Cytidine Monophospho-*N*-Acetylneuraminic Acid Hydroxylase (CMAH)

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N. Taniguchi et al. (eds.), *Handbook of Glycosyltransferases and Related Genes*,
DOI 10.1007/978-4-431-54240-7_133,

Introduction

Sialic acids are a family of more than 50 naturally occurring acidic nine-carbon backbone monosaccharides. The predominant sialic acids in mammals are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), which commonly occupy the terminal positions of various glycan chains (Varki and Schauer 2009). The only known biosynthetic pathway for generation of Neu5Gc takes place in the cytosol and is catalyzed by the cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (Cmah). The Cmah enzyme acts only at the level of activated sugars and catalyzes the conversion of the precursor molecule CMP-Neu5Ac to CMP-Neu5Gc involving a complex mechanism with cofactors NADH, cytochrome b5, b5 reductase, iron, and molecular oxygen (Shaw and Schauer 1988; Muchmore et al. 1989; Kozutsumi et al. 1990; Shaw et al. 1992, 1994; Kawano et al. 1993, 1995; Schneckenburger et al. 1994; Takematsu et al. 1994; Schlenzka et al. 1996). The Cmah enzyme is conserved among animals of the deuterostome lineage (vertebrates and "higher" invertebrates), but no homologous enzymes are known in any other eukaryotic taxa (Varki 2009). Only distantly related bacterial and plant hydroxylases were predicted to have some degree of structural similarity to Cmah (Schmidt and Shaw 2001). Interestingly, *N*-glycolyl groups are very rare in nature. Besides the Cmah enzyme, only one bacterial enzyme is known to be capable of generating *N*-glycolyl groups in the form of *N*-glycolylmuramic acid in mycobacteria (Raymond et al. 2005). Notably, the responsible gene *namH* shows distant homology to *CMAH* and acts on a nucleotide sugar (UDP-*N*-acetylmuramic acid) as well.

Exclusively in humans, the single-copy *CMAH* gene is inactivated by an Alu-mediated genomic deletion of a 92 bp exon (Chou et al. 1998, 2002; Irie et al. 1998; Hayakawa et al. 2006). The complete absence of Neu5Gc in a $Cmah^{-/-}$ mouse model with the humanlike defect in *Cmah* indicates that humans entirely lost Neu5Gc *de novo* biosynthesis and that there is no alternate pathway to synthesize it (Hedlund et al. 2007; Bergfeld et al. 2012b). However, small amounts of Neu5Gc have been shown to be present in various human carcinomas, sites of inflammation, fetal meconium, and in lower extent even in normal human tissues (Malykh et al. 2001; Tangvoranuntakul et al. 2003; Diaz et al. 2009). This is because human metabolism recognizes nonhuman Neu5Gc as "self" and incorporates this sialic acid from Neu5Gc-rich dietary sources such as red meats into human cell surface glycoconjugates (Tangvoranuntakul et al. 2003; Bardor et al. 2005; Banda et al. 2012). In striking contrast, the human immune system recognizes Neu5Gc-containing glycan structures as "foreign," and all humans tested to date have a circulating polyclonal anti-Neu5Gc antibody repertoire (Tangvoranuntakul et al. 2003; Nguyen et al. 2005; Padler-Karavani et al. 2008). The incorporation of exogenous Neu5Gc from dietary sources in the face of an immune response against this nonhuman structure makes Neu5Gc the first known example of a "xeno-autoantigen" (Padler-Karavani et al. 2008; Varki et al. 2011). The resulting chronic inflammation ("xenosialitis") has been proposed to be a novel human-specific pathologic mechanism. In atherosclerotic tissues, the xeno-autoantigen Neu5Gc

was identified in endothelium overlying plaques and in subendothelial regions, providing multiple pathways for accelerating inflammation in this disease (Pham et al. 2009). The enhanced accumulation of Neu5Gc in human cancers in the face of circulating anti-Neu5Gc antibodies was also found to accelerate tumor progression by the resulting low-grade chronic inflammation in Neu5Gc-deficient *Cmah*$^{-/-}$ mice (Hedlund et al. 2008).

Databanks

IUBMB enzyme nomenclature (Enzyme Commission Number), E.C. 1.14.18.2 (formerly 1.14.13.45); CAS registry number, 116036-67-0. No PDB numbers are currently available for Cmah enzymes as no structures have been reported to date.

Cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH)

Species	Gene symbol	GenBank accession number	UniProt ID
Mus musculus	*Cmah*	NM_001111110, NM_007717	Q61419, Q3TLI9
Rattus norvegicus	*Cmah*	NM_001024273 XM_341528	Q501W3
Cricetulus griseus	*Cmah*	XM_003502203	Q9WV23
Felis catus	*CMAH*	NM_001244985	A6Y8N2
Sus scrofa	*CMAH*	NM_001113015, NP_001106486	A8WAC5
Macaca mulatta	*CMAH*	NM_001032856	Q9TUJ2
Pan paniscus	*CMAH*	AF494221, AAN05316, XM_003823210	Q8MJD0
Gorilla gorilla gorilla	*CMAH*	AF494222, AAN05317, AAN05318, AAL56239	Q8MJC8
Pongo pygmaeus	*CMAH*	AF494224, AAN05320, AAL56238	Q8MJC6
Pan troglodytes	*CMAH*	NP_001009041	O97598
Homo sapiens	*CMAHP* (pseudogene)	NR_002174.2	Q9Y471
Asterias rubens	*cmah*	AJ308602, CAC67678	Q95V11
Danio rerio	*cmah*	NM_001002192	Q6GML1
Xenopus laevis	*cmahp* (pseudogene)	NM_001086828	Q8AVF5

Name and History

Names and Synonyms

The systematic name is CMP-*N*-acetylneuraminate, ferrocytochrome-b5: oxygen oxidoreductase (*N*-acetyl-hydroxylating). Other names include CMP-*N*-acetylneuraminic acid hydroxylase, CMP-Neu5Ac hydroxylase, cytidine

monophospho-*N*-acetylneuraminate monooxygenase, *N*-acetylneuraminate monooxygenase, and cytidine-5′-monophosphate-*N*-acetylneuraminic acid hydroxylase. In the following we will use the common abbreviation Cmah.

