Glycoconj J (2009) 26:415–422
DOI 10.1007/s10719-008-9189-6

Biochemical characterization of human and murine isoforms of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE)

Stefan O. Reinke · Colin Eidenschink · Chris M. Jay · Stephan Hinderlich

Received: 25 July 2008 / Revised: 1 September 2008 / Accepted: 4 September 2008 / Published online: 23 September 2008

Abstract The bifunctional enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) is the key enzyme for the biosynthesis of sialic acids, terminal components of glycoconjugates associated with a variety of physiological and pathological processes. Different protein isoforms of human and mouse GNE, deriving from splice variants, were predicted recently: GNE1 represents the GNE protein described in several studies before, GNE2 and GNE3 are proteins with extended and deleted N-termini, respectively. hGNE2, recombinantly expressed in insect and mamalian cells, displayed selective reduction of UDP-GlcNAc 2-epimerase activity by the loss of its tetrameric state, which is essential for full enzyme activity. hGNE3, which had to be expressed in *Escherichia coli*, only possessed kinase activity, whereas mGNE1 and mGNE2 showed no significant differences. Our data therefore suggest a role of GNE1 in basic supply of cells with sialic acids, whereas GNE2 and GNE3 may have a function in fine-tuning of the sialic acid pathway.

S. O. Reinke · C. Eidenschink
Institute of Biochemistry and Molecular Biology,
Charité-University Medicine Berlin,
Campus Benjamin Franklin, Arnimallee 22,
14195 Berlin-Dahlem, Germany

S. O. Reinke · S. Hinderlich (✉)
Department of Life Sciences and Technology,
Laboratory of Biochemistry,
University of Applied Sciences Berlin,
Seestrasse 64,
13347 Berlin, Germany
e-mail: stephan.hinderlich@tfh-berlin.de

C. M. Jay
Gradalis, Inc.,
2545 Golden Bear Dr, suite 110,
Carrollton, TX 75006, USA

Keywords GNE · Isoforms · Sialic acid · UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase

Abbreviations

GNE	UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase
UDP-GlcNAc	UDP-*N*-acetylglucosamine
ManNAc	*N*-acetylmannosamine
Neu5Ac	*N*-acetylneuraminic acid

Introduction

Sialylation of glycoproteins and glycolipids on eukaryotic cell surfaces plays an important role during development and regeneration, and in the pathogenesis of diseases [1]. Terminal sialic acids are highly expressed on the cell surface and involved in a variety of cellular interactions, such as cell–cell adhesion or cell migration [2], metastasis formation, and progression of a variety of tumors [3]. They are also known to be involved in the formation and masking of recognition determinants [4] and the biological stability of glycoproteins [5].

N-Acetylneuraminic acid (Neu5Ac) is the biosynthetic precursor of virtually all of the naturally occurring sialic acids [6]. In mammals, Neu5Ac and its activated nucleotide sugar CMP-Neu5Ac are synthesized in the cytosol from UDP-*N*-acetylglucosamine (UDP-GlcNAc) by five consecutive reactions [7]. The first two steps in this biosynthesis are catalyzed by the bifunctional enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (UDP-GlcNAc 2-epimerase/ManNAc kinase; GNE). The epimerization from UDP-GlcNAc to ManNAc by UDP-GlcNAc 2-epimerase is irreversible and rate-limiting.

ManNAc is then phosphorylated at the 6 position by ManNAc kinase. GNE has been recognized as the key enzyme in the pathway of sialic acids and it is feedback inhibited by CMP-Neu5Ac [8]. Neu5Ac-9-phosphate is then formed by the addition of phospho*enol*pyruvate to ManNAc-6-phosphate, catalyzed by the Neu5Ac-9-phosphate synthase [9, 10]. After release of the phosphate group ends, Neu5Ac is created. Neu5Ac is activated by the CMP-Neu5Ac synthase [11] in the nucleus. The end product of the biosynthesis, CMP-Neu5Ac, serves as substrate for the sialyltransferases in glycoconjugate biosynthesis [12].

The biological importance of GNE is further reflected in a drastic reduction of cellular sialylation upon loss of enzyme activity [13]. Furthermore the knockout of the gene in mice is embryonic lethal at day 8.5 [14]. Two human diseases are referred to point mutations in the GNE gene. Sialuria is characterized by a massive production of free Neu5Ac due to loss of the feedback control of the UDP-GlcNAc 2-epimerase activity [15]. Hereditary Inclusion Body Myopathy is an autosomal recessive neuromuscular disorder characterized by adult-onset, slowly progressive distal and proximal muscle weakness, and a typical muscle pathology including cytoplasm rimmed vacuoles and cytoplasm or nuclear filamentous inclusions composed of tubular filaments [16]. The disease is caused by over 50 different homozygous as well as heterozygous mutations spreading over the whole enzyme, including both domains [17].

