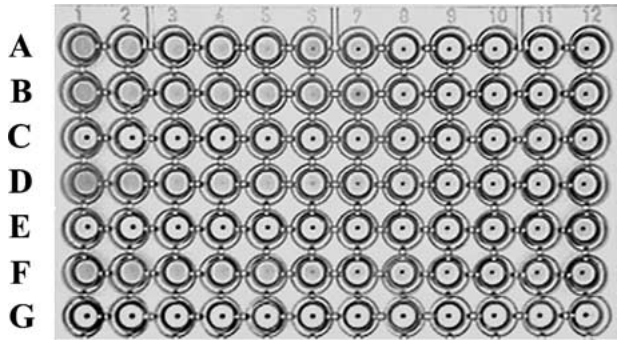

Figure 5. Receptor-destroying activity of recombinant influenza C virus HE-GFP proteins. Chicken erythrocytes were incubated overnight at 37°C with PBS (A), culture supernatant of Sf9 cells infected with wild-type baculovirus (B), HE1-GFP (C), HE1-GFP(S → T) (D), HE1/2-GFP (E), HE1/2-GFP(S → T) (F), or influenza C virus (G). Then erythrocytes were washed and resuspended in PBS, and adjusted to a concentration of 0.5%. Influenza C virus with an HA titer of 6 was added to the first wells of each row and serially diluted. Then the preincubated erythrocytes were added, and allowed to agglutinate for 90 min at 4°C.

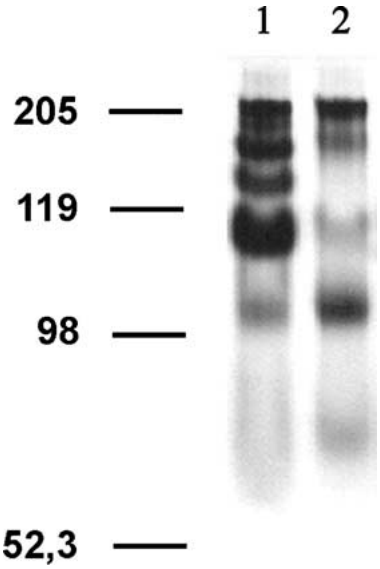

Figure 6. Detection of recombinant SDAV HE-GFP proteins by α-naphtyl acetate. Culture supernatant (1) and a cell lysate (2) of Sf9 cells infected with recombinant baculovirus expressing SDAV HE-GFP protein was analyzed on a 10% non-reducing SDS-PAGE and stained with a commercially available α-naphtylacetate kit. Positions and the apparent molecular weight (kDa) of prestained marker proteins are indicated at the left.

sequence of this esterase is closely related to those of different MHV strains [34]. When we expressed the chimeric SDAV HE-GFP protein in Sf9 cells, we found the recombinant enzyme was retained intracellularly and only partially secreted into the culture supernatant (data not shown). Since in several instances insect cells are unable to proteolytically cleave mammalian signal peptides [35], we replaced the SDAV signal peptide with that of honeybee prepromelittin [26]. Expression of this construct resulted in an efficiently secreted protein, which could easily be detected by its esterase activity in a nonreducing SDS-PAGE. Interestingly, we detected several bands of the recombinant protein, indicating oligomerization (Figure 6). The esterase-positive bands were observed mainly in the culture supernatant.

We then tested the substrate specificity of the SDAV HE-GFP protein and compared it with that of the influenza C virus HE1/2-GFP protein. First, we incubated BSM with the recombinant esterases. While the influenza C virus esterase efficiently hydrolyzed glycosidically bound Neu5,9Ac_2, Neu5,7(8),9Ac_3, and Neu5Gc9Ac (Figure 7b), these sialic acids were not hydrolyzed by the SDAV esterase (Figure 7c). The identity of all peaks indicated was confirmed by ESI-MS analysis of the DMB-derivatized sialic acids (data not shown). In contrast, the rat coronavirus esterase completely de-*O*-acetylated Neu4,5Ac_2 of horse serum glycoproteins (Figure 8c), which was not hydrolyzed by the HE1/2-GFP protein (Figure 8b).