Discovery of Cmah Enzymatic Activity

Many research groups have investigated the biosynthetic pathway of Neu5Gc. The discovery of the hydroxylase/monooxygenase enzyme activity involved originated from studies by Schauer and coworkers (Schauer et al. 1968; Schoop et al. 1969). Subsequent work by Kozutsumi, Schauer, Suzuki, and their coworkers unraveled the details of this enzyme, which converts CMP-Neu5Ac to CMP-Neu5Gc. The complex enzymatic reaction was also found to require various cofactors, including cytochrome b5, cytochrome b5 reductase, iron, NADH, and molecular oxygen (Schauer 1970; Shaw and Schauer 1988; Muchmore et al. 1989; Kozutsumi et al. 1991; Shaw et al. 1992, 1994; Kawano et al. 1994; Takematsu et al. 1994). Thus far, CMP-Neu5Ac hydroxylase activity has been exclusively identified in the deuterostome lineage of animals (vertebrates and the so-called "higher" invertebrates) and therewith likely occurred around the Cambrian expansion (∼500 mya (million years ago)).

Identification of the *Cmah* Gene

Although the Cmah enzymatic activity was known for decades, the *Cmah* gene was only first identified in 1995. Kawano and coworkers initially purified and characterized the Cmah enzyme from the cytosolic fraction of mouse liver to homogeneity (Kawano et al. 1994). In a subsequent study, they went on and determined the amino acid sequence of the isolated enzyme. A mouse cDNA clone was thereby obtained, which contained an open reading frame for a 577 amino acid protein with a predicted molecular mass of 66 kDa (Kawano et al. 1995). Finally, transfection of the respective cDNA construct into COS-1 cells was shown to increase Cmah activity as well as Neu5Gc levels in such cells (Kawano et al. 1995). Later on, the *Cmah* cDNAs from the starfish *Asterias rubens* (Martensen et al. 2001) and from porcine endothelial cells (Ikeda et al. 2012) were also cloned and investigated.

Identification of the Human *CMAHP* Pseudogene

Two research groups identified and reported the human *CMAH* locus to be a pseudogene. The initial publication by Irie and coworkers identified the 92 bp deletion within the human *CMAHP* mRNA using a HeLa cell cDNA library (Irie et al. 1998) and confirmed the presence of the deletion as representing a single exon of the gene within the human genome. Although the 5′region of the cDNA was incomplete, the authors predicted that this deletion would result in translation of an

N-terminally truncated 486 carboxy-terminal protein, lacking the corresponding 104 amino acid N-terminal domain of the mouse enzyme. They expressed the predicted truncated protein and showed that it displayed no residual enzymatic activity when transfected into COS cells (Irie et al. 1998). The same year, a second research group reported the identical 92 bp deletion within the human *CMAHP* cDNA (Chou et al. 1998). These authors newly identified the correct initiator methionine, which was found upstream of the 92 bp deletion. The newly identified intact primary initiator methionine codon corresponds to that within the sequences of the mouse and chimpanzee mRNAs (Chou et al. 1998). By identifying the full-length human *CMAHP* cDNA, Chou and coworkers concluded that it encoded a much shorter truncated polypeptide involving only the amino terminus of the human enzyme (Chou et al. 1998). The initially reported amino acid sequence by Irie and coworkers was thus not based on the correct translation start site. The second publication also used PCR to discover that the missing human exon was present in the genomes of the closely related great apes (chimpanzees, gorillas, and orangutans) showing that the human pseudogenization event occurred after the common ancestor of these species with humans (Chou et al. 1998). Subsequent work reported that the deletion was mediated by an *Alu-Alu* fusion event (Hayakawa et al. 2001), which occurred about 3 Mya (Chou et al. 2002) and may have been fixed in the human ancestral population about 2 Mya (Hayakawa et al. 2006) (Fig. 138.1).

Structure

The structure of Cmah enzymes has not yet been reported. A cytosolic enzyme is found in mammals, whereas a membrane-associated protein with a putative C-terminal transmembrane domain is assumed for echinoderms such as starfish (Martensen et al. 2001). By electron paramagnetic resonance (EPR) spectroscopy and analysis of the primary structure, Cmah was identified as the only iron-sulphur protein of the Rieske type found in the cytosol of eukaryotes, and a putative cytochrome b5 binding site was also predicted (Schlenzka et al. 1996). The Cmah gene is highly conserved among animals of the deuterostome lineage, but the only known homolog proteins are distantly related hydroxylases in plants and bacteria (Schmidt and Shaw 2001). The only known functionally related enzyme is the *N*-acetylmuramic acid hydroxylase (namH), which catalyzes the hydroxylation of UDP-*N*-acetylmuramic acid to UDP-*N*-glycolylmuramic acid in the actinomycete bacteria (Raymond et al. 2005). Remarkably, Cmah and namH are the only known enzymes in nature capable of synthesizing an *N*-glycolyl group.

Enzyme Activity Assay and Substrate Specificity

Cmah activity in homogenates was first investigated using radiolabeled substrates with product analysis by radio-TLC (Bergwerff et al. 1992). In this assay, 6 mg of total protein in HEPES buffer pH 6.7 was incubated for 3 h at 37 °C in the presence

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human       ATGGGCAGCATCGAACAAACAACTGAGATCCTGTTGTGCCTATCACCTGTTGAAGTTGCC 60
orangutan   ATGGGCAGCATCGAACAAACAACTGAGATCCTGTTGTGCCTATCACCTGTTGAAGTTGCC 60
chimp       ATGGGCAGCATCGAACAAACAACTGAGATCCTGTTGTGCCTATCACCTGTTGAAGTTGCC 60
bonobo      ATGGGCAGCATCGAACAAACAACTGAGATCCTGTTGTGCCTATCACCTGTTGAAGTTGCC 60
gorilla     ATGGGCAGCATCGAACAAACAACTGAGATCCTGTTGTGCCTATCACCTGTTGAAGTTGCC 60
rhesus      ATGGGCAGCACTGAACAAACAACTGAGATCCTGTTGTGCCTATCACCTGTGGAAGTTGCT 60
pig         ATGAGCAGCATCGAACAAACGACGGAGATCCTGTTGTGCCTCTCACCTGCCGAAGCTGCC 60
rat         ATGGACAGGA---AACAGACAGCCGAGACCCTGCTGTCCTTGTCTCCTGCTGACGTCGCC 57
mouse       ATGGACAGGA---AACAGACAGCTGAGACCCTGCTGACCCTGTCTCCTGCTGAAGTTGCC 57

human       AGTCTTAAGGAAGGAATCAATTTCTTTCGCAATAAGAGCACTGGCAAAGACTACGTCTTG 120
orangutan   AATCTTAAGGAAGGAATCAATTTCTTTCGCAATAAGAGCACTGGCAAAGACTACATCTTG 120
chimp       AGTCTTAAGGAAGGAATCAATTTCTTTCGCAATAAGAGCACTGGCAAAGACTACATCTTG 120
bonobo      AGTCTTAAGGAAGGAATCAATTTCTTTCGCAATAAGAGCACTGGCAAAGACTACATCTTG 120
gorilla     AGTCTTAAGGAAGGAATCAATTTCTTTCGCAATAAGAGCACTGGCAAAGACTACATCTTG 120
rhesus      AATCTTAAGGAAGGAATCAATTTCTTTCGTAATAAGAGCACTGGCAAAGACTACATCTTG 120
pig         AATCTCAAGGAAGGAATCAATTTTGTTCGAAATAAGAGCACTGGCAAGGACTACATCTTA 120
rat         AACCTCAAGGAAGGAGTCAATTTTTGTCGAAATAAGACTACTGGGAAAGAGTACATTTTA 117
mouse       AACCTCAAGGAAGGGATCAATTTTTTTCGAAATAAGACTACTGGGAAAGAGTACATTTTA 117