Four different splice variants of human GNE mRNA were described [18]. The original GNE gene is complemented by an additional 90 bp exon, named A1, which is located about 20,000 bp upstream of exon 1. The four splice variants result from alternative splicing of the exons A1, 1 and 2. As exon 1 is a non-coding exon, two of the splice variants encode for a protein with 722 amino acids, which represents the original protein, characterized in several former studies [19, 20]. Recently, we predicted novel protein isoforms of human and mouse GNE, deriving from the other two splice variants [21]. Consequently, human GNE exist in three different isoforms, namely hGNE1, hGNE2, and hGNE3 (Fig. 1). hGNE2 and hGNE3 possess extended and deleted N-termini, respectively. The N-terminus of hGNE2 is prolonged by 31 additional amino acids. The first 17 amino acids are encoded by exon A1, the residual 14 amino acids are encoded by the nucleotides of exon 2. The lack of exon 2 in the cDNA encoding for hGNE3 leads to loss of the first 55 amino acids of hGNE1. These amino acids are replaced by 14 new amino acids encoded by another reading frame of exon A1. Mouse GNE only exists in two different isoforms homologous to hGNE1 and hGNE2. GNE2 was also found in other species like apes, rodents, chicken, and fish whereas GNE3 seems to be restricted to primates [21]. GNE1 is expressed ubiquitiously, but GNE2 and GNE3 display tissue specific expression patterns. Kidney, liver, placenta and colon expressed both GNE2 and GNE3, whereas brain, lung and pancreas additionally expressed GNE2 [21]. However, the functional importance of the different GNE isoforms is unknown to date. In this work, we expressed functional human and mouse GNE isoforms and characterized their biochemical properties.

Materials and methods

Construction of the human and murine GNE isoforms

The original cDNA of human GNE [22] was used as template for the amplification of hGNE1 and hGNE3 cDNA. Commercial QUICK-Clone™ human placenta cDNA (BD Biosciences; Heidelberg, Germany) was used as a template for cDNA amplification of hGNE2. The following Forward-Primers were used: hGNE1-For (5′-ATGGAGAAGAAT GGAAATAACCGAAAG-3′), hGNE2-For (5′-ATGGAAACCTATGGTTATCTGCAGAG GGAGTCATGCTTTCAAGGA-3′) and hGNE3-For (5′-*ATGGTTATCTGCAGAGGGAGT CATGCTTTCAAG GACCTCAT*AAATACATATCGAATG-3′); italic bases indicate codons for the hGNE3 specific amino acids. For all PCR reactions the following degenerated Reverse-Primer was used: (5′-*CTAGTGATGGTGATGGTGATGATCGATT GG*GTAGATC CTGCGTGT-3′); italic bases indicate codons for the 6xHis-tag and the stop codon. As template for PCR of mGNE1 and mGNE2 the clone IRAK-p961E1325Q2 (RZPD, Deutsches Ressourcenzentrum für Genomforschung; Berlin, Germany) was used. As Forward-Primers for the cDNA amplification the following were used: mGNE1-For (5′-ATGGAGAAGAAC GGGAACA ACCGAAAGCTC-3′) and mGNE2-For (5′-ATGGAAA CACACGCGCATCTC CACAGG-3′). The following degenerated Reverse-Primer, that contains codons for the 6xHis-tag and the stop codon (underlined) was used: (5′-*CTAGTGATGGTGATGGTGATG ATCGATTGG*GTG GATCCTGCGCGT-3′). For PCR the AccuPrime™ *Pfx* DNA polymerase kit (Invitrogen; Karlsruhe, Germany) was applied. For one PCR reaction 100 ng cDNA, 1 μM Forward-Primer and 1 μM Reverse-Primer were used. Thermocycling was done in a Mastercycler EP Gradient S (Eppendorf; Hamburg, Germany) with the following PCR conditions: 5 min 95°C; 35×(30 s 95°C/30 s 60°C/2 min 30 s 68°C); 5 min 68°C; then hold at 4°C. The PCR products were cloned into a pCR®-Blunt vector (Invitrogen). The inserts were verified via sequencing and thereafter cloned into the pFASTBAC™ 1 vector (Invitrogen) via

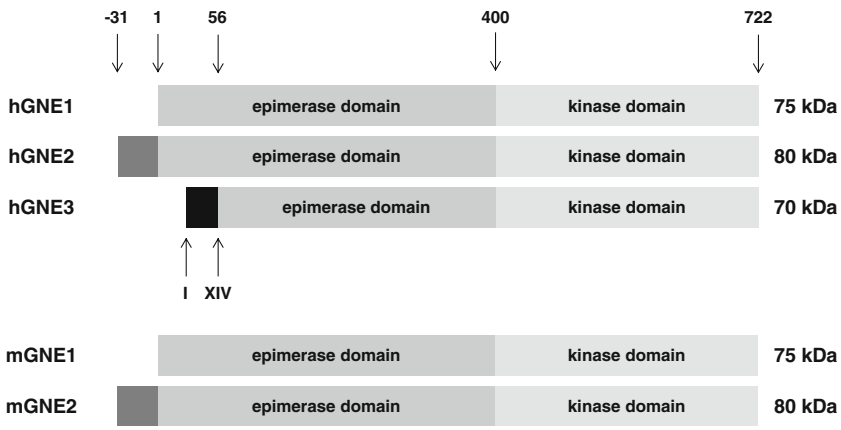

Fig. 1 Schematic diagram of the structure of human and murine GNE isoforms. *Numbers* indicate the amino acids. GNE2 specific amino acids are numbered with *negative arabic numerals*. GNE3 specific amino acids are numbered with *latin numerals*. Theoretical molecular masses are indicated *right*

XhoI/KpnI restriction sites. Competent *Escherichia coli* TOP10 cells (Invitrogen) were transformed and the plasmids isolated.

