The Golgi CMP-sialic acid transporter: A new CHO mutant provides functional insights

Sing Fee Lim, May May Lee, Peiqing Zhang, and Zhiwei Song

A CHO mutant line, MAR-11, was isolated using a cytotoxic lectin, *Maackia amurensis* agglutinin (MAA). This mutant has decreased levels of cell surface sialic acid relative to both wild-type CHO-K1 and Lec2 mutant CHO cells. The CMP-sialic acid transporter (CMP-SAT) gene in the MAR-11 mutant cell has a C→T mutation that results in a premature stop codon. As a result, MAR-11 cells express a truncated version of CMP-SAT which contains only 100 amino acids rather than the normal CMP-SAT which contains 336 amino acids. Biochemical analyses indicate that recombinant interferon-γ (IFN-γ) produced by the mutant cells lack sialic acid. Using MAR-11 as host cells, an EPO/IEF assay for the structure–function study of CMP-SAT was developed. This assay seems more sensitive than previous assays that were used to analyze sialylation in Lec2 cells. Cotransfection of constructs that express CMP-SAT into MAR-11 cells completely converted the recombinant EPO to a sialylation pattern that is similar to the EPO produced by the wild-type CHO cells. Using this assay, we showed that CMP-SAT lacking C-terminal 18 amino acids from the cytosolic tail was able to allow high levels of EPO sialylation. Substitution of the Gly residues with Ile in three different transmembrane domains of CMP-SAT resulted in dramatic decreases in transporter’s activity. The CMP-SAT only lost partial activity if the same Gly residues were substituted with Ala, suggesting that the lack of side chain in Gly residues in the transmembrane domains is essential for transport activity.

Introduction

The biosynthesis of the N-linked carbohydrates is initiated in the ER and completed in the Golgi apparatus. The Glc3Man2GlcNAc2 oligosaccharide assembled on the lipid dolichol carrier in the ER is transferred to the Asn residues of the nascent protein as the core oligosaccharide unit. After the transfer, the oligosaccharide undergoes a series of modifications before it reaches its maturity in the Golgi. Three groups of proteins are involved in the direct modification of the oligosaccharide. The trimming enzymes remove the glucose residues and certain mannose residues from the core oligosaccharide unit (Herscovics 1999). The glycosyl transferases transfer different sugar residues from the sugar nucleotide donors to the oligosaccharide inside the Golgi apparatus (Harduin-Lepers et al. 2001; Coutinho et al. 2003). The nucleotide sugar transporters transport the activated sugar substrates, such as CMP-sialic acid, UDP-galactose, UDP-GlcNAc, and GDP-fucose into the Golgi apparatus (Gerardy-Schahn et al. 2001; Martinez-Dunker et al. 2003).

The nucleotide sugar transporters are type III membrane proteins with 6–10 predicted transmembrane domains. They are responsible to transport different nucleotide sugars into the sealed vesicular structures of the Golgi apparatus. The CMP-sialic acid transporter (CMP-SAT) from Chinese hamster has been cloned and it presumably contains 10 transmembrane domains (Eckhardt et al. 1996, 1999). Many CHO lines that have genetic defects in the glycosylation pathway have been isolated (Patnaik and Stanley 2006), including the Lec2 complementation group which lack functional CMP-SAT (Eckhardt et al. 1998). Three mutant lines in this group, including Lec2, carry a deletion mutation in the gene. Another mutant line, 9D3, carries a point mutation (G189E). All these lines have been shown to have lost the CMP-SAT activity (Eckhardt et al. 1998). The Lec2 cells have been extensively used as a model cell line to express sialic acid-free glycoproteins.

*Maackia amurensis* agglutinin (MAA) is a plant lectin that specifically recognizes α2,3-linked sialic acid to galactose residues on glycoproteins or glycolipids (Wang and Cummings 1988; Knibbs et al. 1991). CHO cells express α2,3-linked sialic acid, not α2,6-linked sialic acid, on their N-linked glycoproteins (Conradt et al. 1987). We discovered that MAA was highly toxic to CHO cells. MAA kills CHO cells possibly by inducing apoptosis after it binds to the cell surface sialic acid. This can be inferred from our earlier work which showed that CHO cells become more resistant to MAA treatment when the proapoptotic proteins Bax and Bak were knocked down by RNA interference (RNAi) (Lim et al. 2006). Bax and Bak are known as the key regulators of the intrinsic apoptotic pathway (Wei et al. 2001). With this lectin, a panel of MAA-resistant CHO cell lines were isolated. One of the mutant lines is MAR-11.