human       TACAAGAATAAGAGCCGACTGAGGGCATGCAAGAATATGTGCAAGCACCAAGGAGGCCTG 180
orangutan   TACAAGAATAAGAGCCGACTGAGGGCATGCAAGAATATGTGCAAGCACCAAGGAGGCCTG 180
chimp       TACAAGAATAAGAGCCGACTGAGGGCATGCAAGAATATGTGCAAGCACCAAGGAGGCCTG 180
bonobo      TACAAGAATAAGAGCCGACTGAGGGCATGCAAGAATATGTGCAAGCACCAAGGAGGCCTG 180
gorilla     TACAAGAATAAGAGCCGACTGAGGGCATGCAAGAATATGTGCAAGCACCAAGGAGGCCTG 180
rhesus      TACAAGAGTAAGAGCCGACTGAGGGCATGCAAGAATGTGTGCAAGCACCAAGGAGGCCTG 180
pig         TTTAAGAATAAGAGCCGCCTGAAGGCATGTAAGAACATGTGCAAGCACCAAGGAGGCCTC 180
rat         TACAAGGAGAAGGACCACCTAAAGGCATGCAAGAACCTCTGCAAGCACCAAGGTGGCCTG 177
mouse       TACAAGGAGAAGGACCATCTAAAGGCATGCAAGAACCTCTGCAAGCACCAGGGAGGCCTG 177

human       TTCATAAAAGATATCGAGGATTTAGCCGGAAG---------------------------- 212
orangutan   TTCATAAAAGATATCGAGGATTTAGCCGGAAGGTCTGTTAGATGCACAAAGCACAACTGG 240
chimp       TTCATAAAAGATATCGAGGATTTAGCCGGAAGGTCTGTTAGATGCACAAAGCACAACTGG 240
bonobo      TTCATAAAAGATATCGAGGATTTAGCCGGAAGGTCTGTTAGATGCACAAAGCACAACTGG 240
gorilla     TTCATAAAAGATATCGAGGATTTAGCCGGAAGGTCTGTTAGATGCACAAAGCACAACTGG 240
rhesus      TTCATAAAAGATATCGAGGATTTAGCTGGAAGGTCTGTTAGATGCACAAAGCATAACTGG 240
pig         TTCATTAAAGACATTGAGGATCTAAATGGAAGGTCTGTTAAATGCACAAAACACAACTGG 240
rat         TTCATGAGAGATATCGAGGATTTAGATGGAAGGTCCGTTAAATGCACAAAGCACAACTGG 237
mouse       TTCATGAAAGACATCGAGGATTTAGATGGAAGGTCCGTTAAATGCACAAAGCACAACTGG 237

human       ------------------------------------------------------------
orangutan   AAATTAGATGTGAGCACCATGAAGTATATCAACCCTCCGGAAAGCTTCTGTCAAGATGAG 300
chimp       AAATTAGATGTGAGCACCATGAAGTATATCAACCCTCCGGAAAGCTTCTGTCAAGATGAG 300
bonobo      AAATTAGATGTGAGCACCATGAAGTATATCAACCCTCCGGAAAGCTTCTGTCAAGATGAG 300
gorilla     AAATTAGATGTGAGCACCATGAAGTATATCAACCCTCCGGAAAGCTTCTGTCAAGATGAG 300
rhesus      AAATTAGATGTGAGCACCATGAAGTATATCAACCCTCCAGAAAGCTTCTGTCAAGATGAG 300
pig         AAGTTAGATGTAAGCAGCATGAAGTATATCAATCCTCCTGGAAGCTTCTGTCAAGACGAA 300
rat         AAGTTAGATGTGAGCACCATGAAGTATACCAACCCTCCAGGGAGCTTCTGTCAAGACGAG 297
mouse       AAGTTAGACGTGAGCACCATGAAATATATCAACCCTCCAGGGAGCTTCTGTCAAGACGAG 297

human       ----TTGTTGAAATGGATGAAAACAACGGACTTTTGCTTTTAGAACTGAATCCTCCTAAC 268
orangutan   CTGGTTGTTGAAATGGATGAAAACAACAGACTTTTACTTTTAGAACTGAATCCTCCTAAC 360
chimp       CTGGTTGTTGAAATGGATGAAAACAACAGACTTTTACTTTTAGAACTGAATCCTCCTAAC 360
bonobo      CTGGTTGTTGAAATGGATGAAAACAACAGACTTTTACTTTTAGAACTGAATCCTCCTAAC 360
gorilla     CTGGTTGTTGAAATGGATGAAAACAACAGACTTTTACTTTTAGAACTGAATCCTCCTAAC 360
rhesus      CTGGTTGTTGAAATGGATGAAAACAACGGACTTTTACTTTTAGAACTGAATCCTCCTAAC 360
pig         CTGGTTGTAGAAAAGGATGAAGAAAATGGAGTTTTGCTTCTAGAACTAAATCCTCCTAAC 360
rat         CTTGTTGTGGAAATGGATGAGAACAATGGGCTTTGCCTTGTAGAACTGAACCCTCCTAAC 357
mouse       CTCGTTATTGAAATGGATGAAAACAATGGGCTTTCCCTGGTAGAACTGAACCCTCCTAAC 357

human       CCTTGGGACTTACAGCCCAGATCTCCTGAAGAGTTGGCTTTTGGAGAAGTACAGA ...
orangutan   CCTTGGGACTTACAGCCCAGATCTCCTGAAGAGTTGGCTTTTGGAGAAGTCCAGA ...
chimp       CCTTGGGACTTACAGCCCAGATCTCCTGAAGAGTTGGCTTTTGGAGAAGTCCAGA ...
bonobo      CCTTGGGACTTACAGCCCAGATCTCCTGAAGAGTTGGCTTTTGGAGAAGTCCAGA ...
gorilla     CCTTGGGTCTTACAGCCCAGATCTCCTGAAGAGTTGGCTTTTGGAGAAGTCCAGA ...
rhesus      CCTTGGGACTCAGAGCCCAGATCTCCTGAAGAGTTGGATTTTGGAGAAGTCCAGA ...
pig         CCGTGGGATTCAGAACCCAGATCTCCTGAAGATTTGGCTTTTGGGGAAGTGCAGA ...
rat         CCCTGGGACTCTGACCCCAGGTCTCCTGAAGAATTAGCTTTTGGGGAGGTACAGA ...
mouse       CCCTGGGACTCTGATCCCAGGTCTCCTGAAGAATTAGCTTTTGGGGAAGTACAGA ...
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Fig. 138.1 Alignment of *Cmah* sequences from various animals. Shown is the first ~400 bp of the coding region for each gene. The human-specific deletion is highlighted in *grey*. Sequence alignment was performed with ClustalW