Expression and purification of GNE proteins in Sf9 insect cells

Generation of recombinant baculovirus and protein expression in *Sf9* cells was done by the BAC-TO-BAC® system (Invitrogen). Briefly, pFASTBAC™ 1 vector was transformed into *E. coli* DH10BAC™ cells. Bacmid DNA was generated by homologous recombination in the DH10BAC™ cells, selected by blue-white screening, isolated and transfected into *Sf9* cells. Five days post transfection, the first virus was harvested. This virus was used for a two step viral amplification after which the virus titer was determined through plaque assays. For protein expression 2×10^6 cells/ml were infected with the viruses with a MOI of 1 and incubated for 48–72 h. Cells were harvested by centrifugation at 700×*g* for 5 min at room temperature, then resuspended in lysis buffer (10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride; 1 ml per 10 ml cell culture) and lysed by a French-Press with a pressure of 0.5 bar. After centrifugation at 20,000×*g* for 30 min at 4°C the supernatant was applied to a Ni-NTA column (0.5 ml; Qiagen; Hilden, Germany). The column was washed with 5 ml of 10 mM NaH_2PO_4, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiotreitol, 20 mM imidazole, pH 8.0, and protein were eluted in 2 ml of 10 mM NaH_2PO_4, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiotreitol, 100 mM imidazole (pH 8.0). For desalting, fractions containing protein were applied to a PD-10 gel filtration column (Amersham; Freiburg, Germany) and eluted with 10 mM NaH_2PO_4, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.1 mM UDP. Fractions were assayed for protein concentration by the Coomassie protein assay kit (BioRad; München, Germany) and for purity by SDS-PAGE using a Mini-Protean III system (BioRad).

Expression and purification of hGNE3 in E. coli

hGNE3 was cloned as a GST-fusion protein into the multiple cloning site of pGEX 4T-1™ vector. The cDNAs were amplified by PCR using the pCR®-Blunt-hGNE3-His vector (see above) as template and the following degenerated primers which encode the cleavage sites for EcoRI and NotI, were used respectively (italic): hGNE3EC-For (5′-*GAATTC GAATTC*ATGGTTATCTGCAGAGGG-3′) hGNE3EC-Rev (5′-*GCGGCCGCGCGGCCGC* GTA GATCCTGCG-3′). The PCR product was digested, ligated into pGEX 4T-1™ vector and the resulting plasmid was transformed into *E. coli* BL21 (DE3) cells (Invitrogen). For protein expression, a 2 L culture of LB medium containing 100 μg/mL ampicillin was inoculated with 20 mL of an overnight culture and grown at 37°C to an A_{600} of 0.6, whereupon IPTG was added to a final concentration of 0.3 mM. After over night expression at 16°C, cells were harvested, resuspended in lysis buffer (10 mM NaH_2PO_4, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, 1 mM EDTA, 1 U/ml DNase, 10 μg/ml lysozyme, pH 7.5) and lysed by a French Press (1.0 bar). After centrifugation at 20,000×*g* for 30 min at 4°C the supernatant was applied to a 500 μl glutathione sepharose column (GE Healthcare; Freiburg, Germany), washed with 5 ml PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4), and eluted with 10 mM glutathione in PBS. Fractions were assayed for protein concentration by the Coomassie protein assay kit (BioRad) and for purity by SDS-PAGE (BioRad).

Determination of enzymatic activities

A colorimetric assay was used to determine the epimerase activity of recombinant GNE. The assay composed of 45 mM Na_2HPO_4, pH 7.5, 10 mM $MgCl_2$, 1 mM UDP-

GlcNAc and 0.2–1 μg of protein in a final volume of 200 μl. The reaction was performed at 37°C for 30 min and stopped by heating at 100°C for 1 min. ManNAc produced in this reaction was detected by mixing 150 μl of sample with 30 μl of 0.8 M H_2BO_3, pH 9.1, and boiling for 3 min. Then 800 μl of DMAB solution (1% (*w*/*v*) 4-dimethylamino benzaldehyde in acetic acid/1.25% 10 N HCl) was added and incubated at 37°C for a further 30 min. The absorbance was read at 578 nm. The assay used to determine the kinase activity of recombinant GNE contained 60 mM Tris/HCl, pH 8.1, 10 mM $MgCl_2$, 5 mM ManNAc, 10 mM ATP, 0.2 mM NADH, 2 mM phospho*enol*pyruvate, 2 U pyruvate kinase, 2 U lactate dehydrogenase, and 0.1–0.5 μg of protein in a final volume of 200 μl. The reaction was performed for 20 min at 37°C and stopped by addition of 800 μl 10 mM EDTA. The decrease of NADH was monitored at 340 nm.

To determine the UDP-GlcNAc 2-epimerase activity of GNE isoforms from *CHO* Lec3 cells a radiometric assay was used [23]. In brief, a UDP-GlcNAc 2-epimerase assay composed of 45 mM Na_2HPO_4, pH 7.5, 10 mM $MgCl_2$, 1 mM UDP-GlcNAc, 50 nCi of UDP-[^{14}C]GlcNAc. The maximum volume of 80 μl GNE protein was used. The final volume of mixture was 145 μl and incubations were carried out at 37°C for 4 h. The resulting radiolabeled [^{14}C] ManNAc were separated by paper chromatography for 16–20 h in 70% (*v*/*v*) 1-propanol/100 mM sodium acetate (pH 5.0). Radioactivity was determined in the presence of Ultima Gold XR (Packard; Groningen, Netherlands) in a Tri-Carb 1900 CA liquid scintillation analyzer (Packard).

Determination of oligomeric states

The oligomeric state of GNE was determined with purified proteins by gel filtration on a Superdex®200 (Amersham). A buffer containing 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl was used as eluent. The extinction was detected at 280 nm at a flow rate of 0.5 ml/min. The molecular masses of the protein were determined by calibration of the column with a standard mixture of thyreoglobulin (670 kDa), IgG (158 kDa) and ovalbumin (44 kDa), and myoglobin (17 kDa).

