Glycoconjugate Journal (1996) **13:** 353–358

A naturally occurring 46-amino acid deletion of cytidine monophospho-*N*-acetylneuraminic acid hydroxylase leads to a change in the intracellular distribution of the protein*

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Received 30 June 1995; revised 25 October 1995

Cytidine monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) hydroxylase is a key enzyme for the expression of *N*-glycolylneuraminic acid. The molecular cloning of this enzyme from mouse liver has been described in our previous report (Kawano T, Koyama S, Takematsu H, Kozutsumi Y, Kawasaki H, Kawashima S, Kawasaki T, Suzuki A (1995) *J Biol Chem* **270**: 16458–63). During the cDNA cloning, a cDNA containing a truncated open reading frame (ORF) was isolated. This clone encodes a protein of 531 amino acids which lacks 46 amino acids in the middle of the normal full-length protein. The percentage of this mRNA containing the truncated ORF out of the total population of CMP-NeuAc hydroxylase mRNA in various mouse tissues was about 10–25%. The truncated protein was expressed in COS-1 cells, but did not show any enzymatic activity. The truncated protein was localized to the region which appeared to be the endoplasmic reticulum, whereas the full-length protein with normal enzymatic activity was detected in the cytosol. These data suggest that this naturally occurring 46-amino acid deletion leads to a change in the intracellular distribution of CMP-NeuAc hydroxylase, and a loss in the activity of this enzyme.

Keywords: cytidine monophospho-*N*-acetylneuraminic acid hydroxylase, *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, membrane transport

Abbreviations: NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; ORF, open reading frame; ER, endoplasmic reticulum; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pI, isoelectric point; bp, base pair(s).

Introduction

Sialic acid derivatives of glycoconjugates participate in various biological functions, such as cell adhesion [1], lymphocyte homing [2], and viral infection [3]. Sialic acids are now known to be a family of compounds, consisting of over 30 members [4, 5]. *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) are two major members of this family of sialic acids.

The expression of NeuGc varies between animal species and between tissues. In mice, NeuGc-containing gangliosides are strongly expressed in liver [6]. High levels of NeuGc are also observed in the bovine thymus and porcine submandibular glands [7, 8], and the poly-

*This paper is dedicated to Dr Sen-ichiro Hakomori in celebration of his 65th birthday.

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morphic expression of NeuGc-containing glycoconjugates in particular tissues has also been reported in dogs [9, 10], cats [11] and rats [12]. However, essentially no NeuGc is found in the brains of most animals [13]. On the other hand, the expression of NeuGc is barely detectable under normal conditions in humans. However, when humans are exposed to animal antisera such as horse serum, the Hanganutziu-Deihcher antibody is produced, which is an antibody against NeuGc-containing glycoconjugates [14]. NeuGc-containing glycoconjugates can also be detected in colon cancer tissues [15], melanoma tissues [16], retinoblastoma cells [17], breast cancer tissues [18], other malignant tumours [19, 20] and fetal tissues [15]. Therefore, NeuGc appears to be an oncofetal antigen in humans. Recently, Kelm *et al.* reported that mouse CD 22 bound preferentially to NeuGc-containing oligosaccharides rather than NeuAc-containing oligosaccharides, and that the specificity of mouse sialoadhesin was specific for NeuAc-containing oligosaccharides [21].

NeuGc is derived from NeuAc by hydroxylation of a methyl moiety in the *N*-acetyl residue. This hydroxylation occurs by the conversion of CMP-NeuAc to CMP-NeuGc, which is carried out by an electron transport system involving NADH-dependent cytochrome b_5 reductase, cytochrome b_5 (b_5), and CMP-NeuAc hydroxylase [22, 23]. CMP-NeuAc hydroxylase is the key enzyme in the hydroxylation of CMP-NeuAc, and functions at the final step of the electron transport system. We have previously purified CMP-NeuAc hydroxylase from the mouse liver [24], and isolated a cDNA for this enzyme which contains an ORF encoding a protein of 577 amino acids [25]. During the cloning process, we also obtained a truncated cDNA clone encoding a protein of 531 amino acids. In this paper, this truncated protein has been characterized using an expression system with COS-1 cells.