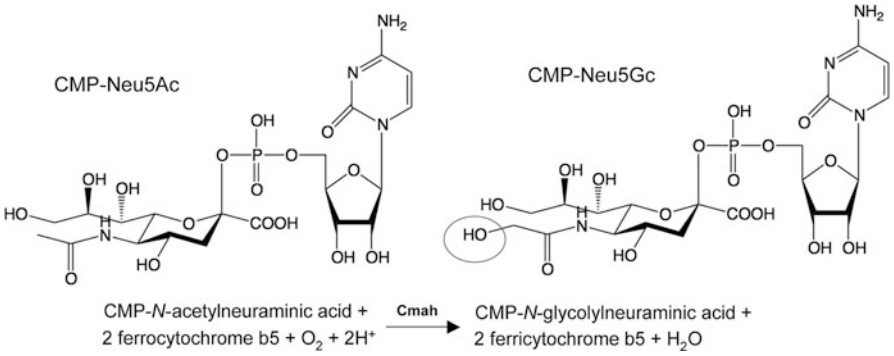

Fig. 138.2 Scheme of enzymatic reaction catalyzed by Cmah

of 1 mM NADH, 0.5 mM $FeSO_4$, and 0.53 μM CMP-[^{14}C]Neu5Ac (0.05 μCi) in a total volume of 300 μl. Incubations were terminated by addition of 30 μl 2 M HCl and the released [^{14}C]sialic acids were analyzed by radio-TLC. As internal standards, nonradioactive Neu5Ac and Neu5Gc were cochromatographed on the same lane. As controls, incubations were also performed using heat-inactivated protein fractions. An HPLC method was later developed to analyze the Cmah enzymatic reaction. Due to their different hydrophobicities, the substrate CMP-Neu5Ac and the product CMP-Neu5Gc could be separated on a C18 reversed-phase HPLC column within 10 min, and absorbance can be monitored at 271 nm (Kozutsumi et al. 1990). Conversion of CMP-Neu5Ac to CMP-Neu5Gc can be calculated by determining the peak areas. The detection limit was estimated to be as low as 0.2 pmoles per injection, and no radioactivity is required (Fig. 138.2).

Fluorimetric HPLC analysis using DMB can also be used to analyze the amount and ratio of Neu5Ac and Neu5Gc in tissue-derived animal samples (Malykh et al. 1998). Briefly, bound sialic acids are released by acid treatment and total (bound and free) sialic acids are derivatized by incubation in the presence of the fluorogen 1,2-diamino-4,5-methylene dioxybenzene (DMB). Subsequent analysis on a reversed-phase C18 column allows separation of Neu5Ac from Neu5Gc by HPLC, which can be detected by fluorescence of the labeled sialic acid. Determination of the peak areas in samples compared to sialic acid standards of known concentration allows calculation of total sialic acid contents.

Preparation

Cmah enzymatic activity was described in various animal tissues, and a few Cmah enzymes have been enriched or even purified over the past decades as described below.

Purification of Cmah from Porcine Submandibular Glands

Cmah enzyme was purified from porcine submandibular glands to apparent homogeneity by precipitation with *N*-acetyl-*N,N,N*-trimethylammonium bromide and fractionation on Q-Sepharose, Cibacron Blue 3GA-Agarose, Reactive Brown 10-Agarose, Hexyl-Agarose, and Superose S.12 (Schlenzka et al. 1994). This procedure resulted in an 8,960-fold enrichment of Cmah with a total recovery of 0.8 %. Analysis by SDS-PAGE revealed a molecular mass of 65 kDa for the purified protein. Gel filtration on Superose S.12 suggests that the enzyme migrates as a monomer with a mass of 60 kDa. The enzyme was activated by $FeSO_4$ and inhibited by various iron-binding reagents. An apparent *Km* of 11 μM was determined for the substrate CMP-Neu5Ac using purified hydroxylase in the presence of Triton X-100-solubilized microsomes. In a reconstituted system consisting of purified hydroxylase, cytochrome b5, cytochrome b5 reductase, and catalase, an apparent *Km* of 3 μM was measured.

Purification of Cmah from Mouse Liver

Cmah was enriched from Triton X-100-solubilized mouse liver microsomes by fractionation on Q-Sepharose, Cibacron Blue 3GA-Agarose, Reactive Brown l0-Agarose, and Superose S.12 (Schneckenburger et al. 1994). This procedure resulted in a 1,000-fold enrichment of Cmah with a total recovery of 0.4 %. SDS-PAGE revealed a molecular weight of 66 kDa and the protein appears to be monomeric as determined by gel filtration. The hydroxylase system was reconstituted with Triton X-100-solubilized mouse liver microsomes and purified soluble or microsomal forms of cytochrome b5 reductase and cytochrome b5.

Purification of Cmah from Mouse Liver

Cmah was purified to apparent homogeneity from the cytosolic fraction of mouse liver by using ion exchange columns, a Red-Sepharose column, and a soluble cytochrome b5-immobilized Sepharose column (Kawano et al. 1994). Analysis by SDS-PAGE revealed a single band with a molecular mass of 64 kDa, and the isolated enzyme migrates as a monomer of 58 kDa in gel filtration. The absorption spectrum did not indicate the presence of a heme prosthetic group in the enzyme. Atomic absorption spectrometry and an inhibition test using an iron chelator indicated that the enzyme contains nonheme iron as the electron acceptor. A reconstitution experiment of isolated Cmah, soluble cytochrome *b5*, and recombinant NADH-cytochrome *b5* reductase revealed that these three factors are essential for the reaction. The hydroxylase exhibited high affinity to the substrate CMP-NeuAc ($K_m = 5$ μM) and was greatly stabilized by CMP-NeuAc.

Cloning and Recombinant Expression of Cmah from Starfish

The *Cmah* gene from the starfish *Asterias rubens* was cloned and an ORF coding for a protein with 653 amino acids (75 kDa) could be identified (Martensen et al. 2001). An expression vector harboring a truncated *Cmah* variant lacking the sequence encoding for the predicted hydrophobic C-terminal transmembrane domain was generated. Rather unstable and low Cmah enzymatic activity was detectable in *E. coli* bacterial lysates after induction of expression at 15 °C.