Purification of the recombinant HE1/2 GFP fusion protein

Finally, we attempted to purify the recombinant esterases from the culture supernatant. The secreted HE1/2-GFP fusion protein was partially purified from 200 ml of the culture supernatant via the following protocol: baculovirus in the cell culture supernatant was removed by ultracentrifugation at 26000 rpm and 4°C for 90 min in a Beckman SW28 rotor. Then the supernatant was passed through a 0.2 μm membrane filter, dialyzed overnight at 4°C against 4 liters of buffer A (20 mM TRIS/HCl pH 7.4), and applied to a Q Sepharose FF column (Amersham-Pharmacia). Bound proteins were eluted with a gradient ranging from 0–1 M NaCl in 20 mM Tris/HCl (pH 7.4). Esterase-positive fractions were pooled and dialyzed overnight at 4°C against 20 volumes of 1 mM NaCl. The sample was then loaded onto a hydroxyapatite column (BioRad) and eluted with a gradient ranging from 1 mM NaCl to 300mM potassium phosphate pH 6.8. Fractions were analyzed for esterase activity by Western blotting. Figure 9 shows two typical elution profiles and a Western blot of the purification of HE1/2-GFP.

Discussion

The original cDNA clone encoding the influenza C virus esterase [16] has been used by several laboratories to study the function of Neu5,9Ac_2 during development and maturation. The laboratory of Dr. Varki has demonstrated that transgenic mice expressing the viral esterase exhibit developmental abnormalities [4]. From the original clone a recombinant soluble chimeric influenza C virus esterase was expressed in this laboratory and used to detect widespread but selective expression of 9-*O*-acetylated sialic acids in specific cell types of rat tissues [5]. In addition, 9-*O*-acetylation was shown to affect tissue homing