Transient protein expression of human and murine GNE isoforms in CHO Lec3 cells

The cDNAs of human and murine GNE isoforms were excised from the pFASTBAC 1™ vectors by SalI and KpnI and ligated into the pUMVC3 vector (Aldevron; Fargo ND, USA). *CHO* Lec3 cells [24] were transient transfected with the isolated plasmids via TransFectin™ (BioRad; München, Germany) according to the instruction of the manufactors. After an incubation time of 48 h, cells were harvested by centrifugation at 900 rpm for 3 min and resuspended in lysis buffer. Cells were lysed by 20 strokes through a syringe with a narrow needle and centrifuged at 16,000×*g* for 20 min. The cytosolic supernatant was then analyzed by the radiometric epimerase assay.

Results

Recombinant expression of human GNE isoforms

The cDNAs of hGNE1 and hGNE2 were amplified by PCR using a degenerated reverse primer which encodes for a 6xHis-tag added to the C-terminus of the resulting protein. For generation of hGNE3 cDNA an additional degenerated forward primer was used containing the isoform-specific codons. The PCR products were ligated into the pCR®-Blunt vector. After confirmation of the DNA sequences, the inserts were excised with XhoI and KpnI and ligated into the pFASTBAC 1™ vector. The plasmids were isolated and used for bacmid generation. After production of baculoviruses in *Sf9* cells, the same cells were used for protein expression. Cells were lysed, and after centrifugation the cytosolic supernatant was analyzed by SDS-PAGE. hGNE1 and hGNE2 were successfully over expressed, but hGNE3 protein was not observed in the fraction containing soluble proteins. However, analysis of the pellet obtained after centrifugation of the lysate of cells expressing hGNE3 by SDS-PAGE and western blot revealed a prominent band of about 70 kDa. This indicates the expression of GNE protein with strong structural alterations, which tends to precipitate when expressed in insect cells [25, 26]. Replacement of the first 59 amino acids by 14 new amino acids obviously did not compensate the structural alteration which led to protein precipitation.

Overexpressed hGNE1 and hGNE2 were purified by Ni-NTA affinity chromatography by their C-terminal 6xHis-tag. SDS-PAGE analysis revealed almost homogenous proteins with apparent molecular masses of 70 and 75 kDa, respectively (Fig. 2). The slight differences of the molecular masses in SDS-PAGE compared to the theoretical predictions (Fig. 1) is typical for GNE, and due to the principle of the SDS-PAGE method [23, 25]. hGNE2 has an extended N-terminus of 31 additional amino acids and its size is therefore increased by 5 kDa compared to hGNE1. hGNE1 was also expressed with an N-terminal 6xHis-tag in insect cells in former studies and found to be a 75 kDa protein [25]. However, this shift of size is due to a larger N-terminus with a cleavable linker between the 6xHis-tag and the GNE protein.

As hGNE3 could not be expressed in insect cells, its cDNA was amplified with degenerated primers creating

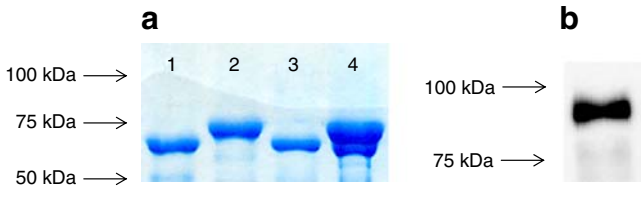

Fig. 2 Recombinant expression of human and mouse GNE isoforms. (**A**) Proteins were expressed in insect (*Sf9*) cells, affinity purified and analyzed by SDS-PAGE. (*1*) hGNE1, (*2*) hGNE2, (*3*) mGNE1, (*4*) mGNE2. (**B**) hGNE3 was expressed in *E. coli* (BL21) cells as a GST fusion protein. The protein was affinity purified and analyzed by western blotting using α-GST antibody

cleavage sites for EcoRI and NotI, which enabled cloning into the pGEX 4T-1™ vector. This vector is suited for expression of proteins in *E. coli* with an N-terminal GST-tag. By this method hGNE3 was successfully expressed and purified by GST affinity chromatography. Analysis of the fractions by SDS-PAGE revealed a prominent 95 kDa band, which was determined by Western blot with α-GST antibody to be the GST-hGNE3 fusion protein (Fig. 2). However, more than 95% of the expressed protein was found in the insoluble fraction after cell lysis and centrifugation. This was already observed for the expression of a rat GST-hGNE1 fusion protein [25], and therefore does not seem to have an effect of the GNE3-specific N-terminus.

Protein expression of murine GNE isoforms

The cDNAs of mGNE1 and mGNE2 were amplified by PCR using degenerated reverse primer which encodes for a 6xHis-tag added to the C-terminus of the resulting protein. The PCR products were ligated into the pCR®-Blunt vector. After confirmation of the DNA sequences, the inserts were excised with XhoI and KpnI and ligated into the pFASTBAC 1™ vector. The plasmids were isolated and DH10BAC™ cells were transformed for bacmid generation. After production of baculoviruses in *Sf9* cells, the same cells were used for protein expression. Cells were lysed via French-Press and after centrifugation the cytosolic supernatant was analyzed by SDS-PAGE. mGNE1 and mGNE2 were successfully over expressed in the same amounts as the human counterparts. The lysates were purified by Ni-NTA affinity chromatography. SDS-PAGE analysis revealed a homogenous mGNE1 protein with a size of 70 kDa (Fig. 2), in agreement with the molecular masses predicted from cDNA sequences.