Isolation of Cmah from Starfish Gonads

The Cmah enzyme from the gonads of starfish *Asterias rubens* was enriched using anion exchange chromatography and immobilized cytochrome *b*5 chromatography (Gollub and Shaw 2003). The enzyme was enriched 137-fold with a yield of 13 %. The isolated protein was analyzed by SDS-PAGE and activity correlated with the major band of 76 kDa as well as with a minor band around 64 kDa. The apparent Km value for the substrate CMP-Neu5Ac was found to be 0.8 μM determined in the presence of 100 mM NaCl. Addition of Fe ions activated isolated Cmah threefold, whereas iron chelators were found to be potent inhibitors.

Biological Aspects

The Cmah enzyme is the only known biosynthetic pathway to generate the major mammalian sialic acid Neu5Gc. Besides namH, a bacterial hydroxylase (Raymond et al. 2005), it is the only known enzyme capable of catalyzing the generation of an *N*-glycolyl group in nature. The *Cmah* gene has been identified in various animals of the deuterostome lineage (vertebrates and the so-called "higher" invertebrates) and thus far appears to be a marker of this lineage, indicating that the enzymatic activity likely first appeared around the time of the Cambrian expansion (~500 mya (million years ago)).

As discussed above ("Identification of the Human CMAHP Pseudogene" section), the human *CMAH* gene uniquely carries an Alu-mediated loss-of-function 92 bp exon deletion, which is fixed in the human population (Chou et al. 1998; Irie et al. 1998; Hayakawa et al. 2001, 2006). Therewith, humans lost the ability to synthesize Neu5Gc. Genomic studies indicate that the inactivation of *CMAH* likely occurred prior to brain expansion during human evolution around 3 mya (million years ago) (Chou et al. 2002). It remains to be resolved why the human *CMAH* gene inactivation was driven to fixation in the population. One theory considers fixation of the human *CMAH* pseudogene by natural selection, potentially resulting in resistance to major pathogens at the time. For example, *P. reichenowi* likely causes malaria in chimpanzees in a Neu5Gc-dependent manner. Inactivation of the *CMAH* gene in the human lineage rendered human ancestors unable to generate the sialic acid Neu5Gc from its precursor Neu5Ac and possibly made humans resistant to

P. reichenowi (Martin et al. 2005b). In keeping with this, phylogenetic analysis indicates that all current *P. falciparum* populations (the pathogen causing malignant malaria in humans today) originated from *P. reichenowi*, likely by a single host transfer, which apparently occurred sometime after *CMAH* inactivation in humans (Varki and Gagneux 2009). Mutations in the dominant invasion receptor EBA 175 in the *P. falciparum* lineage are postulated to have provided the parasite with preference for the overabundant Neu5Ac precursor present in humans, accounting for its extreme human pathogenicity today (Varki and Gagneux 2009). In addition to malaria, infection with Shiga toxigenic *E. coli* was found to be dependent on AB5 toxin binding to Neu5Gc-containing host structures (Byres et al. 2008). Also *E. coli* K99, a major gastrointestinal pathogen of livestock, requires Neu5Gc-ganglioside for invasion (Kyogashima et al. 1989). Thus, loss of Neu5Gc expression might have protected human ancestors from serious disease.

Another not mutually exclusive hypothesis to explain the fixation of the human *CMAH* inactivation in the population involves reproductive incompatibility (Ghaderi et al. 2011). All humans analyzed so far have a polyclonal anti-Neu5Gc antibody repertoire (Nguyen et al. 2005; Padler-Karavani et al. 2008). Such human anti-Neu5Gc antibodies were able of targeting paternally derived antigens and mediate cytotoxicity against Neu5Gc-bearing chimpanzee sperm in vitro (Ghaderi et al. 2011). Female $Cmah^{-/-}$ mice (B) with a humanlike *Cmah* inactivation were immunized to express anti-Neu5Gc antibodies and thereafter showed significantly lower fertility toward Neu5Gc-positive wt males due to pre-zygotic incompatibilities (Ghaderi et al. 2011). Models of populations polymorphic for such antigens show that reproductive incompatibility by female immunity can drive loss-of-function alleles to fixation from moderate initial frequencies.

Interestingly, among the <60 genes known to be involved in sialic acid metabolism, more than 10 were found to have undergone uniquely human genetic changes as compared to our closest evolutionary relatives, the great apes (Varki 2009). Besides the *CMAH* gene inactivation and subsequent loss of Neu5Gc in humans, increased expression of α2-6-linked sialic acids and multiple changes in *SIGLECs* (sialic acid-recognizing Ig-like lectins) have been described. The latter includes altered sialic acid-binding specificities (Siglecs 5, 7, 9, 11, and 12), different expression patterns (Siglecs 1, 5, 6, and 11), gene conversion event (*SIGLEC* 11), and deletion/pseudogenization of genes (*SIGLEC* 13, 14, and 16) (Varki 2010). Given the rarity of genetic differences between humans and great apes, sialic acid biology appears to be a hot spot of genetic and physiological changes during human evolution.

As humans express anti-Neu5Gc antibodies, the question arises when in life and how such antibodies are emerging. Simple feeding of $Cmah^{-/-}$ Neu5Gc-deficient mice with various Neu5Gc-containing glycoconjugates was insufficient to elicit detectable anti-Neu5Gc antibodies. However, immunizations with various purified Neu5Gc-containing glycoconjugates in the presence of adjuvant resulted in an anti-Neu5Gc antibody response in this humanlike mouse model. But this does of course not mimic the human situation. A possible mechanism for development of anti-Neu5Gc antibodies in humans was recently identified (Taylor et al. 2010). It was shown that exogenous free Neu5Gc incorporates into the cell surface

lipooligosaccharides of nontypeable *Haemophilus influenzae* (NTHi), a widespread human-specific commensal/pathogen. Subsequently, infant anti-Neu5Gc antibodies were found to appear coincident with antibodies against NTHi (Taylor et al. 2010). As the nasopharyngeal flora of infants can be in contact with ingested food, a novel model for how NTHi and dietary Neu5Gc cooperate to generate anti-Neu5Gc antibodies in humans was proposed (Taylor et al. 2010). This hypothesis was strengthened by the finding that Neu5Gc-loaded NTHi were sufficient to elicit an anti-Neu5Gc antibody response in *Cmah*$^{-/-}$ mice without the need of adjuvant.

Also, it has been shown very recently that certain ticks take up exogenous Neu5Gc from feeding on animals. This sialic acid was identified to incorporate into the endogenous salivary glycans of the tick (Vancova et al. 2012). As already proposed for the nonhuman xeno-reactive terminal Galα1-3Gal epitopes, such Neu5Gc-containing tick structures might elicit an anti-Neu5Gc immune response following a tick bite. Remarkably, the habitat of the tick used in this study happens to be an area with high incidences of red meat allergy in humans (Van Nunen et al. 2009; Commins et al. 2011; Mullins et al. 2012).