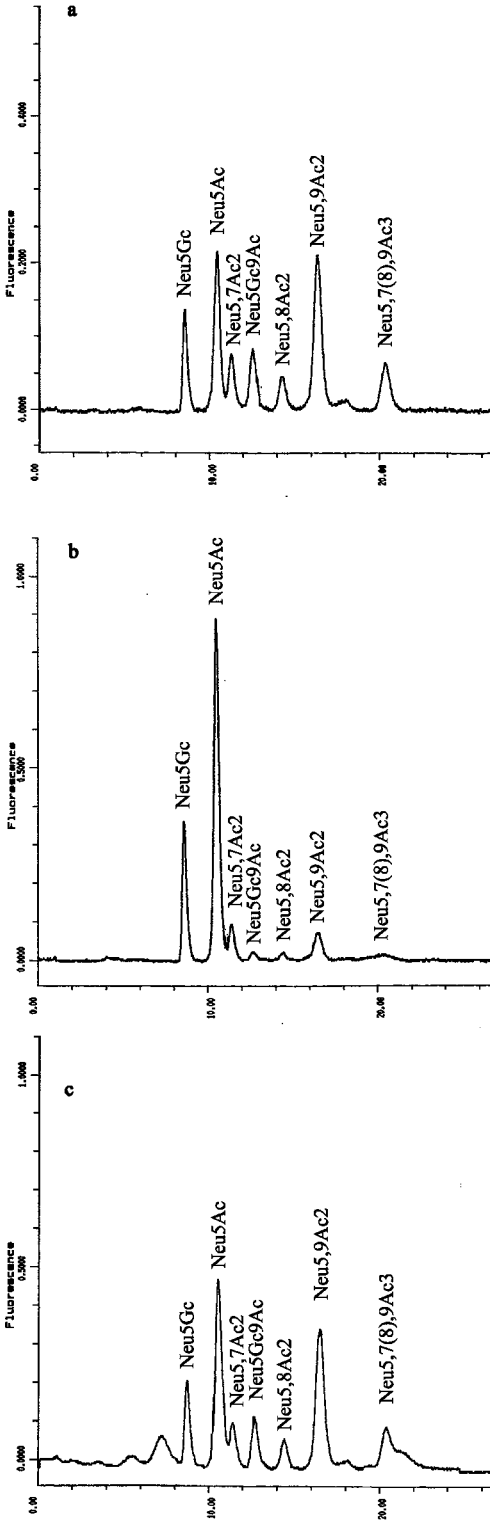

Figure 7. Hydrolysis of glycosidically bound 9-*O*-acetylated sialic acids. BSM was incubated with recombinant influenza C virus HE1/2-GFP (b) or SDAV HE-GFP (c) (15 mU of esterase) overnight at 37°C. For control, BSM was incubated with PBS (a). Then, sialic acids were released by acidic hydrolysis, derivatized with DMB reagent, and analyzed by fluorimetric HPLC. Positions of sialic acids are indicated.

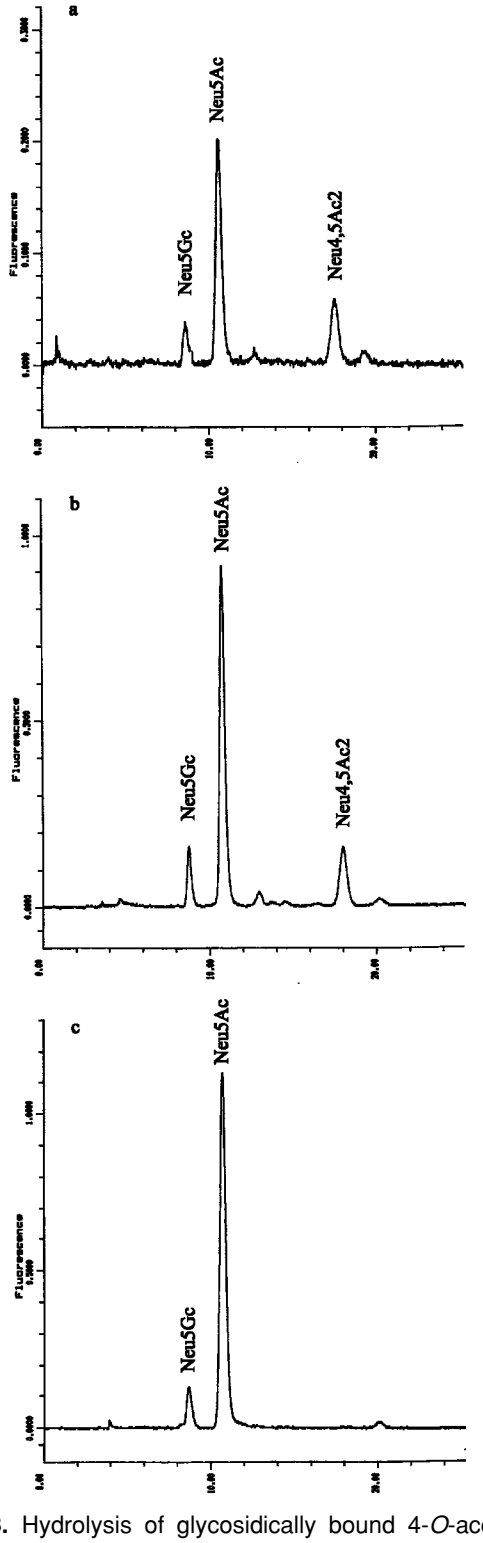

Figure 8. Hydrolysis of glycosidically bound 4-*O*-acetylated sialic acid. Horse serum glycoproteins were incubated with recombinant influenza C virus HE1/2-GFP (b) or SDAV HE-GFP (c) (15 mU of esterase) overnight at 37°C. For control, horse serum was incubated with PBS (a). Then, sialic acids were released by acidic hydrolysis, derivatized with DMB reagent, and analyzed by fluorimetric HPLC. Positions of sialic acids are indicated.

