The human Cas1 protein: A sialic acid-specific *O*-acetyltransferase?

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**Sialic acids are important sugars at the reducing end of
glycoproteins and glycolipids. They are among many other
functions involved in cell–cell interactions, host–pathogen
recognition and the regulation of serum half-life of
glycoproteins. An important modification of sialic acids is
O-acetylation, which can alter or mask the biological prop-
erties of the parent sialic acid molecule. The nature of
mammalian sialate-*O*-acetyltransferases (EC 2.3.1.45)
involved in their biosynthesis is still unknown. We have
identified the human *CasD1* (capsule structure1 domain
containing 1) gene as a candidate to encode the elusive
enzyme. The human *CasD1* gene encodes a protein with a
serine–glycine–asparagine–histidine hydrolase domain and
a hydrophobic transmembrane domain. Expression of
the Cas1 protein tagged with enhanced green fluorescent
protein in mammalian and insect cells directed the
protein to the medial and *trans*-cisternae of the Golgi.
Overexpression of the Cas1 protein in combination with
α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 1 (GD3
synthase) resulted in an up to 40% increased biosynthesis
of 7-*O*-acetylated ganglioside GD3. By quantitative real-
time polymerase chain reaction, we found up to 5-fold
increase in *CasD1* mRNA in tumor cells overexpressing
O-Ac-GD3. *CasD1*-specific small interfering RNA reduced
O-acetylation in tumor cells. These results suggest that the
human Cas1 protein is directly involved in *O*-acetylation
of α2-8-linked sialic acids.**

Keywords: *O*-acetyl transferase / ganglioside GD3 /
lymphocytes / melanoma cells / sialic acid

Introduction

Sialic acids are a family of acidic amino sugars derived from
5-*N*-acetyl-neuraminic acid, typically found in *N*- and
O-linked glycans of glycoproteins and in glycolipids. They
are mostly present at the nonreducing end of glycan chains.
They are capping sugars which prevent further elongation of
glycan chains. An exception is the further elongation by
specific sialyltransferases leading to the formation of oligo- or
polysialic acids. Structural variations occur at carbon 5, which
result in the formation of either 5-*N*-glycolyl-neuraminic acid
or 2-keto-3-deoxynononic acid. In addition to modifications at
carbon 5, substitutions of the hydroxyl groups at carbons 4, 7,
8 and 9 are known. Substituents are *O*-acetyl, sulfate, methyl
and lactyl groups, which occur at one or more of these pos-
itions. Until now, approximately 50 different sialic acid
derivatives have been isolated and characterized from natural
sources. An overview on the biosynthesis, biology and diver-
sity of sialic acids is given in several reviews (Schauer and
Kamerling 1997; Varki 1997, 2007; Schauer 2004, 2009;
Varki and Schauer 2009). Among many other functions, sialic
acids are known to mask glycan epitopes. As an example,
human erythrocytes are covered with sialic acids. Upon aging,
sialidases cleave off sialic acids, and the modified erythro-
cytes are then sequestered by galactose-specific receptors
(Müller et al. 1981; Bratosin et al. 1995), e.g. the asialoglyco-
protein receptor in the liver (Ashwell and Harford 1982) or
similar receptors on macrophages. Different glycoforms of
therapeutica are also cleared from the bloodstream at different
rates. In several instances, therapeutic glycoproteins lacking
sialic acids are rapidly removed from the blood stream by the
asialoglycoprotein receptor and/or the mannose receptors
(Ashwell and Harford 1982; Lee et al. 2002). The serum
half-lives of desialylated glycoproteins decrease in average
from hours to minutes. This has been reported for
pro-urokinase (Henkin et al. 1991), chorionic gonadotropin
(Martinuk et al. 1991), thyrotropin (Szkudlinski et al. 1995a,
1995b), luteinizing hormone (Burgon et al. 1997) and erythro-
poietin (Fukuda et al. 1989). In contrast, the introduction of
additional glycosylation sites into the protein backbone of ery-
thropoietin or follicle-stimulating hormone results in artificial
hypersialylation concomitant with extended half-life and
improved pharmacokinetics (Egrie et al. 2003; Perlman et al.
2003).

The best studied *O*-acetyl esterification is the substitution
of the hydroxyl group at carbon 9. This modification arises
by enzymatic transfer of *O*-acetyl groups to carbon 7 of

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glycosidically bound sialic acids, followed by the migration of the acetyl group to carbon 9 (Kamerling et al. 1987; Vandamme-Feldhaus and Schauer 1998). The biosynthesis of 5-N-acetyl-9-O-acetyl-neuraminic acid is tightly regulated during brain development (Constantine-Paton et al. 1986; Levine et al. 1986; Blum and Barnstable 1987) and the activation of B and T cells (Kniep et al. 1995; Erdmann et al. 2006). Aberrant expression coincides with malignant transformation and metastasis. Different types of changes in the expression of O-acetylated sialic acids exist: In cancer cells derived from the neuroectoderm and in acute lymphoblastic leukemia, the expression levels are significantly increased (Cheresh, Reisfeld, et al. 1984; Cheresh, Varki, et al. 1984; Varki et al. 1991; Sjoberg et al. 1992; Pal et al. 2001; Kohla et al. 2002; Ghosh et al. 2005), whereas in colon cancer the amount of O-acetylated sialic acids, primarily on mucin-type molecules, is reduced (Corfield et al. 1999; Byrd and Bresalier 2004; Shen et al. 2004).

Current models indicate that overexpression of the ganglioside GD3 results in the activation of apoptosis (De Maria et al. 1997; Rippo et al. 2000), whereas O-acetylation of the α2,8-linked sialic acid of GD3 contributes to the inhibition of apoptosis (Chen and Varki 2002; Malisan et al. 2002; Erdmann et al. 2006; Kniep et al. 2006).

Moreover, O-acetylation regulates the activation of the innate immune system (Crocker and Varki 2001; Crocker et al. 2007; Schauer et al. 2010) and the alternate complement pathway (Shi et al. 1996c). Most recently, it was shown that a cellular sialate O-acetyl esterase regulates the function of CD22, a siglec which negatively regulates the B cell receptor (Cariappa et al. 2009). Loss of function of the cellular acetyl-esterase results in autoimmune disease (Pillai et al. 2009; Surolia et al. 2010). Modifications of sialic acids result in altered recognition by bacterial sialidases (Corfield et al. 1986, 1992, 1993) and viral pathogens, including influenza C viruses (Herrler et al. 1985; Muchmore and Varki 1987; Vlasak et al. 1987), coronaviruses (Vlasak et al. 1988; Schultze et al. 1991; Regl et al. 1999; Smits et al. 2005), infectious salmon anemia viruses (Hellebo et al. 2004) and toroviruses (Smits et al. 2005; de Groot 2006).

The search for the enzyme catalyzing the transfer of O-acetyl groups to sialic acids started almost 40 years ago. The latest status on the purification was described recently (Lrhorfi et al. 2007; Srinivasan and Schauer 2009). Two types of transferases apparently exist: The bovine enzyme transfers acetyl groups to carbon 7, whereas the transferase isolated from equine and guinea pig tissues preferentially adds O-acetyl groups to carbon 4. The expression of the 9-O-acetyltransferase is developmentally regulated (Varki and Kornfeld 1980; Muchmore et al. 1987; Shi et al. 1996b; Krishna and Varki 1997). Attempts to identify the gene by expression cloning failed in the past (Shi et al. 1998; Satake et al. 2003). Other laboratories also described efforts toward identifying the gene (Ogura et al. 1996; Kanamori et al. 1997). In summary, the nature of the sialic-acid-specific O-acetyltransferase remains enigmatic.

We have used a rational approach to identify the gene encoding the mammalian sialate-O-acetyltransferase (SOAT) (EC 2.3.1.45). Taking into account the diversity of O-acetylated sialic acids on N- and O-glycans as well as on glycosphingolipids and possibly also on polysialic acids, more than one gene may exist. Alternatively, different specificities may be derived from the action of modulating factors. Genetic evidence suggested the presence of a single human O-acetyl transferase gene related to O-acetylation of mucin sialic acids in the intestine (Campbell et al. 1994). Therefore, we screened the human genome databases for genes with unknown functions, which are predicted to possibly encode acetyl transferases and potentially be located in the Golgi membrane. This search finally resolved the candidate gene CasD1 (capsule structure1 domain containing 1), which is similar to a gene of the fungus Cryptococcus neoformans. Our results indicate a direct involvement of the human Cas1 protein (Cas1p) in the O-acetylation of sialic acids.

Results

Data mining of the human genome

In order to detect candidate genes, we used the search function of the Ensembl programme (www.ensembl.org) to screen for genes with unknown functions, which are predicted to possibly encode acetyl transferases.

The first search for "transferase" in the human genome resulted in 1717 hits. When the search was narrowed to "O-acetyltransferase", 126 entries with a matching term were found. They were then further screened for a potential Golgi location of the encoded protein.

This search finally resolved a single candidate gene, which is similar to a gene of the yeast strain C. neoformans. Strong genetic evidence exists that the yeast Cas1p is an O-acetyl transferase, which adds O-acetyl groups at the C6 position of mannose in a capsid structure composed of glucuronoxylomannans (Janbon et al. 2001). Genes homologous to the human CasD1 gene are present in plants and throughout the animal kingdom.

Structure of the CasD1 gene and its predicted transcripts

The human CasD1 gene is predicted to consist of 18 exons. In the human and chimpanzee genome, the gene is located on chromosome 7q21.3. CasD1 is a maternally expressed imprinted gene in mice (Babak et al. 2008), and it is located next to the paternally expressed imprinted genes SGCE (Grabowski et al. 2003) and PEG10 (Ono et al. 2001). In the drug-resistant human neuroblastoma cell line IGRN-91-R, its expression is significantly upregulated (Flahaut et al. 2009). The predicted length of the transcript is 3942 nucleotides, which encodes a protein of 797 amino acid residues.