Various Neu5Gc-containing glycoconjugates have been described from multiple species of the deuterostome lineage, and Neu5Gc levels vary greatly between species and tissues. The brain represents a remarkable exception as among species brain Neu5Gc expression has been maintained at extremely low levels over hundreds of millions of years of vertebrate evolution (Davies et al. 2012). In contrast to all other tissues, the vertebrate brain is very rich in polysialic acid, and resistance of Neu5Gc-containing polysialic acid to sialidases provides a potential explanation for the rarity of Neu5Gc in the brain (Davies et al. 2012).

Whereas the human *CMAH* gene is inactivated throughout the human population, a subset of cats also carry mutations in the promoter region of the *Cmah* gene. Such feline mutations strongly correlate with the cat blood types A (Neu5Gc-GD3) and B (Neu5Ac-GD3) and may represent the first causative mutation for a blood group in non-primate species (Bighignoli et al. 2007). The lack of Neu5Gc in birds and reptiles has also been suggested to indicate an independent loss of Cmah in the sauropsid lineage (Schauer et al. 2009).

Knockout Mice

Two research groups independently generated *Cmah*$^{-/-}$ mice by using different strategies. Results have been mostly described in a joint publication (Hedlund et al. 2007). One approach (A) generated a *Cmah*$^{-/-}$ strain by Neo cassette insertional mutagenesis in mouse ES cells, which disrupted the open reading frame of the *Cmah* gene. The second approach (B) generated a targeted deletion of exon 6 in the mouse *Cmah* gene to exactly mirror the human-inactivated *CMAH* gene. Both strains were viable and fertile and were fully backcrossed (>10 generations) into the same C57Bl/6 genetic background. No obvious differences between the two strains were noted. A mouse-specific B-cell defect was reported in *Cmah*$^{-/-}$ strain (A), which is not present in humans (Naito et al. 2007) (Table 138.1).

Table 138.1 Summary of phenotypes of the *Cmah*$^{-/-}$ mouse model

Increased 9-*O*-acetylation of sialic acids (Hedlund et al. 2007)	*Cmah*$^{-/-}$mice were found to have an increased level of 9-*O*-acetylation of sialic acids. Sialic acid *O*-acetylation can modulate recognition by intrinsic Sia-binding molecules, such as Siglecs, as well as to various pathogen-binding proteins (Kelm and Schauer 1997; Angata and Varki 2002)
Incorporation of exogenous Neu5Gc into tumors and fetuses (Hedlund et al. 2007)	*Cmah*$^{-/-}$mice were found to be devoid of any detectable Neu5Gc, confirming that there is only a single Cmah-dependent pathway to biosynthesize endogenous Neu5Gc. In agreement with detection of Neu5Gc in humans, exogenous Neu5Gc was taken up by the mice and incorporated well into tumors and fetuses
Disturbed hearing and inner ear morphology (Hedlund et al. 2007)	Older *Cmah*$^{-/-}$ mice (9-month-old but not 3-month-old) exhibited reduced hearing sensitivity across frequencies. Furthermore, abnormalities of the vestibular and auditory inner ears became apparent. Unusual deposits of acellular material were detected on the apical surface of the vestibular otoconial epithelia, among the stereociliary bundles, which might affect the function of stereocilia through mechanical interference. More subtle changes in morphology were also observed in the semicircular canal organs. In addition, some older mice displayed fluid-filled cysts or outer hair cell degeneration throughout the cochlea
Defects in wound healing (Hedlund et al. 2007)	Wound repair is significantly delayed in *Cmah*$^{-/-}$ animals compared to wt mice. Histological analysis of the wounds over time revealed no obvious differences in inflammatory cell infiltrate, angiogenesis, or keratinocyte morphology
Hyperglycemia and glucose intolerance (Kavaler et al. 2011)	*Cmah*$^{-/-}$mice exhibited fasting hyperglycemia and glucose intolerance following a high-fat diet. The phenotype appeared to be due to compromised pancreatic B-cell function associated with a 65 % decrease in islet size and area as well as a 50 % decrease in islet number. In addition, obese *Cmah*$^{-/-}$ mice had a >40 % reduction in the response to insulin secretagogues in vivo. This may have implications for the pathogenesis of type 2 diabetes in obese humans

Additional genes have been disrupted in *Cmah*$^{-/-}$ mouse strain by crossing with other mutant mice to generate double-knockout (DKO) mice for further studies. This includes the α1-3-galactosyltransferase (GalT), which synthesizes terminal Galα1-3Gal epitopes in animals but is inactivated in humans as well (Basnet et al. 2010). Thymocytes of this *galT*$^{-/-}$*Cmah*$^{-/-}$ DKO strain were found to show small but significant reduction of complement-mediated cytotoxicity after exposure to human sera as compared to the *galT*$^{-/-}$ single-knockout mice thymocytes

(Basnet et al. 2010). Furthermore, *Cmah*$^{-/-}$ mouse strain was crossed into the mdx mouse model for Duchenne muscular dystrophy (DMD) (Chandrasekharan et al. 2010). The introduced humanlike change in sialylation was found to contribute to the significant discrepancy in phenotype between the severe human DMD disease and the currently used mdx mouse model. The DMD phenotype of the *Cmah*-deficient mdx mice was found to occur at an earlier age or at a greater degree as compared to the mdx mice, which better approximates the severe human disease phenotype (Chandrasekharan et al. 2010).