Materials and methods

Polymerase chain reaction (PCR)

A cDNA pool was prepared by reverse transcription using random primers from mouse liver total RNA as templates. The PCR was performed using sense and antisense primers (10 pmol each), with 1.3 U of *Taq* polymerase (Promega) in a reaction mixture (25 μl) containing 1 μg cDNA [25]. The primers used for individual detection of the full-length and truncated mRNAs are 5′-ATTTATGTTGGCGACAC-3′ (nucleotides 652–668 of Fig. 2 of [25]) for the sense primer and 5′-TAAGATTGTTGAGCTGA-3′ (nucleotides 1163–1147 of Fig. 2 of [25]) for the antisense primer. The amplification was performed for 30 cycles of: 94 °C for 0.5 min, 40 °C for 1 min and 72 °C for 1.5 min, using a Zymoreactor II thermal cycler (Atto Corp.). The PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide.

DNA sequencing

The DNA sequencing reaction was performed using Dye Deoxy terminators (Applied Biosystems) and Ampli *Taq* DNA polymerase (Applied Biosystems) in a thermal cycler. Gel electrophoresis and analysis of the data were performed with a 373A DNA sequencer (Applied Biosystems). More than three clones were sequenced in order to compensate for misreading by the *Taq* polymerase. Hydropathy indices were estimated by the Kyte-Doolittle method [26], with a sliding window of nine amino acids.

Expression of cDNAs in COS-1 cells

CMP-NeuAc hydroxylase cDNAs containing the full-length or truncated ORF were ligated into the *Bam* HI site of the expression vector pdKCR [27]. COS-1 cells were obtained from the Japanese Cancer Research Bank (Tokyo), and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The COS-1 cells were then transfected with the purified plasmid by the calcium phosphate precipitation method [25]. At 60 h of incubation, the cells were harvested, rinsed and collected by centrifugation. The harvested cells were rinsed again and homogenized in Tris-buffered saline (TBS). The homogenate was centrifuged at 800 × **g** for 5 min, and the resultant pellet (nuclear fraction) was suspended in TBS. The supernatant was further centrifuged at 105 000 × **g** for 60 min. The second pellet and supernatant were designated as the membrane and cytosolic fractions, respectively. The determination of the sialic acid species was performed according to Hara *et al.* [28], after the samples were hydrolysed in 0.05 N H_2SO_4 at 80 °C for 6 h. CMP-NeuAc hydroxylase activity was then measured as previously described [24]. The protein concentrations were determined with a BCA protein assay kit, with bovine serum albumin as the standard.

Preparation and characterization of a rabbit anti-CMP-NeuAc hydroxylase antiserum

Expression of the *N*-terminal portion of mouse CMP-NeuAc hydroxylase in *E. coli* was performed using the Protein Fusion and Purification System (NEB). The *Bam* HI and *Hpa* I fragments of CMP-NeuAc hydroxylase cDNA subcloned into Bluescript II (see Results) were then ligated into the *Bam* HI site and the *Xba* I site, which was filled, of the pMAL c-2 vector. The resultant plasmid has the ability to express a fusion protein containing a maltose binding protein encoded by pMALc-2, a hexapeptide derived from a portion of the polylinker cloning sites of pMALc-2, the *N*-terminal 238 amino acids of CMP-NeuAc hydroxylase, and a leucine residue, in this order. This plasmid was then transfected into *E. coli* (XL-1

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  1 MMDRKQTAETLLTLSPAEVANLKEGINFFRNKTTGKEYILYKEKDHLKACKNLCKHQGGLFMKDI
 66 EDLDGRSVKCTKHNWKLDVSTMKYINPPGSFCQDELVIEMDENNGLSLVELNPPNPWDSDPRSPE
131 ELAFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLCKADLIYISH
196 MHSDHLSYPTLKQLSQRRPDIPIYVGDTERPVFWNLDQSGVGLTNINVVPFGIWQQVDKSLRFMI
261 LMDGVHPEMDTCIIVEYKGHKILNTVFCTRPNGGRLPEKVALMMSLEAGGASGFPMTFSGGKFTE
326 EWKAQFIKAERRKLLNYKAQLVKDLQPRIYCPFAGYFVESHPSDKYIKETNTKNDPNQLNNLIRK
391 NSDVVTWTPRPGAVLDLGRMLKDPTDSKGIVEPPEGTKIYKDSWDFGPYLEILNSAVRDEIFCHS
456 SWIKEYFTWAGFKNYNLVVRMIETDEDFSPFPGGYDYLVDFLDLSFPKERPSREHPYEEIHSRVD
521 VIRYVVKNGLLWDDLYIGFQTRLLRDPDIYHHLFWNHFQIKLPLTPPNWKSFLMHCD*
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Figure 1. Amino acid sequence of both full-length and truncated open reading frames of CMP-NeuAc hydroxylase. There are two potential sites for translation initiation, and the upstream Met is taken as site 1, as previously described [25]. The deletion site for the truncated protein is boxed. The arrows indicate the position and direction of the PCR primers used for individual detection of the full-length and truncated mRNAs.