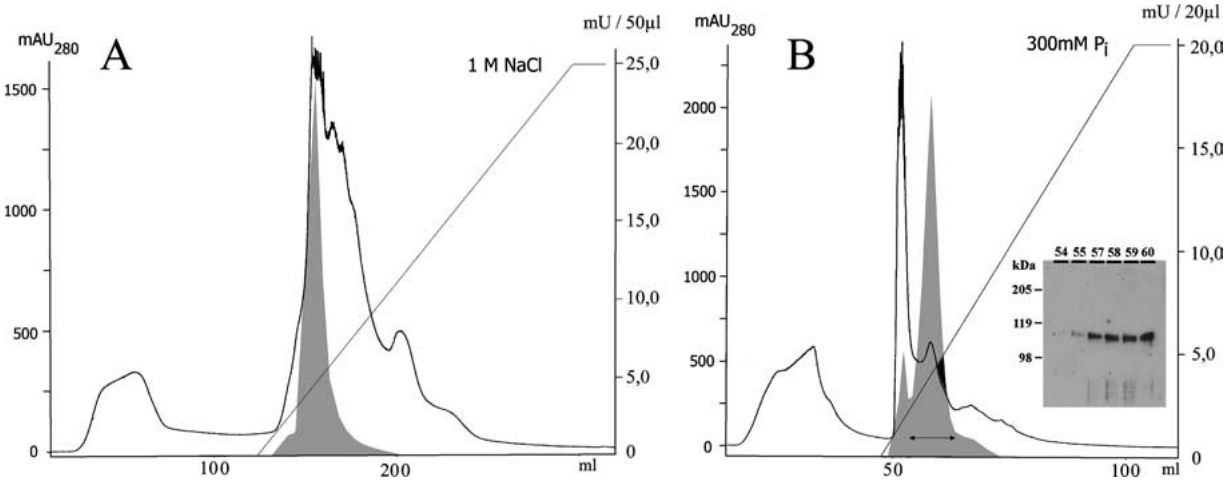

Figure 9. Purification of recombinant influenza C virus HE1/2-GFP. A cell culture supernatant (200ml) of Sf9 cells infected with recombinant baculovirus expressing the influenza C virus HE1/2-GFP protein was subjected to purification of the recombinant protein. A: elution profile from the Q sepharose column. B: elution profile from the hydroxyapatite column. The shaded areas represent fractions containing esterase activity as determined by the *p*NPA assay. Ordinates to the left show the protein absorption units at 280 nm, those to the right the esterase activity units. The elution gradients are indicated. A Western blot inserted in B shows the detection of the HE1/2-GFP protein. Lane numbers on the blot correspond to fraction numbers.

of murine erythroleukemia (MEL) cells in the liver and spleen, and to block binding of complement factor H to those cells [7]. Cell surface expression of Neu5,9Ac_2 was shown to depend on the cell cycle stage of MEL cells: Entry into the G0/G1 stage causes downregulation while arrest at G2/M showed increased expression of cell surface 9-*O*-acetylated sialomucins but not of 9-*O*-acetylated gangliosides [36]. Moreover, this laboratory used the recombinant viral esterase to reveal that 9-*O*-acetylation of sialic acids on cell surface mucins is a novel marker on CD4 T cells that appears during maturation and is modulated downwards upon activation [8].

In addition, to detect *O*-acetylation on glycoproteins, another laboratory used the cloned viral esterase to downregulate the expression of CD60b in murine neuroblastoma cells. Cells transfected with the recombinant esterase exhibited a morphology different from the parental cells with enlarged cell bodies and elongated neurites [37]. By transfecting the viral esterase into hamster melanoma cells, the same laboratory could show a substantial decrease of CD60b expression and detected major effects on proliferation, differentiation, and melanogenesis [38]. Most recently, a direct link between the formation of CD60b and the inhibition of apoptosis was established by transfecting the HE gene into a cell line overexpressing the *O*-acetylated ganglioside [39].