Analysis of the potential full-length transcript revealed a signal sequence, usually found in proteins which are delivered to the endoplasmic reticulum (ER), the Golgi or the plasma membrane. The protein consists of a serine–glycine–asparagine–histidine (SGNH) hydrolase domain and a C-terminal transmembrane domain. The human Cas1p shares some sequence similarity with viral sialic acid-specific O-acetylesterases, particularly around the active site residues (Figure 1). The amino acid sequence flanking the catalytic serine was originally identified for the influenza C virus esterase (Vlasak et al.

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Cas1p       (70)   MMHKYKISEAKNCLV-DKHIAFIGDSRIRQLFYSFVKIINPQFK   (112)
C/JHG/66    (47)   MFELVKPKAGASVLN-QSTWIGFGDSRTDKSNSAFPRSADVSAK   (89)
ISAV         (3)   LFLLLAPVYSRLCLRNYPDTTWMGDSRSDQSRVNPQSLDLVTEF   (47)
BoTV Breda 2 (12)  AFAATPVTPYYGPGHITFDWCGFGDSRSDCTNPQSPMSLDIPQQ   (56)
HCoV OC43   (16)   SLGFYNPPTNVVSHVNG-DWFLFGDSRSDCNHIVNINPHNYSYM   (59)


                                         *   *
Cas1p       (251)  KMFSVSKLI-AQETIMESLDGLHLPESSRETTAMILMNVYCNKI   (294)
C/JHG/66    (347)  LSTPGCMLIQKQKPYIGEADDHHGDQEMRELLSGLDYEARCISQ   (390)
ISAV        (238)  LDEYVDTPNTGGVYPSDGFDSLHGSASVRTFLTDALT---CPDI   (278)
BoTV Breda 2 (306) APYCIFYNKTTPYTVTNGSDANHGDDEVRMMMQGLLRNSSCISP   (349)
HCoV OC43   (307)  CQPPYCYFRNSTTNYVGVYDINHGDAGFTSILSGLLYNSPCFSQ   (350)
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Fig. 1. Alignment of amino acid sequences around the catalytic site of viral sialate-*O*-acetylesterases. The sequence of the human Cas1 protein (accession number NP_075051) was aligned with the sialate-*O*-acetylesterases of influenza C/JHG/66 virus (S07412), infectious salmon anemia virus (ISAV) strain 45–99 (AAL34465), bovine torovirus (BoTV) strain Breda 2 (CAA71819) and human coronavirus (HCoV) OC43 (P30215). The active site residues of the influenza C virus hemagglutinin-esterase are indicated by asterisks. Identical amino acid residues that are present in Cas1p and at least one viral esterase are highlighted in bold.
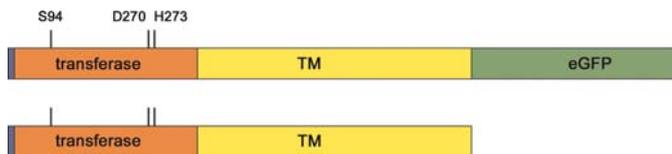

Fig. 2. Schematic representation of the cDNA clones expressing the *CasD1* protein. The signal peptide, transferase domain, transmembrane domain (TM) and the C-terminal eGFP are indicated from left to right. The upper model shows the protein encoded by plasmid pCasD1-eGFP, and the lower structure is encoded by plasmid pCas1stop.

1989) and for cellular sialic acid-specific *O*-acetylesterases (Hayes and Varki 1989). Additional features include an ER export signal (D-X-E) and several *trans*-Golgi network (TGN)–endosome export signals (Y-xx-hydrophobic amino acid or D/E-xxx-L/I). The amino acid sequences connecting the hydrophobic regions exhibit a high content of basic and acidic amino acid residues.

Expression of Cas1p

To allow the expression of Cas1p in mammalian cells, the coding region of the *CasD1* cDNA was amplified by polymerase chain reaction (PCR) and cloned into the expression vector pEGFP-N3. This vector facilitates the expression of chimeric recombinant proteins with a C-terminal enhanced green fluorescent protein (eGFP) tag. Two types of clones were created: pCasD1eGFP allows the detection of the expressed protein by fluorescence microscopy and western blot analysis, whereas pCas1stop encodes the authentic human protein without C-terminal extension (Figure 2). When we expressed the GFP-tagged protein in insect Sf9 cells, the protein was found predominantly in a Golgi-enriched microsomal fraction. It migrated on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with the predicted molecular mass of approximately 115 kDa. In addition to the monomeric protein, two other bands which presumably represent trimers and tetramers were also detected with the GFP-specific antiserum (Figure 3). We also wanted to
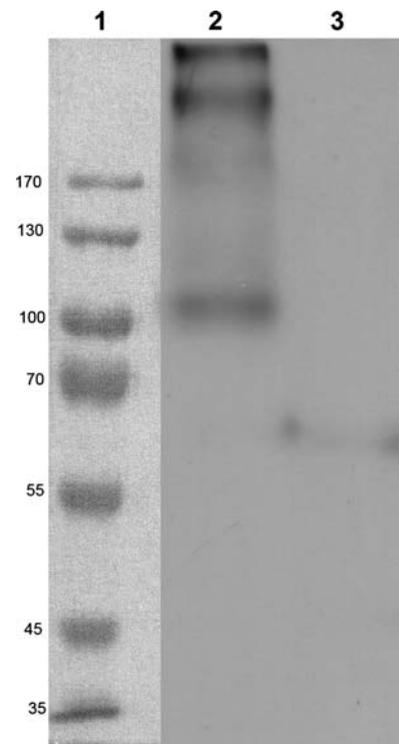

Fig. 3. Expression of the *Cas1-eGFP* fusion protein in Sf9 cells. Sf9 cells were infected with baculovirus CasD1eGFP or wild-type (wt) baculovirus and incubated for 48 h. Proteins were extracted from the Golgi-enriched microsomal fraction of infected cells and separated by 8% SDS–PAGE, blotted onto nitrocellulose and incubated with rabbit anti-GFP antiserum. Bound antiserum was detected with HRP-conjugated goat anti-rabbit antiserum and ECL. (1) Molecular weight marker. Molecular masses are indicated in kDa. (2) Cas1p-eGFP expression. (3) wt baculovirus.

determine the intracellular location of Cas1p. Therefore, we transfected COS cells with plasmid pCasD1eGFP and monitored expression by fluorescence microscopy. The GFP-tagged Cas1p was detected in intracellular compartments close to the nucleus, as shown by counterstaining of nuclei. In addition, Cas1p-eGFP was regularly found as bright spots, which

presumably represent the protein within transport vesicles (Figure 4A–C). To further localize Cas1p, we also transfected COS cells with plasmids pCasD1eGFP and pST6Gal1. The latter plasmid directs expression of sialyltransferase ST6Gal 1, a marker of the TGN (Chen et al. 2003). Cas1p-eGFP was detected directly by fluorescence microscopy, and ST6Gal 1 was detected with mAb STG (Cao et al. 2002). Analysis revealed a partial colocalization in cells expressing both Cas1p and ST6Gal1 (β-galactosamide α-2,6-sialyltransferase 1) (Figure 4D–F).

CasD1 expression results in increased O-acetylation of ganglioside GD3

We then tested whether the Cas1 protein was involved in the O-acetylation of ganglioside GD3. We therefore expressed α-N-acetyl-neuraminide α-2,8-sialyltransferase 1 (ST8Sia1), which directs the formation of GD3 from ganglioside GM3. To allow the expression of ST8Sia1, we constructed plasmid pGD3synthHistag. When we cotransfected this plasmid with pCas1stop, we detected 7-O-acetylated ganglioside GD3 (7-O-Ac-GD3) by immunostaining with MAb U5 in a patched distribution at the cell surface, possibly located in "lipid rafts" (Figure 5A). For quantitative determination of the expression of 7-O-Ac-GD3 in COS cells, we used flow cytometric analysis. Control cells and cells transfected with either plasmid pGD3synthHistag alone or double transfected with pGD3synthHistag and pCas1stop were labeled with either the GD3-specific MAb R24 or MAb U5, which is specific for 7-O-Ac-GD3. A background expression of GD3 (CD60a) and 7-O-Ac-GD3 (CD60c) was found in control cells. It should be mentioned that we selected COS cells for these
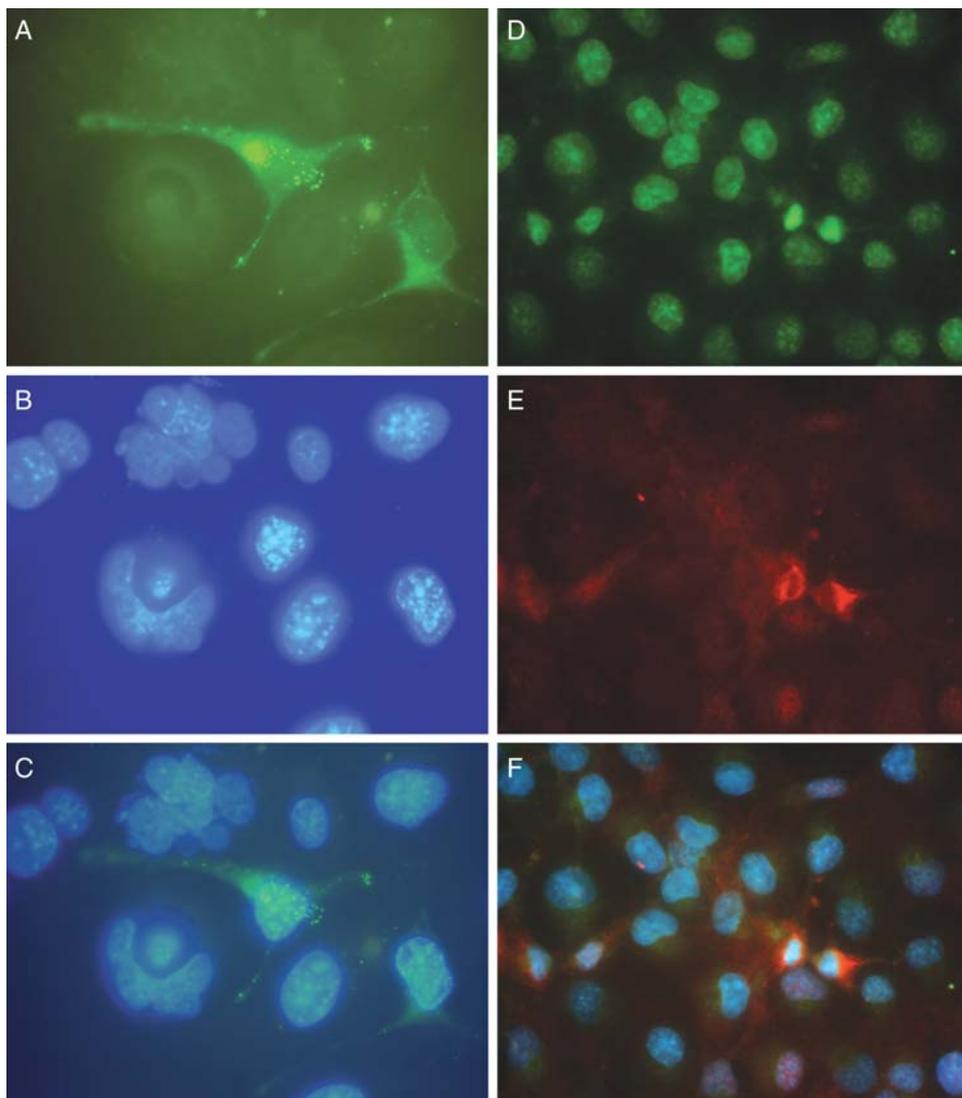