blue), and the fusion protein was purified from cell lysates using an amylose column according to the manufacturer's protocol. The purified fusion protein was injected subcutaneously into two rabbits with an equal volume of either complete (first injection) or incomplete (subsequent injections) Freund's adjuvant. A total of three injections were given at 2 week intervals before the serum samples were collected. The sera were tested for their ability to recognize CMP-NeuAc hydroxylase from mouse liver on immunoblots following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified CMP-NeuAc hydroxylase and the cytosolic fraction from mouse liver gave rise to a single band, and non-specific bands were not detected on the blots under the conditions used (data not shown).

Indirect immunofluorescence

The intracellular localization of CMP-NeuAc hydroxylase expressed in COS-1 cells was examined by indirect immunofluorescence using permeabilized cells, as previously described [29], at 24 h after the transfection. Rabbit anti-CMP-NeuAc hydroxylase antiserum (diluted 1:250) was used as the primary antibody, and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins were used as the secondary antibody.

Immunoblot analysis

SDS-PAGE was performed essentially as described by Laemmli [30], and immunoblot analysis was carried out as described by Harlow and Lane [31] using rabbit anti-CMP-NeuAc hydroxylase antisera (diluted 1:200) as the primary antibody and peroxidase-conjugated goat anti-rabbit immunoglobulins as the secondary antibody.

Results

DNA sequence and deduced amino acid sequence of the cDNA with a truncated open reading frame

To obtain fragments containing the CMP-NeuAc hydroxylase ORF, two primers were used to PCR-amplify with a cDNA pool prepared from mouse liver total RNA. The primers contained either the 5′- or the 3′-end of the coding region plus a *Bam* HI recognition sequence at both ends. The amplified fragment, which was shown as a doublet by agarose gel electrophoresis (data not shown), was subcloned into Bluescript II. Twenty-seven clones were picked and analysed; 22 clones contained the full-length ORF and five clones had the truncated ORF. The amino acid sequence of the clone with the truncated ORF is shown in Fig. 1*. The truncated cDNA lacks 138 bp in the middle of the full-length ORF, without a codon frame-shift downstream, and encodes a protein of 531 amino acids. This protein has a predicted molecular mass of 62 kDa, which is 5 kDa shorter than the full-length protein.

Tissue distribution

To examine the tissue distributions of both truncated and full-length ORF-containing mRNAs, a reverse transcription-polymerase chain reaction (RT-PCR) was carried out with primers flanking the deleted sequence (Fig. 1). As shown in Fig. 2, two fragments from each of the truncated and full-length mRNAs were amplified. The ratio of two PCR products coamplified using the same primers is thought to be constant throughout the amplification, when both amplified cDNAs share the same DNA sequences to

*The nucleotide sequence reported in this paper has been submitted to the GenBankTM/EMBL Data Bank under accession number D21826.

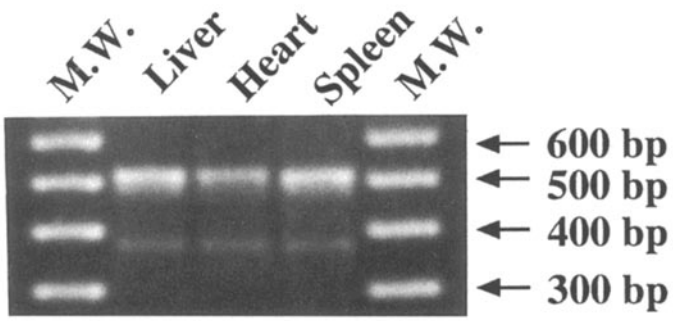

Figure 2. Tissue distribution of both full-length and truncated mRNAs. RT-PCR analysis was performed using the two primers shown in Fig. 1 to compare the amounts of both mRNA species present. The amplified fragments were analysed by agarose gel electrophoresis and quantified by a densitometer. 510 bp and 372 bp fragments were amplified for the full-length and truncated mRNAs, respectively.

which the PCR primers anneal [32–34]. Therefore, the ratio of the truncated and full-length mRNA can be estimated by agarose gel electrophoresis after PCR. The band intensity of the truncated mRNA showed it to be a minor component, but it comprised about 15, 25 and 10% of the total CMP-NeuAc hydroxylase mRNA in the liver, heart and spleen, respectively.