In contrast to mGNE1, purified mGNE2 displayed two bands of 75 and 70 kDa in SDS-PAGE analysis (Fig. 2). Whereas the 75 kDa band is in agreement with the 31 amino acids extension leading to a 5 kDa increase in size compared to mGNE1, the nature of the lower band remained unclear. Therefore, first a peptide mass fingerprint was performed, which confirmed that both bands represent recombinant mouse GNE protein (data not shown). We then considered that the cDNA sequence of mGNE2 contained a potential alternative start codon, which is equivalent to the start codon of mGNE1, resulting in expression of a 70 kDa protein. Consequently, we mutated the potential alternative start codon, but expression of the mGNE2 M32A mutant again revealed the double band (data not shown). As further control experiments, we performed protein expression in the presence of two protease inhibitors (Trasylol, MG 132), and analyzed the proteins for modification by phosphate, applying alkaline phosphatase treatment. In both cases the 70 and 75 kDa bands were still found in SDS-PAGE. Finally, immunoblot analysis revealed no protein ubiquitinylation.

Determination of enzyme activities and oligomeric structures

The epimerase and kinase activities of the purified GNE isoforms were determined by colorimetric assays (Fig. 3). The specific epimerase activity of hGNE1 is approximately 1100 mU/mg, which is almost the same activity as the hGNE1 with N-terminal 6xHis-tag described in former studies [25]. The specific epimerase activity of hGNE2 is reduced approximately 80%. For hGNE3 no epimerase activity could be measured. The epimerase activities of the murine isoforms are equivalent to the hGNE1 epimerase activity. hGNE1 has a specific kinase activity of approximately 2500 mU/mg, which is almost the same as N-terminal 6xHis tagged GNE from former works [22]. Furthermore, the specific kinase activities of hGNE2 and hGNE3 as well as mGNE1 and mGNE2 have approximately the same range.

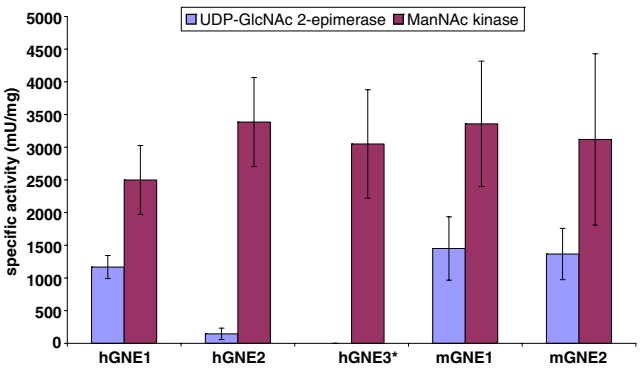

Fig. 3 Determination of enzymatic activities of recombinant GNE isoforms. The cells were lysed, proteins were affinity purified, and assayed for *UDP-GlcNAc 2-epimerase* and *ManNAc kinase* activities by colorimetric methods. Values are means ± SD of eight independent experiments. *Note, that hGNE3 was expressed in *E. coli* cells

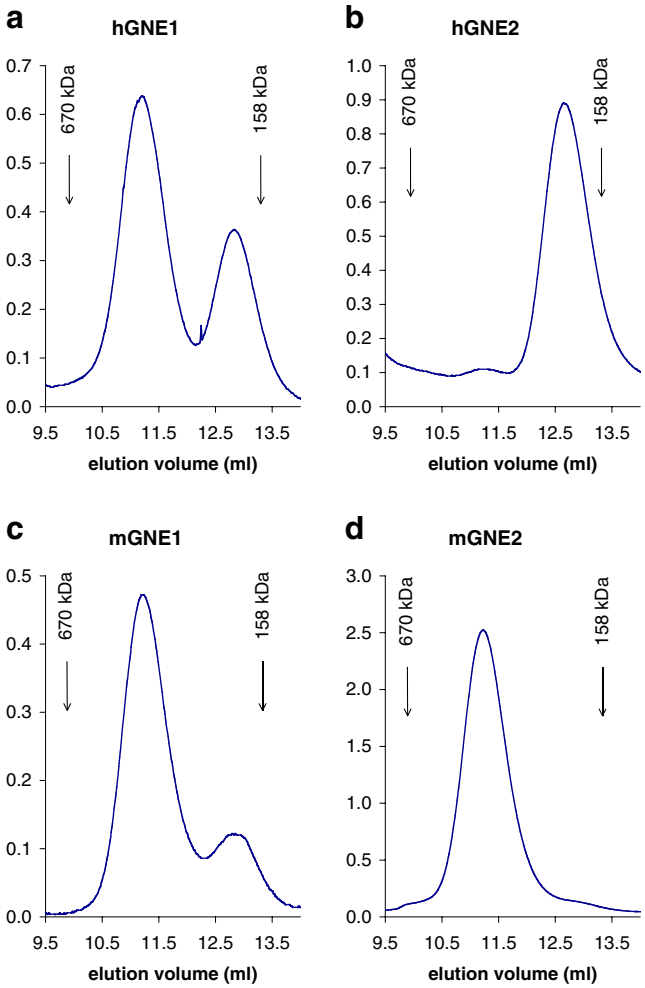

Fig. 4 Determination of oligomeric states of recombinant GNE isoforms. Purified proteins were analyzed on a Sephadex 200 column. *Arrows* indicate elution volumes of standard proteins. Peaks at about 11 ml represent tetramers, peaks at about 13 ml represent dimers

Different biophysical methods revealed that hGNE1 exists as a tetramer and dimer [27], whereby the tetrameric state has full enzyme activity, however the dimer only possesses kinase activity. We confirmed by size exclusion chromatography that recombinant purified hGNE1 and mGNE1 are mixtures of tetramers and dimers (Fig. 4). The ratios of the two oligomeric states were 3:1 and 4:1, respectively (Fig. 4), whereas mGNE2 displays fully tetrameric state and hGNE2 occurs only as a dimer. hGNE3 was not analyzed due to its low expression level.