Fig. 4. Colocalization of Cas1p and ST6Gal1. COS-7 cells were transfected with plasmids pCasD1eGFP and pST6Gal1 and incubated for 72 h. Then cells were fixed, and Cas1p-eGFP was detected by fluorescence of the eGFP domain of the fusion protein. ST6Gal1 was detected by the specific monoclonal antibody STG (Cao et al. 2002). Nuclei were counterstained with Hoechst 33342 DNA dye (**B**, **C** and **E**). Slides were mounted using Mowiol (**A**–**C**) or Fluoromount G (Southern Biotec, Birmingham, AL). (A) COS-1 cells expressing Cas1p-eGFP. (B) Nuclear staining. (C) Overlay of (A) and (B). (**D**) and (**E**) COS-7 cells expressing Cas1p-eGFP (green) and sialyltransferase ST6Gal1 (red). (**F**) Overlay of (D) and (E).
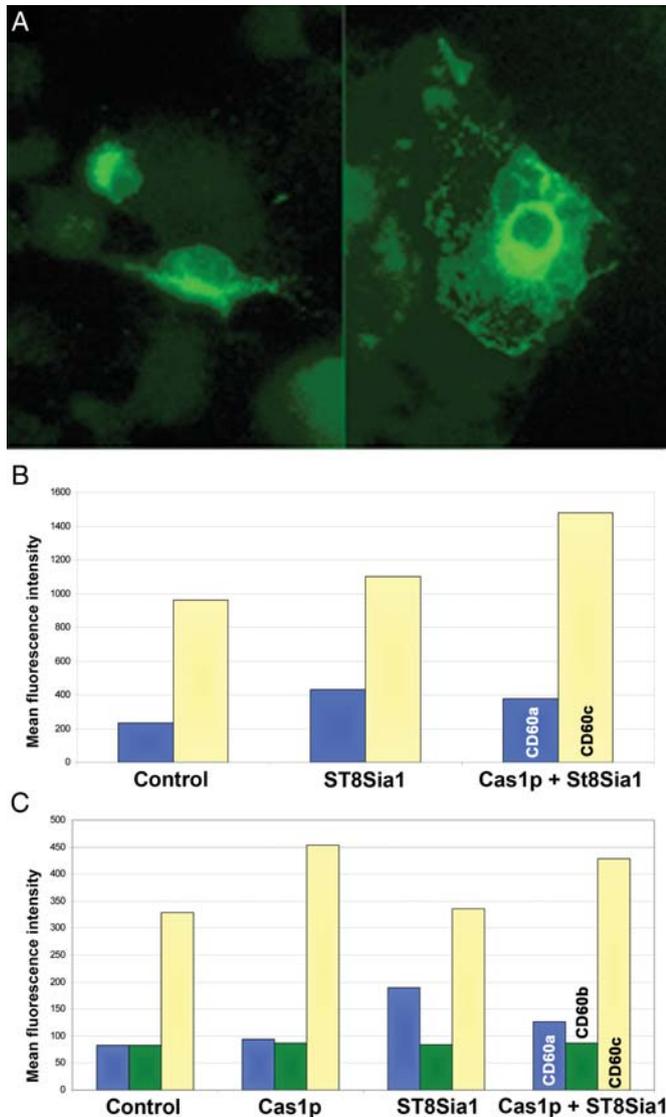
Fig. 5. Expression of 7-O-Ac-GD3 (CD60c) in COS-7 cells. Cells were either transfected with plasmid pCas1stop and/or pGD3synthHisTag. (**A**) The cells were incubated for 3 days, fixed, stained with anti-CD60c mAb U5 and secondary FITC-coupled goat-anti-mouse IgG antibody and monitored by fluorescence microscopy. No immunofluorescence was detected in control cells (data not shown). (**B**) and (**C**) Transfected cells were incubated for 3 days and probed with mAbs R24 (anti-CD60a, blue bars), MT6004 (anti CD60b, green bars) or U5 (anti-CD60c, yellow bars). Bound antibodies were detected with secondary fluorescent antibodies. The mean fluorescence intensities were determined by FACS analysis.

experiments, because it was shown earlier that in contrast to Chinese Hamster Ovary (CHO) cells these cells do not express significant amounts of intrinsic SOAT (Shi et al. 1996a). As shown in Figure 5B, the expression of ST8Sia1 (GD3 synthase) resulted in an 86% increase in GD3 and a small increase (14%) in 7-O-Ac-GD3. The increase in the 7-O-Ac-GD3 may be due to the activation of a pre-existing intrinsic O-acetyltransferase. Co-expression of GD3 synthase and Cas1p led to an increase in both GD3 (62%) and 7-O-Ac-GD3 (54%). In another experiment (Figure 5C), we

also determined the expression of 9-O-Ac-GD3 (CD60b) with MAb MT6004. Whereas the expression of 7-O-Ac-GD3 (CD60c) was increased in the presence of Cas1p, no significant change in 9-O-Ac-GD3 could be detected. This experiment indicates that Cas1p transfers O-acetyl groups exclusively to carbon 7 of sialic acids.

CasD1 expression is upregulated in CD60b⁺ cells

We also determined the expression of *CasD1* mRNA in a number of primary B and T lymphocytes and in cell lines derived from human melanomas and lymphomas. T and B cells of tonsillar and peripheral blood lymphocytes were subjected to 3D flow cytometric analysis, where B cells were characterized by an anti-CD19+ mAb conjugated to PE and T cells by an anti-CD3 secondary mAb conjugated to Cy3. As shown in Figure 6, all freshly extracted normal lymphocytes of various stages and lineages express the *CasD1* gene albeit in different quantities. Non-stimulated B and T lymphocytes from peripheral blood had the highest values followed by tonsillar T and B cells which represent lymphocytes undergoing various activation stages and thymocytes which represent T cells of early T cell differentiation. Human leukemia cell lines express the *CasD1* gene, and the erythroleukemia cell line K562 had the lowest values. The highest values were observed by the T cell leukemia cell line Jurkat, followed cell lines KG1 and KG1a derived from malignant acute myeloblastic leukemia. Interestingly, the cell line KG1a which was found to have a higher state of surface sialylation when compared with the parental cell line KG1 (Schwartz-Albiez et al. 2004) also had a higher expression of CD60c (data not shown) and of *CasD1*. Melanoma cell lines, which are known to express the CD60a and b surface structures, also expressed the *CasD1* gene. The cell line MeWo, a melanoma cell line established and *in vitro* cultivated already for a long time, had lowest *CasD1* values, whereas the cell lines Ma-Mel 95 and Ma-Mel 123 which were freshly established from malignant melanoma patients during the recent years have a high degree of *CasD1* expression. This indicates that *CasD1* may be a marker for malignant melanoma cells and secondly that during long cell cultivation the O-acetylation of gangliosides becomes diminished.