CMP-NeuAc hydroxylase activity

Elevated CMP-NeuAc hydroxylase activity was not detected in the cytosolic fraction from COS-1 cells transfected with the truncated cDNA, in the presence of exogenous b_5 (Fig. 3). In contrast, transfection with the full-length cDNA resulted in the elevation of enzymatic activity in the presence of b_5 as previously described [24]. Increased cellular NeuGc content was also detected in cells transfected with the full-length cDNA, but not in cells transfected with the truncated cDNA (Fig. 4).

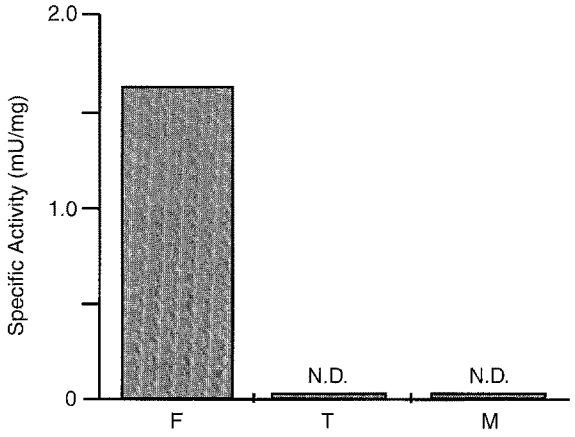

Figure 3. CMP-NeuAc hydroxylase activity in the cytosolic fractions of transfected and non-transfected COS-1 cells. CMP-NeuAc hydroxylase activity in the cytosolic fractions was measured with the addition of b_5 as described in Materials and methods; hydroxylase activity could not be detected without the addition of b_5 (data not shown). One unit of enzyme is defined as the amount of enzyme which catalyses the conversion of 1 μmol of CMP-NeuAc to CMP-NeuGc per min at 37 °C. F, full-length cDNA; T, truncated cDNA; M, mock transfection; ND, not detected.