The observation of dimeric hGNE2 was in contrast to the fact that epimerase activity was measured for this enzyme. This could be due to a reformation of epimerase-active tetramers in the presence of the substrate UDP-GlcNAc, as already observed before [23]. We therefore performed kinetic measurements of hGNE2 and found that there was no initial epimerase velocity of the enzyme. Epimerase activity was observed in later stages of the experiment (data not shown). This indicates that the assay conditions, which include saturating concentrations of UDP-GlcNAc [23], promote partial reformation of the enzymatically active tetramer, as observed in size exclusion chromatography of an aliquot subsequent to the assay (data not shown).

Transient protein expression of human and murine GNE isoforms in CHO Lec3 cells

The human and murine GNE isoforms were transient expressed in *CHO* Lec3 cells, because of their lack of endogenous GNE activity [24]. The cDNA constructs were ligated into the pUMVC3 vector. *CHO* Lec3 cells were transfected with the isolated plasmids via TransFectin™. After an incubation time of 48 h, cells were harvested, lysed, and after centrifugation the cytosolic supernatants were analyzed by the radiometric epimerase assay (Fig. 5). The epimerase activity of the different GNE isoforms is equivalent to the expressed recombinant proteins. The specific activity of hGNE1 and mGNE1 has approximately the same range, whereas for hGNE3 no epimerase activity was detected, in agreement with the results of hGNE3 expression in *E. coli*. The epimerase activity of hGNE2 and mGNE2 are significantly different. mGNE2 exceeds hGNE1 2.5-fold in epimerase activity, and therefore represents the most active GNE isoform. hGNE2 showed a drastically reduced UDP-GlcNAc 2-epimerase activity, in accordance with the results from the protein expressed in *Sf9* cells. This is likely due to the same impaired tetramer formation. However, analogous size exclusion chromatography analysis of hGNE2 expressed in *CHO* cells failed because of the overall low expression level. A fourfold excess of UDP-GlcNAc compared to the saturating concentration of GNE1 [23] in the hGNE2 enzyme assay did

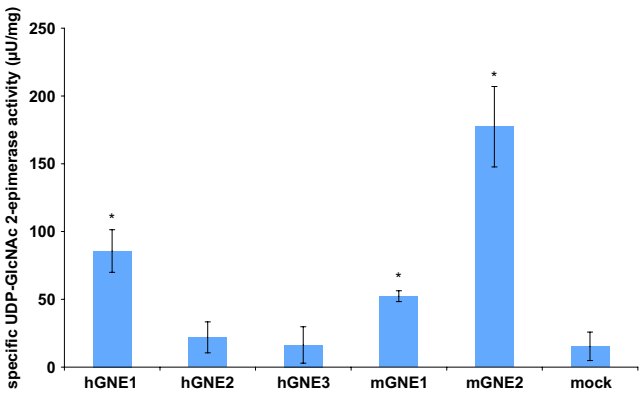

Fig. 5 UDP-GlcNAc 2-epimerase activity of *CHO* Lec3 cells transiently transfected with GNE isoforms. The cells were lysed and epimerase activity was determined by the radiometric assay. Values are means ± SD of for independent experiments (*$p<0.05$)

not significantly increase epimerase activity. We therefore conclude, that tetramer reformation of hGNE2 could not be promoted by increased UDP-GlcNAc concentration. Otherwise the amount of recombinant hGNE2 from *CHO* Lec3 cells seems to be too low for tetramer reformation, which also depends on the protein concentration [23].

Discussions

The human isoforms hGNE1 and hGNE2 and the two murine isoforms mGNE1 and mGNE2 were expressed in *Sf9* insect cells as soluble active enzymes and purified by affinity chromatography in amounts of 2–4 mg/100 ml cell culture. This yield is in agreement with former studies of GNE expression in insect cells. The SDS-PAGE analysis of purified hGNE1, hGNE2, and mGNE1 revealed a distinct protein band. In contrast, mGNE2 displayed two bands in SDS-PAGE and western blot analysis. A peptide mass fingerprint showed that both bands are mGNE proteins. Although we have performed several control experiments the reason for the shift in size of the lower band remained unclear. Although the size of the lower band fits with the size of mGNE1, we can exclude that our mGNE2 preparation contains mGNE1 for the following reasons: (1) Mutation of the mGNE1 start codon in the mGNE2 cDNA did not result in loss of the 70 kDa band and (2) Size exclusion chromatography of mGNE2 revealed no dimeric protein, as it was observed for mGNE1. However, we cannot exclude that the mGNE2 double band is an artifact of the insect cell expression system. We therefore expressed all proteins additionally in a mammalian cell line, namely *CHO* Lec3. The epimerase activities of the Lec3 expressed proteins confirmed the data of the proteins expressed in insect cells, in particular the full enzymatic activity of mGNE2 and the lack of epimerase activity of hGNE2. We therefore conclude that hGNE2 and mGNE2 have different biochemical properties, likely due to independent evolutionary pathway. It can be assumed, that the two proteins have roles outside of sialic acid biosynthesis, *e.g.* in ganglioside biosynthesis, proliferation, and apoptosis as already shown in an independent study [28]. A further example of proteins with different functions is the photolyase/cryptochrome family. Except for humans the function of this DNA-photolyases is the UV-induced repair of DNA damage and the formation of cyclobutan-pyrimidine dimers. In humans photolyases do not contribute to DNA repair, but is involved in the circadian rhythm [29].