Downregulation of O-acetylation by CasD1-specific small interfering RNA

In order to determine whether the *CasD1* gene product is directly involved in the O-acetylation of sialic acids, we used small interfering RNA (siRNA) to downregulate the intrinsic *CasD1* mRNA. In this assay, we used Ma-Mel 123 cells, which express high levels of *CasD1* mRNA (Figure 6) and CD60c (data not shown). First we determined whether transfection of *CasD1*-specific siRNA resulted in decreased amounts of detectable mRNA. By reverse transcription (RT)–PCR, we found that the transfection procedure alone or with unspecific siRNA caused a drop in *CasD1* mRNA expression by approximately 20%, compared with untreated cells. In contrast, transfection of specific siRNA resulted in a more than 80% reduction of detectable *CasD1* mRNA (Figure 7A). We then measured the amount of CD60b-expressing cells by fluorescence-activated cell sorter (FACS). Compared with cells
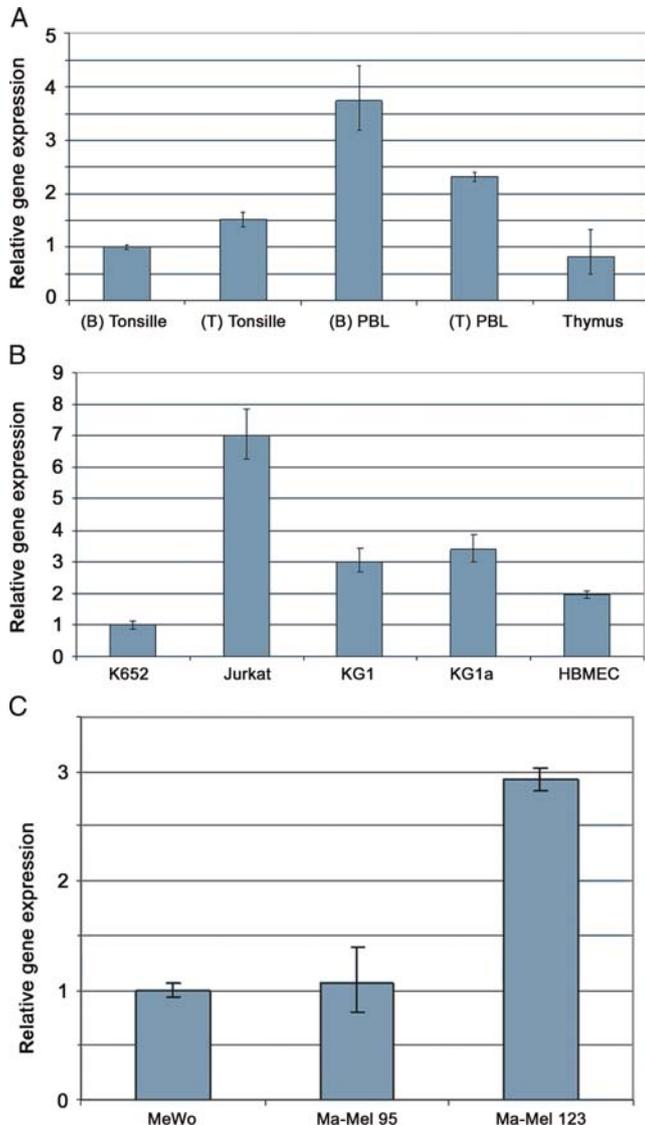
Fig. 6. Expression profiles of *CasD1* mRNA. Cell lines were cultured in Roswell Park Memorial Institute (RPMI) medium, harvested and subjected to RNA extraction and cDNA synthesis. *CasD1* mRNA expression was determined by real-time PCR as described in Materials and methods. Results are shown as the relative gene expression (normalized to β-actin) where the lowest expression level was set to 1. *CasD1* gene expression patterns in native leucocytes (**A**), leukemic cell lines (**B**) and melanoma cell lines (**C**) are shown.

transfected with unspecific siRNA, a significant reduction in CD60b$^+$ cells was observed 24 h after transfection of *CasD1*-specific siRNA. *O*-Acetylation gradually re-appeared in a time-dependent manner and approached original values after 96 h (Figure 7B).

Discussion

O-Acetylation is a common modification of sialic acids. It is found from bacteria to man. Human-pathogenic bacteria such as *Escherichia coli* K1, *Neissera meningitidis* and streptococci known to infect the central nervous system express different forms of *O*-acetylated sialic acids. Recently, several bacterial
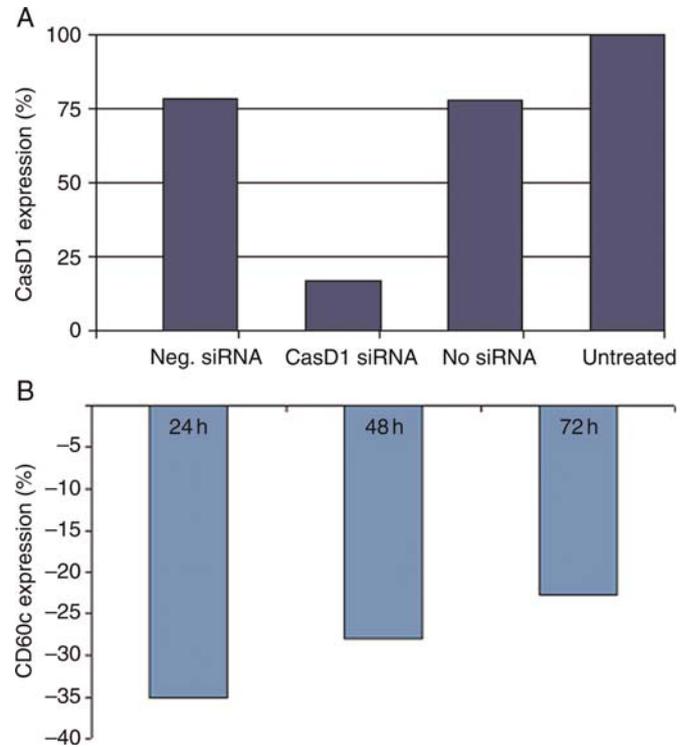

Fig. 7. *CasD1* siRNA reduces *CasD1* gene expression and *O*-acetylation of ganglioside GD3. (**A**) *CasD1*-specific siRNA was transfected into MA-Mel123 cells, and expression levels were monitored by RT–PCR. Expression of *CasD1* in untreated cells was set to 100%, and reduction in specific mRNA levels was determined following the transfection of unspecific and *CasD1*-specific siRNA. As a negative control, transfection reagent without siRNA was used. (**B**) reduction (%) of CD60c (7-*O*-Ac-GD3) by *CasD1* siRNA, when compared with cells transfected with unspecific siRNA was monitored by FACS analysis.

genes encoding *O*-acetyl transferases were identified in human-pathogenic *E. coli* K1 (Deszo et al. 2005; Steenbergen et al. 2006; Vimr and Steenbergen 2006), *Campylobacter jejuni* (Houliston et al. 2006), group B streptococci (Lewis et al. 2004, 2006) and *Neissera meningitides* (Bergfeld et al. 2009; Lee et al. 2009). The bacterial genes and their encoded proteins do not exhibit significant similarities to animal genes or gene products.

Despite numerous attempts to identify the specific *O*-acetyltransferase(s) responsible for the transfer of acetyl groups, the nature of the enzyme up to now remained elusive. Forty years ago, the first reports on a specific *O*-acetyltransferase in bovine and equine submandibular glands were published. Whereas the bovine enzyme catalyzed the transfer to positions 7 and 9 (Schauer 1970b), the equine transferase delivered the acetyl groups to position 4 of the sialic acids (Schauer 1970a). Then it was shown with rat liver that incubation of purified Golgi vesicles with ^{3}H-acetyl-coenzyme A (AcCoA) resulted in a rapid accumulation of radioactivity within the lumen of the Golgi (Diaz et al. 1989). Apparently, no transport of AcCoA into the Golgi was required, only the labeled free acetate was found inside the vesicles. The finding that solubilization of the Golgi membrane with detergents immediately abolished acetyl-transferase activity turned out as a major hurdle towards future

isolation of the intact enzyme. (Diaz et al. 1989) In a further study, it was shown that histidine and lysine residues are essential for the transmembrane transfer of acetyl groups (Higa et al. 1989).

It was also shown that the *O*-acetyltransferase activity is located in Golgi subcompartments which are beyond the block caused by brefeldin, indicating a location in late Golgi vesicles. In addition, the data indicated that *O*-acetylated ganglioside GD2 is synthesized either from the precursor ganglioside *O*-Ac-GD3 or by direct transfer of the *O*-acetyl group to GD2 (Sjoberg et al. 1992; Sjoberg and Varki 1993).

Later, CHO cell lines were created which stably expressed either GD3 synthase (ST8Sia1) or ST6Gal1 (Shi et al. 1996a). Most surprisingly, the expression of each of these sialyltransferases alone was sufficient to allow *O*-acetylation of GD3 or α2,6-linked sialic acids on *N*-glycans, respectively. Thus, an endogenous *O*-acetyltransferase was presumably activated by the expression of the heterologous sialyltransferases. When the same sialyltransferases were expressed in COS cells, no acetylation of sialic acids could be detected, indicating that these cells did not express the endogenous *O*-acetyltransferase present in CHO cells. Interestingly, the expression of ST3Gal3, which transfers sialic acids in an α2,3 linkage to lactosamine, did not result in the formation of *O*-acetylated sialic acids in CHO cells. In other publications, it was shown that the ganglioside GD3 can induce its own *O*-acetylation (Chen et al. 2006; Kniep et al. 2006).

Vandamme-Feldhaus and Schauer (1998) described the partial purification of a 7-*O*-acetyl transferase from bovine submandibulary glands and proposed the existence of a "migrase", which directs the transfer of acetyl groups from carbon 7 to carbon 9. A partially purified enzyme was also prepared from guinea pig liver and equine submandibular glands, which preferentially directs *O*-acetylation at carbon 4 (Iwersen et al. 1998, 2003; Tiralongo et al. 2000). The most recent results on the characterization of the mammalian *O*-acetyl transferase are summarized in three publications (Lrhorfi et al. 2007; Mandal et al. 2009; Srinivasan and Schauer 2009).