Human Disease

Although human ancestors lost the ability to biosynthesize Neu5Gc due to the *CMAH* inactivating mutation ~2–3 mya (million years ago), human metabolic pathways still accept this foreign structure as "self." Human cells supplemented with exogenous Neu5Gc were shown to incorporate this sialic acid into endogenous glycan structures on the cell surface (Tangvoranuntakul et al. 2003; Bardor et al. 2005). However, the human immune system recognizes Neu5Gc as "foreign." Heterophile human antibodies that agglutinate animal red cells were already described over 100 years ago by Hanganutziu and Deicher ("HD antibodies"), as occurring in patients injected with animal serum. Subsequently, HD antibodies were detected in disease-related human sera, without the patients having ever received injections of animal sera. Prominent among these were patients with cancer, leprosy, or rheumatoid arthritis. It was then shown that these HD antibodies were directed against Neu5Gc (Nishimaki et al. 1979; Morito et al. 1986). With the development of more sensitive assay systems, all humans analyzed so far were found to have a circulating polyclonal anti-Neu5Gc antibody repertoire with sometimes high titers (Padler-Karavani et al. 2008). Meanwhile, dietary Neu5Gc from animal-derived foods was shown to incorporate into tissues as recently demonstrated in *Cmah*$^{-/-}$ mice with a humanlike *Cmah* defect (Banda et al. 2012). Given that Cmah appears to be the exclusive pathway for Neu5Gc biosynthesis (Hedlund et al. 2007; Varki 2009; Bergfeld et al. 2012b), uptake of dietary Neu5Gc very likely explains the low-level expression of Neu5Gc in normal human tissues (Diaz et al. 2009; Pham et al. 2009). Furthermore, higher accumulation of the xeno-autoantigen Neu5Gc in human cancers has been reported in multiple publications over the past decades (Malykh et al. 2001). In addition, increased Neu5Gc accumulation can be found at sites of inflammation and in fetal tissues. As a result of metabolic incorporation of diet-derived nonhuman Neu5Gc, a novel xeno-autoantigen reaction and chronic inflammation ("xenosialitis") is postulated as a likely outcome in humans (Varki et al. 2011). Taken together, Neu5Gc may very well be an additional factor explaining the well-established correlation of consumption of red meat (very rich in Neu5Gc) with overall mortality (Pan et al. 2012). With regard to atherosclerosis, Neu5Gc was found to be present both in endothelium overlying plaques and in subendothelial regions, providing multiple potential pathways for accelerating inflammation in this disease (Pham et al. 2009).

The accumulation of Neu5Gc in human cancers in the face of a circulating anti-Neu5Gc antibody repertoire may facilitate tumor progression by the resulting low-grade chronic inflammation. Indeed, murine tumors expressing humanlike levels of Neu5Gc show accelerated growth in syngeneic Neu5Gc-deficient *Cmah*$^{-/-}$ mice, coincident with the induction of anti-Neu5Gc antibodies and increased infiltration of inflammatory cells (Hedlund et al. 2008). In addition, isolated human anti-Neu5Gc antibodies were also found to accelerate growth of Neu5Gc-containing tumors in Neu5Gc-deficient *Cmah*$^{-/-}$ mice (Hedlund et al. 2008). On the other hand, the highly elevated Neu5Gc accumulation in human cancers compared to normal healthy tissue is exploited for specific tumor targeting and represents a starting point for the development of potential cancer vaccines (Blanco et al. 2011; Fernandez-Marrero et al. 2011; Segatori et al. 2012).

Besides dietary sources, Neu5Gc contamination in biotherapeutics represents another source for this nonhuman sialic acid. Recombinant glycoprotein therapeutics are commonly produced in established nonhuman mammalian cell lines, which are often even cultured in the presence of animal sera. It is well documented that the resulting biotherapeutics are therefore often modified with high amounts of Neu5Gc (Hokke et al. 1990). Recently, it has been shown that humanlike Neu5Gc-deficient *Cmah*$^{-/-}$ mice generate antibodies against Neu5Gc upon injection of a Neu5Gc-containing biotherapeutic drug (cetuximab) and that such antibodies impact drug clearance (Ghaderi et al. 2010). Neu5Gc contamination on biotherapeutics may therefore influence half-life, efficacy, and immunogenicity of such biotherapeutic drugs among patients (Fig. 138.3).

As mentioned above ("Knockout Mice" section), additional human diseases such as type 2 diabetes and Duchenne muscular dystrophy might be correlated to the nonhuman xeno-autoantigen Neu5Gc by using the Neu5Gc-deficient *Cmah*$^{-/-}$ mouse model. This includes the phenotypic description that *Cmah*$^{-/-}$mice (B) exhibited fasting hyperglycemia and glucose intolerance following a high-fat diet. Additionally, obese mice showed a reduced insulin response in vivo, which makes the Neu5Gc-deficient *Cmah*$^{-/-}$ mice an interesting model to further study pathogenesis of type 2 diabetes in obese humans (Kavaler et al. 2011). Also, Neu5Gc-deficient *Cmah*$^{-/-}$ mice were crossed into the mdx mouse model for Duchenne muscular dystrophy (DMD). The DMD phenotype of the *Cmah*-deficient mdx mice was found to have an earlier onset or to occur at a greater degree as compared to the mdx mice alone, which better approximates the severe human disease phenotype (Chandrasekharan et al. 2010).

Future Perspectives

Based on recent findings, human-specific "xenosialitis" may be orchestrated by the incorporation of antigenic exogenous Neu5Gc in the presence of circulating anti-Neu5Gc antibodies against this nonhuman epitope. Thus reducing the "Neu5Gc burden" in humans is the desirable goal. However, exclusion of animal-derived products from the standard human diet does not represent a feasible strategy, and

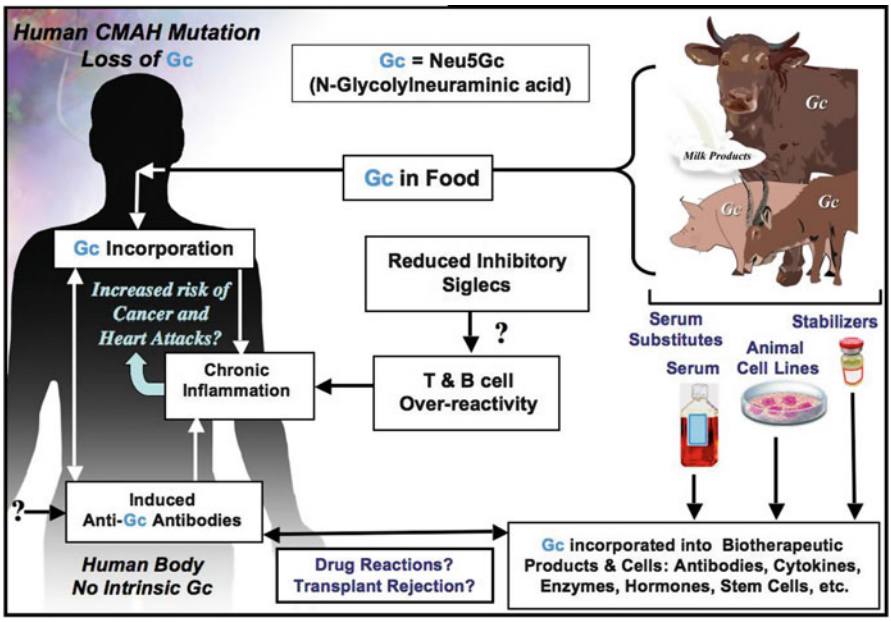

Fig. 138.3 Incorporation of immunogenic Neu5Gc into humans (Duplicated with permission from Varki 2010). Two mechanisms for enhanced chronic inflammation and immune reactions in humans. Metabolic incorporation of dietary Neu5Gc (Gc) from animal-derived food products in the face of circulating anti-Neu5Gc antibodies may contribute to chronic inflammation in endothelia lining blood vessels and in epithelia lining hollow organs, perhaps contributing to the increased risks of cardiovascular disease and carcinomas associated with these foods. The apparent T- and B-cell over-reactivity of humans associated with decreased inhibitory Siglec expression may contribute further toward chronic inflammation. Also shown is that the fact that some molecular and cellular products of biotechnology are likely contaminated with Neu5Gc from multiple sources, potentially contributing to untoward reactions in some individuals