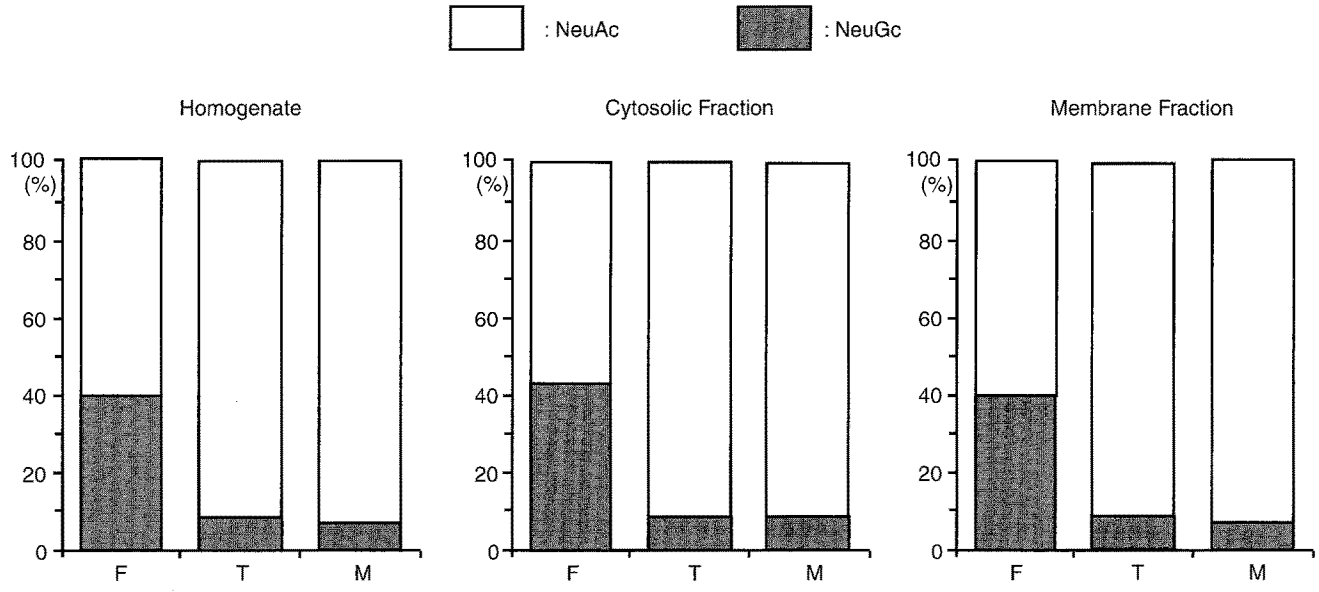

Figure 4. Determination of sialic acid species in transfected COS-1 cells. The amount of NeuGc and NeuAc in total homogenate, in the membrane fraction and in the cytosolic fraction from transfected cells was determined as described in Materials and methods. The DNA transfection did not alter the sum of both sialic acid in each fraction (data not shown). The percentages of NeuAc and NeuGc are shown by the open and closed boxes, respectively, when the sum of both sialic acids is designated as 100%. F, full-length cDNA; T, truncated cDNA; M, mock transfection.

Intracellular distribution

The intracellular distribution of the full-length and truncated proteins expressed in COS-1 cells was explored using an antiserum directed against the *N*-terminal portion shared by both proteins. As shown in Fig. 5, histochemical analysis revealed that the full-length protein localized to the cytosol in accordance with the intracellular distribution of the enzyme in mouse liver [22]. In sharp contrast, the truncated protein was distributed in the juxta-nuclear membrane which appeared to be the endoplasmic reticulum (ER). These findings were confirmed by immunoblot analysis using subcellular fractions isolated from the COS-1 cells (Fig. 6). The full-length protein co-migrated with CMP-NeuAc hydroxylase isolated from the mouse liver on SDS-PAGE, and was recovered mostly from the cytosolic fraction, whereas the truncated protein was recovered from both the nuclear and membrane fractions.

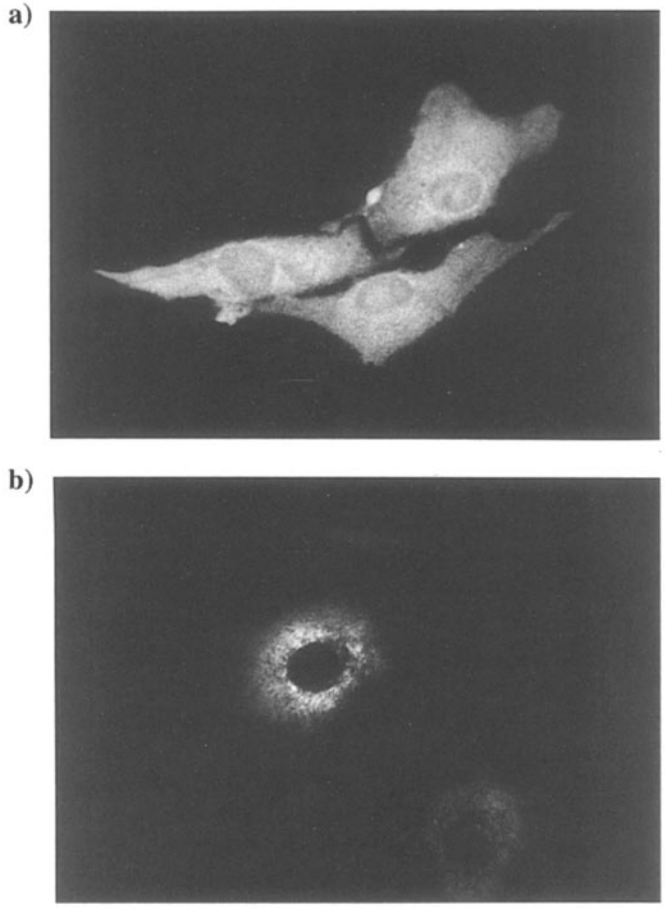

Figure 5. Immunolocalization of mouse CMP-NeuAc hydroxylase expressed in COS-1 cells. The immunofluorescent study was carried out using cells transfected with the full-length cDNA (a) and the truncated cDNA (b).