In this study, we describe new approaches toward identifying the mammalian enzyme catalyzing the transfer of *O*-acetyl groups from acetyl-CoA to sialic acids. By data mining, we identified the product of the human *CasD1* gene as a candidate protein which might represent the elusive sialic acid-specific *O*-acetyltransferase. The Cas1 protein is a protein with a predicted molecular mass of 87.5 kDa composed of up to 12 transmembrane domains. The expression of Cas1p with a C-terminal eGFP domain resulted in the formation of monomeric and oligomeric proteins. The peptides connecting the transmembrane domains contain 9 histidine and 22 lysine residues. Thus, the human Cas1p fulfills the requirements for an acetyl-CoA transporter/antiporter. Our data indicate that it is located in specific intracellular compartments, most likely in the TGN as shown by partial colocalization with ST6Gal1, a marker enzyme for TGN (Chen et al. 2003). Furthermore, Cas1p possesses an SGNH hydrolase domain with sequence similarity to viral sialic acid-specific *O*-acetyl esterases. Co-expression of Cas1p and ST8Sia1 (GD3 synthase) resulted in the formation of 7-*O*-Ac-GD3. We observed a slight increase in the presence of 7-*O*-Ac-GD3 in COS cells transfected with *ST8Sia1* alone, indicating that these cells also express a low background level of intrinsic SOATs. However, the co-expression of ST8Sia1 and Cas1p resulted in significantly increased amounts of 7-*O*-Ac-GD3. It should also be noted that we did not observe a significant increase in *O*-acetylation of GD3 in all experiments. The reasons are currently unclear. They may be a result of variations of transfection efficiencies. On the other hand, our data cannot completely exclude the possibility that the expression of Cas1p stimulates the expression of intrinsic SOATs, while Cas1p might have other functions. In previous work, it was hypothesized that *O*-acetyltransferase activity may be associated within a membrane-bound complex composed of the acetyl-CoA transporter, sialyltransferase and acetyltransferase activities and possibly an acetylated intermediate (Diaz et al. 1989; Lrhorfi et al. 2007; Schauer et al. 2010). In addition, a soluble cofactor may also be required for activity (Srinivasan and Schauer 2009). Thus, the metabolic status of cells may contribute to the observed differences in the amount of *O*-acetylation of GD3. On the other hand, the transfection of *CasD1*-specific siRNA into melanoma cell line Ma-Mel123 resulted in an approximately 82% reduction of *CasD1* mRNA and a concomitant 35% reduction of 7-*O*-Ac-GD3 within 24 h of incubation. In conclusion, we hypothesize that the human Cas1p represents a sialic acid-specific *O*-acetyltransferase. The results indicate that the expression of Cas1p in COS cells directs acetyl groups to carbon 7 of sialic acid. Upon co-expression of Cas1p and ST8Sia1 increased amounts of 7-*O*-Ac-GD3 were detected, whereas 9-*O*-Ac-GD3 levels remained essentially unchanged. This observation is in accordance with published data, which indicate that acetylation at carbon 9 results from the migration of the acetyl group at carbon 7 (Vandamme-Feldhaus and Schauer 1998). The CD60c antigen (7-*O*-Ac-GD3) was shown to be differently expressed from CD60b (9-*O*-Ac-GD3) in human B and T lymphoblasts, indicating differences in their biosynthesis and function (Erdmann et al. 2006). Most recently, the SOAT activity in lymphoblasts derived from patients with acute lymphoblastic leukemia was characterized, and again this enzyme predominantly catalyzed the formation of 7-*O*-acetylated sialic acid (Mandal et al. 2009). We therefore suggest that the human Cas1p may represent a sialic acid-specific *O*-acetyltransferase, which transfers acetyl groups to carbon 7.

Most interestingly, the *CasD1* gene is imprinted in mice. Imprinted genes are expressed from one allele derived either maternally or paternally. Several imprinted genes are essential to mammalian embryogenesis. Genomic imprinting influences mammalian development, growth and behavior. A number of human genetic diseases are related to imprinted genes that exist in different chromosomal regions. Aspects on the evolution of imprinting and the impact for human health have been reviewed recently (Das et al. 2009). *CasD1* exhibits equal biallelic expression in neonatal mouse brain. In extra-embryonic tissues, the gene is expressed ubiquitously, and a weak maternal bias was observed (Ono et al. 2003). The finding that *CasD1* is a maternally expressed imprinted gene was substantiated by transcriptome sequencing (Babak et al. 2008). For humans, no evidence of imprinting of *CasD1* has

been published, and no human genetic disease has yet been described to be associated. In summary, current evidence indicates that *CasD1* in postnatal mice is predominantly expressed from the maternal allele. Mapping of the adult mouse brain showed high expression levels of *CasD1* in the hippocampus [Allen Mouse Brain Atlas (Internet), Seattle (WA): Allen Institute for Brain Science ©2009. Available from http ://mouse.brain-map.org]. In this region of the mouse brain, a number of sialyltransferases, including ST3Gal1, ST3Gal3, ST3Gal4, ST3Gal5, ST6Gal2, ST6GalNAc5, ST8Sia1, ST8Sia2, ST8Sia3 and ST8Sia5 are also highly expressed.

In the future, we want to perform enzyme tests with the purified Cas1p. Preliminary data indicate that purification of Cas1p alone may be not sufficient to determine enzymatic activity (data not shown), suggesting that Cas1p may require interactions with other cellular components in order to become active. Interaction partners may either be the substrates themselves, other proteins, e.g. sialyltransferases or other glycosyltransferases within the Golgi, or cytoplasmic proteins that may modulate the transport of AcCoA into the Golgi. We preferentially want to test the role of sialyltransferases, specific substrates and the putative cofactor/activator.

Materials and methods

Cloning and expression of the human genes encoding CasD1, sialyl-2,6-Gal-transferase 1 and sialyl-2,8-Sia-transferase 1 (GD3 synthase)

The clones with the *CasD1* cDNA (IMAGE clone 5286382) and the cDNA for *ST8Sia1* (IMAGE clone 40125836) were obtained from Geneservice Ltd. (UK), the cDNA clone for ST6Gal1 (IRATp970A0993D) was purchased from RZPD Deutsches Resourcenzentrum für Genomforschung GmbH (Berlin).

Plasmid pCasD1-eGFP. From the plasmid pIMAGE5286382, two PCR fragments were generated. Fragment 1 was obtained with primers CasD1fwd and CasD1-rev-*Esp*3I. Fragment 2 was generated with primers CasD1-fwd2-*Esp*3I and CasD1-*Bam*HI rev (Table I). Both PCR products were digested with *Esp*3I, ligated and digested with *Acc*65I and *Bam*HI. The digested ligation product was inserted into the *Acc*65I/*Bam*HI window of plasmid pEGFP-N3 (Takara Bio Europe/Clontech, France), resulting in plasmid pCas1-eGFP.

Plasmid pCas1stop. From the plasmid pIMAGE5286382 a PCR product encoding the entire ORF and the stop codon

was generated with primers Cas IF fwd and Cas stop IF rev. The resulting PCR product was digested with *Acc*65I and *Bam*HI and ligated into the *Acc*65I/*Bam*HI window of plasmid pEGFP-N3 (Takara Bio Europe/Clontech, France), resulting in plasmid pCas1stop.

Plasmid pGD3synthHistag. From plasmid pIMAGE40125836, a PCR product covering the entire ORF for GD3 synthase was generated with primers GD3 fwd and GD3–6His rev. The resulting PCR product was digested with *Acc*65I and *Sal*I and inserted into the *Acc*65I/*Sal*I window of plasmid pCI (Promega, Germany), resulting in plasmid pGD3synthHistag.

Cells and cell lines

COS-1 and COS-7 cells were cultured as monolayer at 37°C/ 5% CO_2 in the DMEM medium (PAA Laboratories, Austria) containing 10% fetal calf serum. COS-1 cells were used in Salzburg and COS-7 cells were used in Heidelberg. The human leukemia cell lines K562, Jurkat, KG1 and KG1a were a kind gift of Dr. G. Moldenhauer, German Cancer Research Center, Heidelberg, Germany. Cell line MeWo was kindly provided by Dr. S. Eichmueller, German Cancer Research Center, cell lines Ma-Mel 95 and Ma-Mel123 were a kind gift by Dr. D. Schadendorf, Dermatology Department, University Heidelberg-Mannheim. Non-inflammatory tonsillar lymphocytes were extracted from tissue after tonsillectomy and purified as described (Erdmann et al. 2006), and lymphocytes from peripheral blood were separated by standard Ficoll-Paque centrifugation. Thymocytes were prepared from thymic tissue obtained in the course of corrective cardiac surgery. All biological material from patients was obtained after having received informal consent by the patients or their parents and after approval of the ethical committee on the use of human tissue in research at the Universities of Heidelberg and Heidelberg-Mannheim.

Recombinant baculoviruses

Plasmid pCasD1-eGFP was cleaved with *Acc*65I and *Not*I to obtain a fragment representing CasD1-eGFP. This fragment was ligated into the baculovirus transfer vector pBacPAK 8 (Takara Bio Europe/Clontech, France.), resulting in the construct pBacPAK CasD1-eGFP. The integrity of the fusion site was confirmed by DNA sequencing on both strands. Recombinant baculoviruses were prepared by the transfection of 500 ng of pBacPAK CasD1-eGFP with 100 ng of Baculo Gold DNA (BD Biosciences Pharmingen, Germany) into Sf9 cells using Cellfectin (Invitrogen, Karlsruhe, Germany) according to the

Table I. List of primers used for cloning of *CasD1* and *ST8Sia1* cDNA into expression vectors

Cas IF fwd	TGCAGTCGACGGTACCATGGCGGCTCTGGC
Cas stop IF rev	TGGTGGCGATGGATCCCTAATGTTTTGATTTAT
CasD1-fwd2-*Esp*3I	TAGCCGTCTCCATGATTCTTATGAATGTG
CasD1-rev-*Esp*3I	ACACCGTCTCGTCATAGCAGTAGTTTCTCTGC
CasD1-*Bam*HI rev	GTGCGGATCCATGTTTTGATTTATCTTGAATGG
CasD1 fwd	TATAGGTACCATGGCGGCTCTGGC
GD3-fwd	TATAGGTACCATGAGCCCCTGCGGG
GD3-6His rev	AATCGTCGACCTAATGATGATGATGATGATGTCCGGAAGTGGGCTGGAGTG

Oligonucleotides are shown in 5′–3′ orientation.

manufacturer's protocol. Sf9 cells were incubated at 27°C in Insect Express Sf9 medium (PAA Laboratories, Austria).