hGNE1, mGNE1, and mGNE2 formed UDP-GlcNAc 2-epimerase active tetramers, whereas hGNE2 showed a drastically reduced UDP-GlcNAc 2-epimerase activity. This was due to the formation of dimers, and residual epimerase activity of hGNE2 in some assays was promoted by tetramer reassociation in the presence of excess UDP-GlcNAc. In contrast, all recombinant proteins, including hGNE3, displayed ManNAc kinase activity in the same range. Obviously, the structural alterations of the N-terminus have no effect on the C-terminal kinase domain. However, the influence of additional amino acids on the N-terminus of the GNE2 protein, which participate in tetramer formation of GNE [26], seems to be unique to the additional peptide sequence. The homology of the additional N-termini of hGNE2 and mGNE2 is only 58%, and the different amino acids appear to have a strong influence on tetramer formation. Therefore mutagenesis experiments are planned to investigate the role of single amino acids on the N-terminus of human and murine GNE function.

The results of this study lead to the assumption that hGNE1 in general is responsible for the basic supply of the cells with sialic acids. hGNE2 and hGNE3 may have functions in particular fine-tuning of the sialic acid pathway, which is involved in regulation of the sialylation of glycoconjugates via the intracellular concentration of sialic acid and CMP-sialic acid [30]. For example, when an increased sialic acid amount is required in cells, hGNE2 and hGNE3 may increase overall ManNAc kinase activity and contribute to Neu5Ac production and fill up the sialic acid pool. However, the differences between hGNE2 and mGNE2, as discussed above, also suggested functions of at least some isoforms independent from sialic acid biosynthesis. Further studies including specific knock-down of the isoforms by RNAi or the generation of animal models with altered isoform expression may give a more detailed view into the particular functions of the GNE isoforms.

Acknowledgements This work was supported by the German-Israeli Foundation for Science Research & Development, Jerusalem, Israel. We thank Werner Reutter for helpful discussion and Christiane Kilian for excellent technical assistance. *CHO* Lec3 cells were kindly provided by Pamela Stanley.

References

1. Corfield, A.P., Schauer, R.: Occurrence of sialic acids. In: Schauer, R. (ed.) Sialic Acids. Chemistry, Metabolism, and Function, pp. 5–50. Springer, Wien (1982)
2. Varki, A.: Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature **446**, 1023–1029 (2007). doi:10.1038/nature05816
3. Bhavanandan, V.P.: Cancer-associated mucins and mucin-type glycoproteins. Glycobiology **1**, 493–503 (1991). doi:10.1093/glycob/1.5.493
4. Schauer, R., Kelm, S., Reuter, G., Roggentin, P., Shaw, L.: Biochemistry and role of sialic acids. In: Rosenberg, A. (ed.) Biology of the Sialic Acids, pp. 7–67. Plenum, New York (1995)
5. Rens-Domiano, S., Reisine, T.J.: Structural analysis and functional role of the carbohydrate component of somatostatin receptor. Biol. Chem. **266**, 20094–20102 (1991)