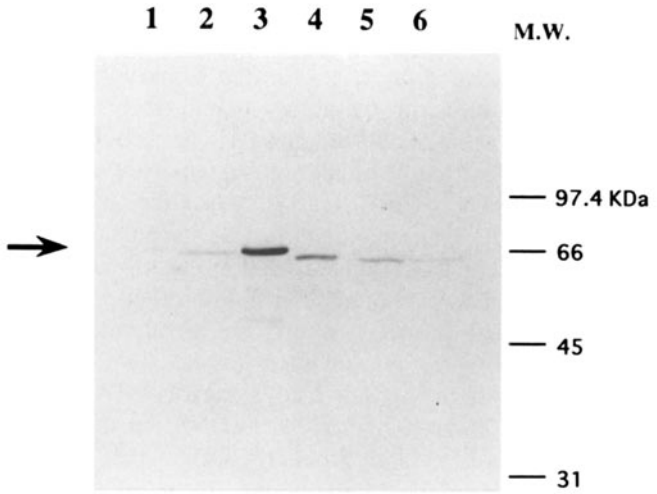

Figure 6. Immunoblot analysis of mouse CMP-NeuAc hydroxylase expressed in COS-1 cells. COS-1 cells were transfected with the full-length cDNA (lanes 1–3) and the truncated cDNA (lanes 4–6). Equimolar aliquots of the nuclear fraction (lanes 1 and 4), the membrane fraction (lanes 2 and 5) and the cytosolic fraction (lanes 3 and 6) isolated from COS-1 cells were then analysed by SDS-PAGE, followed by immunoblot analysis, as described in Materials and methods. The arrow on the left denotes the migration position of the CMP-NeuAc hydroxylase purified from mouse liver [24]. The lines on the right indicate the positions of the following molecular mass standard: phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

Discussion

CMP-NeuAc hydroxylase converts CMP-NeuAc to CMP-NeuGc, and is the key enzyme in regulating the expression of NeuGc-containing glycoconjugates. Two mRNA species were detected in mouse tissues. The longer message contains a full-length ORF, and encodes a protein of 577 amino acids with a predicted molecular mass of 67 kDa. The shorter mRNA consists of a truncated ORF in the middle, and encodes a protein of 531 amino acids with a predicted molecular mass of 62 kDa. The deletion of these 46 amino acids resulted in an alteration of the intracellular distribution of this protein. The full-length protein localizes to the cytosol, as does the nascent mouse liver CMP-NeuAc hydroxylase, whereas the truncated protein localizes to the ER. No information was obtained to support the contention of membrane transport of the truncated protein based on hydropathy plots of both proteins (data not shown). No signal sequence leading to the membrane localization was generated by this deletion. Therefore, it remains to be elucidated how this deletion of 46 amino acids can change the intracellular distribution of CMP-NeuAc hydroxylase.

CMP-NeuAc hydroxylation is performed by an electron transport system consisting of b_5, b_5 reductase, and the terminal enzyme CMP-NeuAc hydroxylase [22, 23]. Upon

initiation of this reaction, CMP-NeuAc hydroxylase binds to the substrate CMP-NeuAc, and then the resultant complex associates with b_5 to form a ternary complex [35]. Both soluble and membrane-bound forms of b_5 occur in mammals. Since no soluble b_5 activity was detected in COS-1 cells (data not shown), the electron transport system for CMP-NeuAc hydroxylation of the isolated cytosolic fraction could not be reconstituted *in vitro* without exogenously added b_5 (Fig. 3). However, an elevation in NeuGc content was observed in the transfected cells (Fig. 4). Therefore, the membrane-bound form of b_5 seems to be involved in the CMP-NeuAc hydroxylation system in COS-1 cells transfected with the full-length CMP-NeuAc hydroxylase cDNA. In this case, CMP-NeuAc hydroxylase probably forms the ternary complex with the membrane-bound form of b_5 and CMP-NeuAc on the ER membrane. After the conversion from CMP-NeuAc to CMP-NeuGc, the CMP-NeuAc hydroxylase molecule in the ternary complex would dissociate and translocate back to the cytosol. Thus, the truncated CMP-NeuAc hydroxylase may remain associated with membrane-bound b_5 due to its lack of catalytic activity to convert CMP-NeuAc to CMP-NeuGc. Alternatively, the shortened protein may be able to bind to membrane-bound b_5 without CMP-NeuAc. Interestingly, the isoelectric point (pI) of the deleted 46-amino acid sequence was calculated to be 9.82, whereas the pI of the truncated protein was 5.72 (data not shown). This suggests that there may be an electrostatic interaction between the deleted sequence and CMP-NeuAc, which is an acidic compound. Further work is needed to resolve this problem.

The truncated form of CMP-NeuAc hydroxylase appears to have lost its enzymatic activity. However, the altered intracellular distribution and the small but significant amount of truncated mRNA in mouse tissues imply that the shortened protein may possess some biological functions. Further study is required to elucidate this point.

Acknowledgements

This work was supported partly by a Grant-in-Aid for scientific Research from the Ministry of Education, Science and Culture of Japan.

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