Flow cytometric analysis

For flow cytometric analysis of surface expression of GD3 (CD60a) and its *O*-acetylated variants 9-*O*-Ac-GD3 (CD60b) and 7-*O*-Ac-GD3 (CD60c), the following monoclonal antibodies (mAbs) were used: for CD60a: mAb R24 (IgG3 isotype), for CD60b mAb MT6004 (IgM isotype) and CD60c: U5 (GD3 isotype). mAbs R24 and U5 were a kind gift of Dr. C. Claus, University of Mainz, Germany, and were purified in our laboratory (RSA), mAb MT6004 was kindly donated by Dr. B. Kniep, University of Dresden, Germany. Preparation and the binding capacity of the mAbs have been described elsewhere (Erdmann et al., 2006). Cells (1×10^6 cells/mL) were resuspended after careful washing in PBS + 1% BSA + 0.01% NaN$_3$ and incubated with the respective mAbs for 30 min on ice. Purified mAbs (R24, U5, 1 mg/mL) were diluted 1:100 for the staining procedure and hybridoma supernatants (mAb MT6004) were applied undiluted (100 µL/cell preparation). Cells were washed three times in PBS + 1% BSA + 0.01% NaN$_3$ and incubated for further 30 min on ice with secondary anti-mouse IgG/IgM antibody conjugated to fluorescein isothiocyanate (FITC) in a dilution of 1:100. Then, the preparations were washed again three times, and cells were resuspended in 300 µL of PBS. For the exclusion of dead cells, Viaprobe$^©$ (BD Biosciences Pharmingen, Germany) was added to the cell preparations according to the manufacturer's instructions shortly before cytometric measurement. Viaprobe-stained dead cells were then determined at FL3 and excluded from staining with the respective mAbs. Flow cytometric analysis was performed using an FACS Canto II (BD Biosciences Pharmingen, Germany).

Transfection and immunofluorescence microscopy

Transfection of plasmid DNA into COS cells was performed using Lipofectamine 2000 (Invitrogen, Germany) according to the manufacturer's recommendations. For transfection of 10^7 cells 10 µL of lipofectamine and 10 µg of plasmid DNA were used. After transfection, cells were incubated for 3–5 days at 37°C/5% CO$_2$ until further investigations were performed.

Transfection efficiency was determined by fluorescence microscopy. For immunofluorescence, mAb U5 was used in a 1:50 dilution and mAb UM4D4 was diluted 1:300. mAb STG against the human ST6Gal1 was produced by one of the authors (Cao et al. 2002).

RNA isolation and cDNA synthesis

Cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). Total RNA (300 ng) was oligo(dT)-primed and first-strand cDNA synthesis was performed according to the manufacturer's guidelines (Super Script^{TM} First-Strand Synthesis System for RT–PCR; Invitrogen).

Real-time quantitative RT–PCR

For the quantification of *CasD1* mRNA expression, cDNA samples were analyzed by real-time quantitative PCR. A total of 125 ng of cDNA was amplified in 25 µL using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in the presence of 900 nmol of the specific *CasD1* primers (fwd: gtggatttctgtggcatcc, rev: aagcgcttcactgctaccat) using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). For GD3 synthase, PCR was performed with primers ST8Sia1 fw (gcgatgcaatctccctcct) and ST8Sia1 rev (ttgccgaattatgctgggat). Oligonucleotide specificity, synthesized by MWG Biotech (Ebersberg, Germany), was computer-tested (BLAST, NCBI) by homology search with the human genome. Samples were run in triplicate and experiments were repeated twice. The thermal profile for the reaction was 2 min at 50°C, followed by 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. In order to exclude unspecific amplification, dissociation curve analysis and agarose gel electrophoresis of the PCR products were performed at the end of the run. The endogenous reference gene β-actin was chosen for normalization. Primers were β-actin fw (gctcctcctgagcgcaag) and β-actin rev (catctgc tggaaggtggaca). Relative gene expression was calculated using the comparative C_t method (Livak and Schmittgen 2001).

siRNA assays

For transient siRNA transfection, we used the ON-TARGETplus SMARTpool siRNA system of Dharmacon (Bonn, Germany) containing four target sequences (GAUGGAGGUUAGACCG UUA; CGUAAUGCUCAUCGGAAGA; UAGAGAACAAA CAGACGAA; GGAUAUGCCCGUUCAGUUU) and an ON-TARGETplus negative-control non-targeting siRNA. Before transfection, cells were cultured for 2 days and brought to approximately 70% confluency. Transfection was performed using the AMAXA nucleofector kit V, programme T27 according to the manufacturer's instructions (Amaxa, Cologne, Germany). After 24 h of cultivation, RT–PCR analysis for the suppression of the *CasD1* gene was performed. In addition, effective inhibition of CD60c expression as a consequence of *CasD1* gene inhibition was monitored by flow cytometric analysis as described in Results.

Conflict of interest statement

None declared.

Abbreviations

AcCoA, acetyl-coenzyme A, *CasD1*, capsule structure1 domain containing 1; Cas1p, Cas1 protein; eGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody; *O*-Ac-GD3, *O*-acetylated ganglioside GD3; 7-*O*-Ac-GD3,

7-*O*-acetylated ganglioside GD3; 9-*O*-Ac-GD3, 9-*O*-acetylated ganglioside GD3; RT–PCR, reverse transcription–polymerase chain reaction; SGNH, serine–glycine–asparagine–histidine; siRNA, small interfering RNA; SOAT, sialate-*O*-acetyltransferase; ST6Gal1, β-galactosamide α-2,6-sialyltransferase 1; ST8Sia1, α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 1; TGN, *trans*-Golgi network

References

Ashwell G, Harford J. 1982. Carbohydrate-specific receptors of the liver. *Annu Rev Biochem*. 51:531–554.

Babak T, Deveale B, Armour C, Raymond C, Cleary MA, van der Kooy D, Johnson JM, Lim LP. 2008. Global survey of genomic imprinting by transcriptome sequencing. *Curr Biol*. 18:1735–1741.

Bergfeld AK, Claus H, Lorenzen NK, Spielmann F, Vogel U, Muhlenhoff M. 2009. The polysialic acid-specific O-acetyltransferase OatC from Neisseria meningitidis serogroup C evolved apart from other bacterial sialate O-acetyltransferases. *J Biol Chem*. 284:6–16.

Blum AS, Barnstable CJ. 1987. O-Acetylation of a cell-surface carbohydrate creates discrete molecular patterns during neural development. *Proc Natl Acad Sci USA*. 84:8716–8720.

Bratosin D, Mazurier J, Debray H, Lecocq M, Boilly B, Alonso C, Moisei M, Motas C, Montreuil J. 1995. Flow cytofluorimetric analysis of young and senescent human erythrocytes probed with lectins. Evidence that sialic acids control their life span. *Glycoconj J*. 12:258–267.

Burgon PG, Stanton PG, Pettersson K, Robertson DM. 1997. Effect of desialylation of highly purified isoforms of human luteinizing hormone on their bioactivity in vitro, radioreceptor activity and immunoactivity. *Reprod Fertil Dev*. 9:501–508.

Byrd JC, Bresalier RS. 2004. Mucins and mucin binding proteins in colorectal cancer. *Cancer Metastasis Rev*. 23:77–99.

Campbell F, Appleton MA, Fuller CE, Greeff MP, Hallgrimsson J, Katoh R, Ng OL, Satir A, Williams GT, Williams ED. 1994. Racial variation in the O-acetylation phenotype of human colonic mucosa. *J Pathol*. 174:169–174.

Cariappa A, Takematsu H, Liu H, Diaz S, Haider K, Boboila C, Kalloo G, Connole M, Shi HN, Varki N, Varki A, Pillai S. 2009. B cell antigen receptor signal strength and peripheral B cell development are regulated by a 9-O-acetyl sialic acid esterase. *J Exp Med*. 206:125–138.

Cao Y, Merling A, Crocker PR, Keller R, Schwartz-Albiez R. 2002. Differential expression of beta-galactoside alpha2,6 sialyltransferase and sialoglycans in normal and cirrhotic liver and hepatocellular carcinoma. *Lab Invest*. 82:1515–1524.

Chen HY, Challa AK, Varki A. 2006. 9-O-Acetylation of exogenously added ganglioside GD3. The GD3 molecule induces its own O-acetylation machinery. *J Biol Chem*. 281:7825–7833.

Chen TL, Chen C, Bergeron NQ, Close BE, Bohrer TJ, Vertel BM, Colley KJ. 2003. The two rat alpha 2,6-sialyltransferase (ST6Gal I) isoforms: Evaluation of catalytic activity and intra-Golgi localization. *Glycobiology*. 13:109–117.

Chen HY, Varki A. 2002. O-Acetylation of GD3: An enigmatic modification regulating apoptosis? *J Exp Med*. 196:1529–1533.

Cheresh DA, Reisfeld RA, Varki AP. 1984. O-Acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant. *Science*. 225:844–846.

Cheresh DA, Varki AP, Varki NM, Stallcup WB, Levine J, Reisfeld RA. 1984. A monoclonal antibody recognizes an O-acylated sialic acid in a human melanoma-associated ganglioside. *J Biol Chem*. 259:7453–7459.

Constantine-Paton M, Blum AS, Mendez-Otero R, Barnstable CJ. 1986. A cell surface molecule distributed in a dorsoventral gradient in the perinatal rat retina. *Nature*. 324:459–462.

Corfield AP, Myerscough N, Warren BF, Durdey P, Paraskeva C, Schauer R. 1999. Reduction of sialic acid O-acetylation in human colonic mucins in the adenoma–carcinoma sequence. *Glycoconj J*. 16:307–317.

Corfield AP, Sander-Wewer M, Veh RW, Wember M, Schauer R. 1986. The action of sialidases on substrates containing O-acetylsialic acids. *Biol Chem Hoppe Seyler*. 367:433–439.