generation of "humanized" Neu5Gc-deficient $Cmah^{-/-}$ cows, pigs, sheep, or goats may be hampered by the public fears regarding genetically modified food products. Furthermore, well-controlled studies on big cohorts of red meat consumers compared with others are not completed to date but would be required to confirm the current hypothesis. In contrast to dietary Neu5Gc, multiple feasible strategies for reduction of the "Neu5Gc burden" of biotherapeutic products are conceivable. For example, a CHO producer cell line with 80 % reduction in hydroxylase activity was already achieved after RNAi stable transfection (Chenu et al. 2003). Ideally, Neu5Gc-deficient $Cmah^{-/-}$ cell lines would be generated and optimized to be cultured in Neu5Gc-free culture conditions as the next-generation producer cell lines in biopharma. Furthermore, substantial amounts of the xeno-autoantigen Neu5Gc were found on human stem cells originating from culture medium and from feeder layers (Martin et al. 2005a). This finding highlights once again the need for completely xeno-autoantigen free culturing conditions in biopharma. Desirable pig-to-human xenograft transplantation represents another major field, which is

hampered by xeno-autoantigens (Padler-Karavani and Varki 2011). The well-studied Galα1-3-Gal epitope is widely expressed by most mammals other than old world primates and recognized by universal human anti-α-Gal antibodies. Difficulties in pig-to-human xeno-transplantation even resulted in the production of α1-3-galactosyltransferase gene-knockout pigs as a potential solution (Kuwaki et al. 2005). However, such knockout pigs still express nonhuman Neu5Gc-containing glycoconjugates, which may likely cause further xenograft rejection (Padler-Karavani and Varki 2011). It has even been described that α1-3-galactosyltransferase deficiency increases sialyltransferase activity and thereby potentially raises Neu5Gc immunogenicity in such knockout pigs (Park et al. 2011). Using the Neu5Gc-deficient $Cmah^{-/-}$ mouse model, it was also recently shown that $Cmah^{-/-}$ mice rejected islets transplanted from syngeneic $Cmah^{+/+}$ mice (Tahara et al. 2010). This finding represents the first direct evidence that anti-Neu5Gc antibody response may be crucially involved in xenograft loss. Thus, a double-knockout animal is needed.

Furthermore, Neu5Gc can also be metabolized in human cells, which yields to *N*-glycolylmannosamine (ManNGc), *N*-glycolylglucosamine (GlcNGc), and *N*-glycolylglucosamine-6-phosphate (GlcNGc-6P) followed by irreversible de-*N*-glycolylation to result the ubiquitous metabolites glucosamine-6-phosphate and glycolate (Bergfeld et al. 2012b). It has also been demonstrated very recently that GlcNGc synthesized during Neu5Gc breakdown can be converted to GalNGc, and both monosaccharides incorporate into cellular glycoconjugates (Bergfeld et al. 2012a; Macauley et al. 2012). Future studies will reveal if Neu5Gc-derived *N*-glycolylated amino sugars represent additional xeno-autoantigens in humans.

Acknowledgments Supported by NIH grant R01GM32373.

Cross-References

- ▶ Cytidine Monophosphate *N*-Acetylneuraminic Acid Synthetase (CMAS)
- ▶ *N*-Acetylneuraminic Acid Synthase (NANS)
- ▶ Solute Carrier Family 35 (CMP-Sialic Acid Transporter), Member A1 (SLC35A1)
- ▶ ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 1 (ST3GAL1)
- ▶ ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 2 (ST3GAL2)
- ▶ ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 3 (ST3GAL3)
- ▶ ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 4 (ST3GAL4)
- ▶ ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 5 (ST3GAL5)
- ▶ ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 6 (ST3GAL6)
- ▶ ST6 Beta-Galactoside Alpha-2,6-Sialyltranferase 1 (ST6GAL1)
- ▶ ST6 Beta-Galactoside Alpha-2,6-Sialyltranferase 2 (ST6GAL2)
- ▶ ST6 *N*-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 1 (ST6GALNAC1)
- ▶ ST6 *N*-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 2 (ST6GALNAC2)

- ST6 *N*-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 3 (ST6GALNAC3)
- ST6 *N*-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 4 (ST6GALNAC4)
- ST6 *N*-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 5,6 (ST6GALNAC5,6)
- ST8 Alpha-*N*-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 1 (ST8SIA1)
- ST8 Alpha-*N*-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 2 (ST8SIA2)
- ST8 Alpha-*N*-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 3 (ST8SIA3)
- ST8 Alpha-*N*-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 4 (ST8SIA4)
- ST8 Alpha-*N*-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 5 (ST8SIA5)
- ST8 Alpha-*N*-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 6 (ST8SIA6)

Further Reading

Schauer et al. (1968): First proposed an enzyme capable of catalyzing the oxidative conversion of *N*-acetyl groups to *N*-glycolyl groups involved in the biosynthesis of Neu5Gc.

Kawano et al. (1995): Describe the first successful cloning of a Cmah gene. They determined the amino acid sequence of the Cmah enzyme isolated from mouse liver, obtained a matching cDNA clone, and showed that the sequence indeed encoded a functional 577 amino acid Cmah enzyme.

Irie et al. (1998): First describe the 92 bp loss-of-function deletion in the human *CMAHP* gene. However, the 5′ region of their cDNA clone was incomplete, and an N-terminally truncated protein was predicted as human CMAHP.

Chou et al. (1998): Detected the presence of the 92 bp loss-of-function deletion in the human *CMAHP* mRNA. They also correctly identified the full coding sequence of human *CMAHP* including the initiator methionine, thus predicting the correct much shorter CMAHP protein in humans. This work also shows that the missing human exon is present in closely related great apes such as chimpanzees, gorillas, and orangutans.

Hedlund et al. (2007): Two research groups independently generated a $Cmah^{-/-}$ mouse model to study the human loss of Neu5Gc. One mouse has a disrupted Cmah gene, whereas the other mouse carries the human 92 bp deletion of exon 6. In this study, Hedlund and coworkers (2007) describe initial phenotypes of the mice and provide the platform for further research.

Varki (2009): A comprehensive review on the human *CMAHP* pseudogene, and the evolutionary impact of Neu5Gc loss and consequences in humans.

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