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 properties 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 structure 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-neuraminic α-2,8-sialyltransferase 1 (GD3 synthase) resulted in an up to 40% increased biosynthesis of 7-O-acetylated ganglioside GD3. By quantitative realtime 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 positions. Until now, approximately 50 different sialic acid derivatives have been isolated and characterized from natural sources. An overview on the biosynthesis, biology and diversity 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 erythrocytes are then sequestered by galactose-specific receptors (Mueller et al. 1981; Bratosin et al. 1995), e.g. the asialoglycoprotein receptor in the liver (Ashwell and Harford 1982) or similar receptors on macrophages. Different glycoforms of therapeutics 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 erythropoietin (Fukuda et al. 1989). In contrast, the introduction of additional glycosylation sites into the protein backbone of erythropoietin 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...
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 (Herrier et al. 1985; Muchmore and Varki 1987; Vlasak et al. 1987), coronaviruses (Vlasak et al. 1988; Schultz et al. 1991; Regl et al. 1999; Smits et al. 2005), infectious salmon anemia virus (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 (Lhrorfi 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 structure 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 glucuronoxylanomannans (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-acetylttransferases, 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. 2010).
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(1989) and for cellular sialic acid-specific O-acetyltransferases (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: pCasD1-eGFP 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 determine the intracellular location of Cas1p. Therefore, we transfected COS cells with plasmid pCasD1-eGFP 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 pGD3 synth Histag. 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 pGD3 synth Histag alone or double transfected with pGD3 synth Histag 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).
express signifi-
cant 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 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-CD2 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 that express high levels of CasD1, the amount of CD60b-expressing cells is 30% lower (data not shown) as a result of CasD1 downregulation by specific siRNA transfection.
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 genes encoding O-acetyl transferases were identified in human-pathogenic *E. coli* K1 (Deszo et al. 2005; Steenbergen et al. 2006; Vinr and Steenbergen 2006), *Campylobacter jejuni* (Houlston 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 submandibulary 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 3H-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 acetyltransferase activity turned out as a major hurdle towards future...
Co-expression of Cas1p and ST8Sia1 (GD3 synthase) resulted in increased similarity to viral sialic acid-specific marker enzyme for TGN (Chen et al. 2003). Furthermore, the TGN as shown by partial colocalization with ST6Gal1, a carbohydrate acetyl-CoA transporter/antiporter. Our data indicate that it is due to the human Cas1p fulfills the requirements for an O-acetyltransferase activity 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; Knipe 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 submandibulary 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 SOAT. 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 differentially 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 extraembryonic 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 reviewed recently (Das et al. 2009).
been published, and no human genetic disease has yet been described to be associated. In summary, current evidence indicates thatCasD1in postnatal mice is predominantly expressed from the maternal allele. Mapping of the adult mouse brain showed high expression levels ofCasD1in 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. sialyltransferrase or other glycosyltransferases within the Golgi, or cytoplasmic proteins that may modulate the transport of AccO 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 encodingCasD1, sialyl-2,6-Gal-transferase 1 and sialyl-2,8-Sia-transferase 1 (GD3 synthase)
The clones with theCasD1cDNA (IMAGE clone 5286382) and the cDNA forST8Sia1 (IMAGE clone 40125836) were obtained from Geneservice Ltd. (UK), the cDNA clone forST6Gal1 (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 Cas1fwd and Cas1rev–3I and was generated with primersCas IF fwd and Cas stop IF rev. The resulting PCR product was digested withAcc65I andBamHIand ligated into theAcc65I/BamHIwindow 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 withAcc65I andSalIand inserted into theAcc65I/SalIwindow of plasmid pC1 (Promega, Germany), resulting in plasmid pGD3synthHistag.

Cells and cell lines
COS-1 and COS-7 cells were cultured as monolayer at 37°C/5% CO2 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 tonsilary 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 withAcc65I andNodI to obtain a fragment representingCasD1-eGFP. This fragment was ligated into the baculovirus transfer vector pBacPAK 8 (Takara Bio Europe/Clontech, France), resulting in the constructpBacPAK 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 S9 cells using Cellfectin (Invitrogen, Karlsruhe, Germany) according to the

| Table I. List of primers used for cloning ofCasD1andST8Sia1cDNA into expression vectors |
|---------------------------------------------------------------|
| Cas IF fwd | TGCAGTCGACGTACCATGGCGCGTCTGCG |
| Cas stop IF rev | TGGTGGCGATGACCTTAAATGTGGTAAT |
| CasD1-fwd2–3I | TAGCCGTCCTCCATGATTCTTATGATG |
| CasD1-rev–3I | ACCCGTCTCGCATAGCGATGTGGTTCGCT |
| CasD1–BamHI rev | GTGCGGATCGCTAGTTGATTCTGTGAG |
| CasD1 fwd | TATAGTACATGGCGGCTCTGCG |
| GD3–fwd | TATAGTACATGCGGCCTGCG |
| GD3–6His rev | AACCTGCGACCTTAATGATGATGATGATGATGATGACGTCGGGAGT |

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 (1×10⁶ cells/mL) were resuspended after careful washing in PBS + 1% BSA + 0.01% NaN₃ and incubated 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% NaN₃ 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 Pharminster, 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 Pharminster, 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 10⁵ 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% CO₂ until further investigations were performed.

Transfection efficiency was determined by fluorescence microscopy. For immunofluorescence microscopy, 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™ 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: gttggattttggtggtgcc, rev: aagcetactgactcctcct) using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). For GD3 synthase, PCR was performed with primers ST8Sia1 fw (gcgcattctctcctcctt) and ST8Sia1 rev (ttggcggatactctctgct). 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 (gcgcctctgccgccag) and β-actin rev (ctctg gagaacctcca). Relative gene expression was calculated using the comparative C₇ 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; CGUAAUGCUCAGGAAGA; UAGAGAACAAA CAGACGAA; GGAUAUGCCCUGACGUUU) and an ON-TARGETplus negative-control non-targeting siRNA. Before transfection, cells were cultured for 2 days and brought to approximately 70% confluence. 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.

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**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; sRNA, small interfering RNA; SOAT, sialate-O-acetyltansf erase; ST6Gal1, β-galactosamidase α-2,6-sialyltransferase 1; ST8Sia1, α-N-acetylneuraminide α-2,8-sialyltransferase 1; TGN, trans-Golgi network

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