Corfield AP, Wagner SA, Clamp JR, Kriaris MS, Hoskins LC. 1992. Mucin degradation in the human colon: Production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. *Infect Immun*. 60:3971–3978.

Corfield AP, Wagner SA, O'Donnell LJ, Durdey P, Mountford RA, Clamp JR. 1993. The roles of enteric bacterial sialidase, sialate O-acetyl esterase and glycosulfatase in the degradation of human colonic mucin. *Glycoconj J*. 10:72–81.

Crocker PR, Paulson JC, Varki A. 2007. Siglecs and their roles in the immune system. *Nat Rev Immunol*. 7:255–266.

Crocker PR, Varki A. 2001. Siglecs in the immune system. *Immunology*. 103:137–145.

Das R, Hampton DD, Jirtle RL. 2009. Imprinting evolution and human health. *Mamm Genome*. 20:563–572.

de Groot RJ. 2006. Structure, function and evolution of the hemagglutinin-esterase proteins of corona- and toroviruses. *Glycoconj J*. 23:59–72.

De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, Rippo MR, Testi R. 1997. Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis [published erratum appears in Science 1998 Apr 17;280(5362):363]. *Science*. 277:1652–1655.

Deszo EL, Steenbergen SM, Freedberg DI, Vimr ER. 2005. *Escherichia coli* K1 polysialic acid O-acetyltransferase gene, neuO, and the mechanism of capsule form variation involving a mobile contingency locus. *Proc Natl Acad Sci USA*. 102:5564–5569.

Diaz S, Higa HH, Varki A. 1989. Glycoprotein sialate 7(9)-O-acetyltransferase from rat liver Golgi vesicles. *Methods Enzymol*. 179:416–422.

Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA. 2003. Darbepoetin alfa has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. *Exp Hematol*. 31:290–299.

Erdmann M, Wipfler D, Merling A, Cao Y, Claus C, Kniep B, Sadick H, Bergler W, Vlasak R, Schwartz-Albiez R. 2006. Differential surface expression and possible function of 9-*O*- and 7-*O*-acetylated GD3 (CD60 b and c) during activation and apoptosis of human tonsillar B and T lymphocytes. *Glycoconj J*. 23:627–638.

Flahaut M, Meier R, Coulon A, Nardou KA, Niggli FK, Martinet D, Beckmann JS, Joseph JM, Muhlethaler-Mottet A, Gross N. 2009. The Wnt receptor FZD1 mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway. *Oncogene*. 28:2245–2256.

Fukuda MN, Sasaki H, Lopez L, Fukuda M. 1989. Survival of recombinant erythropoietin in the circulation: The role of carbohydrates. *Blood*. 73:84–89.

Ghosh S, Bandyopadhyay S, Mallick A, Pal S, Vlasak R, Bhattacharya DK, Mandal C. 2005. Interferon gamma promotes survival of lymphoblasts overexpressing 9-*O*-acetylated sialoglycoconjugates in childhood acute lymphoblastic leukaemia (ALL). *J Cell Biochem*. 95:206–216.

Grabowski M, Zimprich A, Lorenz-Depiereux B, Kalscheuer V, Asmus F, Gasser T, Meitinger T, Strom TM. 2003. The epsilon-sarcoglycan gene (SGCE), mutated in myoclonus-dystonia syndrome, is maternally imprinted. *Eur J Hum Genet*. 11:138–144.

Hayes BK, Varki A. 1989. O-acetylation and de-O-acetylation of sialic acids. Sialic acid esterases of diverse evolutionary origins have serine active sites and essential arginine residues. *J Biol Chem*. 264:19443–19448.

Hellebo A, Vilas U, Falk K, Vlasak R. 2004. Infectious salmon anemia virus specifically binds to and hydrolyzes 4-*O*-acetylated sialic acids. *J Virol*. 78:3055–3062.

Henkin J, Dudlak D, Beebe DP, Sennello L. 1991. High sialic acid content slows prourokinase turnover in rabbits. *Thromb Res*. 63:215–225.

Herrler G, Rott R, Klenk HD, Muller HP, Shukla AK, Schauer R. 1985. The receptor-destroying enzyme of influenza C virus is neuraminate-O-acetylesterase. *EMBO J*. 4:1503–1506.

Higa HH, Butor C, Diaz S, Varki A. 1989. O-acetylation and de-O-acetylation of sialic acids. O-acetylation of sialic acids in the rat liver Golgi apparatus involves an acetyl intermediate and essential histidine and lysine residues—a transmembrane reaction? *J Biol Chem*. 264:19427–19434.

Houliston RS, Endtz HP, Yuki N, Li J, Jarrell HC, Koga M, van Belkum A, Karwaski MF, Wakarchuk WW, Gilbert M. 2006. Identification of a sialate O-acetyltransferase from *Campylobacter jejuni*: Demonstration of direct transfer to the C-9 position of terminalalpha-2, 8-linked sialic acid. *J Biol Chem*. 281:11480–11486.

Iwersen M, Dora H, Kohla G, Gasa S, Schauer R. 2003. Solubilisation and properties of the sialate-4-O-acetyltransferase from guinea pig liver. *Biol Chem*. 384:1035–1047.

Iwersen M, Vandamme-Feldhaus V, Schauer R. 1998. Enzymatic 4-O-acetylation of N-acetylneuraminic acid in guinea-pig liver. *Glycoconj J*. 15:895–904.

Janbon G, Himmelreich U, Moyrand F, Improvisi L, Dromer F. 2001. Cas1p is a membrane protein necessary for the O-acetylation of the *Cryptococcus neoformans* capsular polysaccharide. *Mol Microbiol*. 42:453–467.

Kamerling JP, Schauer R, Shukla AK, Stoll S, Van Halbeek H, Vliegenthart JF. 1987. Migration of *O*-acetyl groups in *N,O*-acetylneuraminic acids. *Eur J Biochem*. 162:601–607.

Kanamori A, Nakayama J, Fukuda MN, Stallcup WB, Sasaki K, Fukuda M, Hirabayashi Y. 1997. Expression cloning and characterization of a cDNA encoding a novel membrane protein required for the formation of O-acetylated ganglioside: A putative acetyl-CoA transporter. *Proc Natl Acad Sci USA*. 94:2897–2902.

Kniep B, Claus C, Peter-Katalinic J, Monner DA, Dippold W, Nimtz M. 1995. 7-*O*-Acetyl-GD3 in human T-lymphocytes is detected by a specific T-cell-activating monoclonal antibody. *J Biol Chem*. 270:30173–30180.

Kniep B, Kniep E, Ozkucur N, Barz S, Bachmann M, Malisan F, Testi R, Rieber EP. 2006. 9-*O*-Acetyl GD3 protects tumor cells from apoptosis. *Int J Cancer*. 119:67–73.

Kohla G, Stockfleth E, Schauer R. 2002. Gangliosides with O-acetylated sialic acids in tumors of neuroectodermal origin. *Neurochem Res*. 27:583–592.

Krishna M, Varki A. 1997. 9-O-Acetylation of sialomucins: A novel marker of murine CD4T cells that is regulated during maturation and activation. *J Exp Med*. 185:1997–2013.

Lee SJ, Evers S, Roeder D, Parlow AF, Risteli J, Risteli L, Lee YC, Feizi T, Langen H, Nussenzweig MC. 2002. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science*. 295:1898–1901.

Lee HJ, Rakic B, Gilbert M, Wakarchuk WW, Withers SG, Strynadka NC. 2009. Structural and kinetic characterizations of the polysialic acid O-acetyltransferase OatWY from *Neisseria meningitidis*. *J Biol Chem*. 284:24501–24511.

Levine JM, Beasley L, Stallcup WB. 1986. Localization of a neurectoderm-associated cell surface antigen in the developing and adult rat. *Brain Res*. 392:211–222.

Lewis AL, Hensler ME, Varki A, Nizet V. 2006. The group B streptococcal sialic acid O-acetyltransferase is encoded by neuD, a conserved component of bacterial sialic acid biosynthetic gene clusters. *J Biol Chem*. 281:11186–11192.

Lewis AL, Nizet V, Varki A. 2004. Discovery and characterization of sialic acid O-acetylation in group B Streptococcus. *Proc Natl Acad Sci USA*. 101:11123–11128.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 25:402–408.

Lrhorfi LA, Srinivasan GV, Schauer R. 2007. Properties and partial purification of sialate-O-acetyltransferase from bovine submandibular glands. *Biol Chem*. 388:297–306.

Malisan F, Franchi L, Tomassini B, Ventura N, Condo I, Rippo MR, Rufini A, Liberati L, Nachtigall C, Kniep B, et al. 2002. Acetylation suppresses the proapoptotic activity of GD3 ganglioside. *J Exp Med*. 196:1535–1541.

Mandal C, Srinivasan V, Chowdhury S, Chandra S, Mandal C, Schauer R, Mandal C. 2009. High level of sialate-O-acetyltransferase activity in lymphoblasts of childhood acute lymphoblastic leukaemia (ALL): Enzyme characterization and correlation with disease status. *Glycoconj J*. 26:57–73.

Martinuk SD, Manning AW, Black WD, Murphy BD. 1991. Effects of carbohydrates on the pharmacokinetics and biological activity of equine chorionic gonadotropin in vivo. *Biol Reprod*. 45:598–604.

Muchmore EA, Varki A. 1987. Selective inactivation of influenza C esterase: A probe for detecting 9-*O*-acetylated sialic acids. *Science*. 236:1293–1295.

Muchmore EA, Varki NM, Fukuda M, Varki A. 1987. Developmental regulation of sialic acid modifications in rat and human colon. *FASEB J*. 1:229–235.

Müller E, Franco MW, Schauer R. 1981. Involvement of membrane galactose in the in vivo and in vitro sequestration of desialylated erythrocytes. *Hoppe Seylers Z Physiol Chem*. 362:1615–1620.