We have now cloned and expressed a series of soluble recombinant viral HE proteins, specific for either Neu5,9Ac_2 or Neu4,5Ac_2. We could demonstrate that the HE1 domain of the influenza C virus is folding independently from the HE2 domain as shown by the correct specificity of the recombinant protein. When we expressed the HE1 alone, the protein was found intracellularly. In contrast, when we expressed a chimeric HE1 fused to GFP, the protein was secreted into the cell culture supernatant, and it exhibited an enzymatic activity indistinguishable from that of the authentic membrane-bound viral protein. The chimeric SDAV HE protein fused to the GFP domain was also secreted into the culture supernatant of insect cells, provided that the authentic signal peptide was replaced by the homologous insect signal peptide derived from honeybee melittin. The recombinant chimeric SDAV esterase containing the melittin signal sequence was secreted into the culture supernatant, and it exhibited a specificity for glycosidically bound Neu4,5Ac_2. Earlier, we had proposed that the group 2 coronaviruses be subdivided into group 2a and 2b according to the genomic organization and their substrate specificities for Neu4,5Ac_2 and Neu5,9Ac_2, respectively [19]. At first glance, our finding appears to further strengthen the hypothesis that all esterases related to the proposed group 2a coronaviruses are specific for Neu4,5Ac_2. It should be mentioned that we have in the meantime analyzed additional coronavirus esterases and found that coronaviruses regularly exchange their HE genes during evolution (Smits *et al.*, in preparation).

The recombinant esterases described in this report are capable of de-*O*-acetylating sialic acids. Techniques involving fluorescent RP-HPLC cannot unambiguously discriminate between Neu5,9Ac_2 and Neu4,5Ac_2 due to their similar Rf values [9]. Currently, the only method to distinguish these sialic acid derivatives are restricted to the use of mass spectrometry in the impact mode. Due to the required expensive equipment, these techniques are not commonly available. Application of the described recombinant enzymes offers an alternative to identify unambiguously Neu5,9Ac_2 and Neu4,5Ac_2 in many different applications, including HPLC techniques, histochemical de-*O*-acetylation and subsequent staining with probes specific for the de-*O*-acetylated derivatives of sialic acids, de-*O*-acetylation of

gangliosides on HPTLC plates, etc. Such applications may in the future facilitate experiments aiming to increase knowledge of the functions of viral esterases during virus replication, and of the biological functions of *O*-acetylation in the control of the cell cycle in the progression of malignant and metastasizing cancer (*e.g.* melanoma), and during the development of the central nervous system. In the past, the influenza C virus HE1-GFP protein has already been successfully applied to the identification of Neu5,9Ac_2 at the surface of *Leishmania* promastigotes [40]. With the expression of a recombinant esterase specific for Neu4,5Ac_2, we now provide a tool to identify another sialic acid derivative. Data on the function of Neu4,5Ac_2 are still limited. It is found in high amounts in horses and guinea pigs [41]. The finding that Neu4,5Ac_2 is the substrate for group 2a coronaviruses [19] suggested that it must be detectable in mice. Indeed, we recently found Neu4,5Ac_2 by GC-MS analysis in virtually all mouse organs susceptible for MHV (Rinninger *et al*., in preparation). We now present data that a recombinant rat coronavirus esterase also hydrolyzes Neu4,5Ac_2 indicating that rats may also express substantial amounts of this sialic acid. By using a combination of HPLC and ESI-MS, Morimoto *et al*. recently reported its presence in the sublingual and submaxillary glands of rats and in the intestine of mice [42]. Moreover, it presumably also exists in teleosts [22,23]. In a recent investigation, Neu4,5Ac_2 was also found in human erythrocytes [43].

Acknowledgments

We thank Dr. Huemer (University of Innsbruck, Austria) for supplying horse serum, and Dr. Briza (University of Salzburg) for ESI-MS analysis of DMB-derivatized sialic acids. This work was supported by grants P09945-Med and P14104-Med from the Austrian Science Fund.

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