6. Angata, T., Varki, A.: Chemical diversity in the sialic acids and related α-keto acids: an evolutionary perspective. Chem. Rev. **102**, 439–469 (2002). doi:10.1021/cr000407m
7. Hinderlich, S., Oetke, C., Pawlita, M.: Biochemical engineering of sialic acids. In: Yarema, K.J. (ed.) Handbook of Carbohydrate Engineering, pp. 387–405. Taylor & Francis, Boca Raton (2005)
8. Kornfeld, S., Kornfeld, R., Neufeld, E.F., O'Brien, P.J.: The feedback control of sugar nucleotide biosynthesis in liver. Proc. Natl. Acad. Sci. USA **52**, 371–379 (1964). doi:10.1073/pnas.52.2.371
9. Watson, D.R., Jourdian, G.W., Roseman, S.: The sialic acids. 8. Sialic acid 9-phosphate synthetase. J. Biol. Chem. **241**, 5627–5636 (1966)
10. Chen, H., Blume, A., Zimmermann-Kordmann, M., Reutter, W., Hinderlich, S.: Purification and characterization of *N*-acetylneuraminic acid-9-phosphate synthase from rat liver. Glycobiology **12**, 65–71 (2002). doi:10.1093/glycob/12.2.65
11. Kean, E.L., Roseman, S.: The sialic acids. X. Purification and properties of cytidine 5′-monophosphosialic acid synthetase. J. Biol. Chem. **241**, 5643–5650 (1966)
12. Harduin-Lepers, A., Mollicone, R., Delannoy, P., Oriol, R.: The animal sialyltransferases and sialyltransferase-related genes: a phylogenetic approach. Glycobiology **15**, 805–817 (2005). doi:10.1093/glycob/cwi063
13. Keppler, O.T., Hinderlich, S., Langner, J., Schwartz-Albiez, R., Reutter, W., Pawlita, M.: UDP-GlcNAc 2-Epimerase: a regulator of cell surface sialylation. Science **284**, 1372–1376 (1999). doi:10.1126/science.284.5418.1372
14. Schwarzkopf, M., Knobeloch, K.-P., Rohde, E., Hinderlich, S., Wiechens, N., Lucka, L., *et al.*: Sialylation is essential for early development in mice. Proc. Natl. Acad. Sci. U S A **99**, 5267–5270 (2002). doi:10.1073/pnas.072066199
15. Seppala, R., Lehto, V.P., Gahl, W.A.: Mutations in the human UDP-*N*-acetylglucosamine 2-epimerase gene define the disease sialuria and the allosteric site of the enzyme. Am. J. Hum. Genet. **64**, 1563–1569 (1999). doi:10.1086/302411
16. Eisenberg, I., Grabov-Nardini, G., Hochner, H., Korner, M., Sadeh, M., Bertorini, T., *et al.*: Mutations spectrum of GNE in hereditary inclusion body myopathy sparing the quadriceps. Hum. Mutat. **21**, 99–105 (2002). doi:10.1002/humu.9100
17. Eisenberg, I., Avidan, N., Potikha, T., Hochner, H., Chen, M., Olender, T., *et al.*: The UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase gene is mutated in recessive hereditary inclusion body myopathy. Nat. Genet. **29**, 83–87 (2001). doi:10.1038/ng718
18. Watts, G.D.J., Thorne, M., Kovach, M.J., Pestronk, A., Kimonis, V.E.: Clinical and genetic heterogeneity in chromosome 9p associated hereditary inclusion body myopathy: exclusion of GNE and three other candidate genes. Neuromuscul. Disord. **13**, 559–567 (2003). doi:10.1016/S0960-8966(03)00070-1
19. Lucka, L., Krause, M., Danker, K., Reutter, W., Horstkorte, R.: Primary structure and expression analysis of human UDP-*N*-acetyl-glucosamine-2-epimerase/*N*-acetylmannosamine kinase, the bifunctional enzyme in neuraminic acid biosynthesis. FEBS Lett. **454**, 341–344 (1999). doi:10.1016/S0014-5793(99)00837-6
20. Effertz, K., Hinderlich, S., Reutter, W.: Selective loss of either the epimerase or kinase activity of UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase due to site-directed mutagenesis based on sequence alignments. J. Biol. Chem. **274**, 28771–28778 (1999). doi:10.1074/jbc.274.40.28771
21. Reinke, S.O., Hinderlich, S.: Prediction of three different isoforms of the human UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase. FEBS Lett. **581**, 3327–3331 (2007). doi:10.1016/j.febslet.2007.06.026
22. Hinderlich, S., Salama, I., Eisenberg, I., Potikha, T., Mantey, L.R., Yarema, K.J., *et al.*: The homozygous M712T mutation of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase results in reduced enzyme activities but not in altered overall cellular sialylation in hereditary inclusion body myopathy. FEBS Lett. **566**, 105–109 (2004). doi:10.1016/j.febslet.2004.04.013
23. Hinderlich, S., Stäsche, R., Zeitler, R., Reutter, W.: A bifunctional enzyme catalyzes the first two steps in *N*-acetylneuraminic acid biosynthesis of rat liver. Purification and characterization of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase. J. Biol. Chem. **272**, 24313–24318 (1997). doi:10.1074/jbc.272.39.24313
24. Hong, Y., Stanley, P.: Lec3 Chinese hamster ovary mutants lack UDP-N-acetylglucosamine 2-epimerase activity because of mutations in the epimerase domain of the Gne gene. J. Biol. Chem. **278**, 3045–3054 (2003)
25. Blume, A., Ghaderi, D., Liebich, V., Hinderlich, S., Donner, P., Reutter, W., *et al.*: UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase, functionally expressed in and purified from Escherichia coli, yeast, and insect cells. Protein Expr. Purif. **35**, 387–396 (2004). doi:10.1016/j.pep.2004.02.013
26. Blume, A., Weidemann, W., Stelzl, U., Wanker, E.E., Lucka, L., Donner, P., *et al.*: Domain-specific characteristics of the bifunctional key enzyme of sialic acid biosynthesis, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase. Biochem. J. **384**, 599–607 (2004). doi:10.1042/BJ20040917
27. Ghaderi, D., Strauss, H.M., Reinke, S., Cirak, S., Reutter, W., Lucka, L., *et al.*: Evidence for dynamic interplay of different oligomeric states of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase by biophysical methods. J. Mol. Biol. **369**, 746–758 (2007). doi:10.1016/j.jmb.2007.03.037
28. Wang, Z., Sun, Z., Li, A.V., Yarema, K.J.: Roles for UDP-GlcNAc 2-epimerase/ManNAc 6-kinase outside of sialic acid biosynthesis: modulation of sialyltransferase and BiP expression, G_{M3} and G_{D3} biosynthesis, proliferation, and apoptosis, and ERK1/2 phosphorylation. J. Biol. Chem. **281**, 27016–27028 (2006). doi:10.1074/jbc.M604903200
29. Christmann, M., Tomicic, M.T., Roos, W.P., Kaina, B.: Mechanisms of human DNA repair: an update. Toxicology **193**, 3–34 (2003). doi:10.1016/S0300-483X(03)00287-7
30. Bork, K., Reutter, W., Gerardy-Schahn, R., Horstkorte, R.: The intracellular concentration of sialic acid regulates the polysialylation of the neural cell adhesion molecule. FEBS Lett. **579**, 5079–5083 (2005). doi:10.1016/j.febslet.2005.08.013