Ogura K, Nara K, Watanabe Y, Kohno K, Tai T, Sanai Y. 1996. Cloning and expression of cDNA for O-acetylation of GD3 ganglioside. *Biochem Biophys Res Commun*. 225:932–938.

Ono R, Kobayashi S, Wagatsuma H, Aisaka K, Kohda T, Kaneko-Ishino T, Ishino F. 2001. A retrotransposon-derived gene, PEG10, is a novel imprinted gene located on human chromosome 7q21. *Genomics*. 73:232–237.

Ono R, Shiura H, Aburatani H, Kohda T, Kaneko-Ishino T, Ishino F. 2003. Identification of a large novel imprinted gene cluster on mouse proximal chromosome 6. *Genome Res*. 13:1696–1705.

Pal S, Chatterjee M, Bhattacharya DK, Bandhyopadhyay S, Mandal C. 2001. *O*-Acetyl sialic acid specific IgM in childhood acute lymphoblastic leukaemia. *Glycoconj J*. 18:529–537.

Perlman S, van den Hazel B, Christiansen J, Gram-Nielsen S, Jeppesen CB, Andersen KV, Halkier T, Okkels S, Schambye HT. 2003. Glycosylation of an N-terminal extension prolongs the half-life and increases the in vivo activity of follicle stimulating hormone. *J Clin Endocrinol Metab*. 88:3227–3235.

Pillai S, Cariappa A, Pirnie SP. 2009. Esterases and autoimmunity: The sialic acid acetylesterase pathway and the regulation of peripheral B cell tolerance. *Trends Immunol*. 30:488–493.

Regl G, Kaser A, Iwersen M, Schmid H, Kohla G, Strobl B, Vilas U, Schauer R, Vlasak R. 1999. The hemagglutinin-esterase of mouse hepatitis virus strain S is a sialate-4-O-acetylesterase. *J Virol*. 73:4721–4727.

Rippo MR, Malisan F, Ravagnan L, Tomassini B, Condo I, Costantini P, Susin SA, Rufini A, Todaro M, Kroemer G, et al. 2000. GD3 ganglioside directly targets mitochondria in a bcl-2-controlled fashion. *FASEB J*. 14:2047–2054.

Satake H, Chen HY, Varki A. 2003. Genes modulated by expression of GD3 synthase in Chinese hamster ovary cells. Evidence that the Tis21 gene is involved in the induction of GD3 9-O-acetylation. *J Biol Chem*. 278:7942–7948.

Schauer R. 1970a. Biosynthesis of *N*-acetyl-*O*-acetylneuraminic acids. I. Incorporation of (14C) acetate into sections of the submaxillary salivary gland of ox and horse. *Hoppe Seylers Z Physiol Chem*. 351:595–602.

Schauer R. 1970b. Biosynthesis of *N*-acetyl-*O*-acetylneuraminic acids. II. Substrate and intracellular localization of bovine acetyl-coenzyme A: *N*-acetylneuraminate-7- and 8-O-acetyltransferase. *Hoppe Seylers Z Physiol Chem*. 351:749–758.

Schauer R. 2004. Sialic acids: Fascinating sugars in higher animals and man. *Zoology (Jena)*. 107:49–64.

Schauer R. 2009. Sialic acids as regulators of molecular and cellular interactions. *Curr Opin Struct Biol*. 19:507–514.

Schauer R, Kamerling JP. 1997. Chemistry, biochemistry and biology of sialic acids. In: Montreuil J, Vliegenthart JFG, et al. editors. *Glycoproteins II*. Amsterdam: Elsevier, p. 243–402.

Schauer R, Srinivasan GV, Wipfler D, Kniep B, Schwartz-Albiez R. 2010. O-Acetylated sialic acids and their role in immune defence. In: Wu AM, editor. *Molecular Immunology of Complex Carbohydrates-3 (MICC-3), Adv. Exp. Med. Biol.*, New York: Kluwer Academic/Plenum Publishers, in press.

Schultze B, Gross HJ, Brossmer R, Herrler G. 1991. The S protein of bovine coronavirus is a hemagglutinin recognizing 9-*O*-acetylated sialic acid as a receptor determinant. *J Virol*. 65:6232–6237.

Schwartz-Albiez R, Merling A, Martin S, Haas R, Gross HJ. 2004. Cell surface sialylation and ecto-sialyltransferase activity of human CD34 progenitors from peripheral blood and bone marrow. *Glycoconj J*. 21:451–459.

Shen Y, Kohla G, Lrhorfi AL, Sipos B, Kalthoff H, Gerwig GJ, Kamerling JP, Schauer R, Tiralongo J. 2004. O-Acetylation and de-O-acetylation of sialic acids in human colorectal carcinoma. *Eur J Biochem*. 271:281–290.

Shi WX, Chammas R, Varki A. 1996a. Linkage-specific action of endogenous sialic acid O-acetyltransferase in Chinese hamster ovary cells. *J Biol Chem*. 271:15130–15138.

Shi WX, Chammas R, Varki A. 1996b. Regulation of sialic acid 9-O-acetylation during the growth and differentiation of murine erythroleukemia cells. *J Biol Chem*. 271:31517–31525.

Shi WX, Chammas R, Varki A. 1998. Induction of sialic acid 9-O-acetylation by diverse gene products: Implications for the expression cloning of sialic acid O-acetyltransferases. *Glycobiology*. 8:199–205.

Shi WX, Chammas R, Varki NM, Powell L, Varki A. 1996c. Sialic acid 9-O-acetylation on murine erythroleukemia cells affects complement activation, binding to I-type lectins, and tissue homing. *J Biol Chem*. 271:31526–31532.

Sjoberg ER, Manzi AE, Khoo KH, Dell A, Varki A. 1992. Structural and immunological characterization of O-acetylated GD2. Evidence that GD2 is an acceptor for ganglioside O-acetyltransferase in human melanoma cells. *J Biol Chem*. 267:16200–16211.

Sjoberg ER, Varki A. 1993. Kinetic and spatial interrelationships between ganglioside glycosyltransferases and O-acetyltransferase(s) in human melanoma cells. *J Biol Chem*. 268:10185–10196.

Smits SL, Gerwig GJ, van Vliet AL, Lissenberg A, Briza P, Kamerling JP, Vlasak R, de Groot RJ. 2005. Nidovirus sialate-O-acetylesterases: Evolution and substrate specificity of coronaviral and toroviral receptor-destroying enzymes. *J Biol Chem*. 280:6933–6941.

Srinivasan GV, Schauer R. 2009. Assays of sialate-O-acetyltransferases and sialate-O-acetylesterases. *Glycoconj J*. 26:935–944.

Steenbergen SM, Lee YC, Vann WF, Vionnet J, Wright LF, Vimr ER. 2006. Separate pathways for O acetylation of polymeric and monomeric sialic acids and identification of sialyl O-acetyl esterase in *Escherichia coli* K1. *J Bacteriol*. 188:6195–6206.

Surolia I, Pirnie SP, Chellappa V, Taylor KN, Cariappa A, Moya J, Liu H, Bell DW, Driscoll DR, Diederichs S, et al. 2010. Functionally defective germline variants of sialic acid acetylesterase in autoimmunity. *Nature*. 466:243–247.

Szkudlinski MW, Thotakura NR, Tropea JE, Grossmann M, Weintraub BD. 1995a. Asparagine-linked oligosaccharide structures determine clearance and organ distribution of pituitary and recombinant thyrotropin. *Endocrinology*. 136:3325–3330.

Szkudlinski MW, Thotakura NR, Weintraub BD. 1995b. Subunit-specific functions of N-linked oligosaccharides in human thyrotropin: role of terminal residues of alpha- and beta-subunit oligosaccharides in metabolic clearance and bioactivity. *Proc Natl Acad Sci USA*. 92:9062–9066.

Tiralongo J, Schmid H, Thun R, Iwersen M, Schauer R. 2000. Characterisation of the enzymatic 4-O-acetylation of sialic acids in microsomes from equine submandibular glands. *Glycoconj J*. 17:849–858.

Vandamme-Feldhaus V, Schauer R. 1998. Characterization of the enzymatic 7-O-acetylation of sialic acids and evidence for enzymatic O-acetyl migration from C-7 to C-9 in bovine submandibular gland. *J Biochem (Tokyo)*. 124:111–121.

Varki A. 1997. Sialic acids as ligands in recognition phenomena. *FASEB J*. 11:248–255.

Varki A. 2007. Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. *Nature*. 446:1023–1029.

Varki A, Hooshmand F, Diaz S, Varki NM, Hedrick SM. 1991. Developmental abnormalities in transgenic mice expressing a sialic acid-specific 9-O-acetylesterase. *Cell*. 65:65–74.

Varki A, Kornfeld S. 1980. An autosomal dominant gene regulates the extent of 9-O-acetylation of murine erythrocyte sialic acids. A probable explanation for the variation in capacity to activate the human alternate complement pathway. *J Exp Med*. 152:532–544.

Varki A, Schauer R. 2009. Sialic acids. In: Varki A, Cummings RD, et al. editors. *Essentials of Glycobiology*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, p. 199–217.

Vimr ER, Steenbergen SM. 2006. Mobile contingency locus controlling *Escherichia coli* K1 polysialic acid capsule acetylation. *Mol Microbiol*. 60:828–837.

Vlasak R, Krystal M, Nacht M, Palese P. 1987. The influenza C virus glycoprotein (HE) exhibits receptor-binding (hemagglutinin) and receptor-destroying (esterase) activities. *Virology*. 160:419–425.

Vlasak R, Luytjes W, Spaan W, Palese P. 1988. Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. *Proc Natl Acad Sci USA*. 85:4526–4529.

Vlasak R, Muster T, Lauro AM, Powers JC, Palese P. 1989. Influenza C virus esterase: Analysis of catalytic site, inhibition, and possible function. *J Virol*. 63:2056–2062.