The structure–function relationship studies on CMP-sialic acid and UDP-Gal transporters have been reported before (Aoki et al. 2001, 2003; Zhao et al. 2006). In those studies, the activities of the transporters in the cells were assessed by lectin staining of the cell surface carbohydrates. Cells that lack functional CMP-SAT or UDP-Gal transporter, such as Lec2 or Lec8 cells, are unable to express β-galactose or sialic acid on their surface N-glycans. Therefore, lectins that are specific for these sugar structures cannot bind the cell surface. Transient transfection of these mutant cells with constructs that express the...
missing transporters will restore the expression of the sugars on the cell surface and result in a positive staining by the lectins (Aoki et al. 2001, 2003). However, with this type of assay, it is difficult to study a mutant protein if it has only partially lost its activity.

Recombinant human erythropoietin (EPO) has been widely used as a model molecule for studying protein glycosylation. It is a heavily glycosylated protein which contains three N-glycans and one O-glycan. A relatively simple analysis on the sialylation pattern of EPO has been reported and it employed isoelectric focusing (IEF) followed by Western blot (Lasne et al. 2002). In this report, we demonstrate that the MAR-11 CHO cells lack a functional CMP-SAT. Using MAR-11 cells as host cells, we developed an EPO/IEF assay that enables us to analyze the relative activities of different mutant transporters. This more sensitive assay allows us to study detailed structure–function relationships of CMP-SAT.

Results

Isolation of an MAA-resistant CHO mutant cell line, MAR-11

Thirteen CHO clones that survived MAA treatment were isolated from 1 × 10^7 CHO-K1 cells. One of the clones, MAR-11 (for Maackia amurensis agglutinin resistant), represents a group that was not agglutinated with MAA but agglutinated strongly with RCA-I, a lectin specific for terminal β-linked galactose residues (Bhattacharyya and Brewer 1988). MAR-11 cells displayed a similar growth rate and morphology to wild-type CHO cells. These mutant cells can easily be adapted to suspension culture in serum-free medium. To quantify the differences in total cell surface sialic acid between the wild-type and MAR-11 CHO cells, a cell surface sialic acid assay was carried out. In order to accurately measure the sialic acid on the cell surface and minimize the influence by the sialic acid present in the serum glycoproteins, CHO-K1 cells and MAR-11 cells were adapted to the suspension culture and grown in a serum-free HyQ medium. The results for cell surface sialic acid assay are shown in Figure 1A. Compared to wild-type cells, MAR-11 cells only expressed residual amounts of sialic acid on the cell surface. The surface sialic acid on Lec2 cells, a well-characterized CHO mutant line that lacks the functional CMP-SAT (Deutscher et al. 1984; Eckhardt et al. 1998), was also analyzed for comparison. Lec2 cells contain slightly higher amounts of surface sialic acid than the MAR-11 cells, both in the presence and absence of serum (Figure 1A).

Molecular cloning of CMP-SAT cDNAs from MAR-11 cells

In order to identify the genetic defect responsible for the lack of the sialic acid phenotype, RT-PCR was carried out to investigate the expression of CMP-SAT mRNA in MAR-11 cells. The RT-PCR results revealed that the CMP-SAT mRNA levels in MAR-11 mutant cells are similar to that of the wild-type CHO (data not shown). The PCR products from both cell lines were cloned into the TOPO vector and sequenced. The sequencing data revealed a point mutation in the CMP-SAT cDNA in the MAR-11 cells. As shown in Figure 1B, a C–T mutation at position 301 of the CMP-SAT coding region in the mutant was identified. This mutation results in changing the Gln (CAG) at position 101 of the protein to a stop codon (TAG). Ten independent CMP-SAT cDNA clones isolated from MAR-11 cells were sequenced and they all contained the same C–T mutation. Therefore, the MAR-11 mutant cells only express a truncated version of CMP-SAT which contains 100 amino acids rather than the normal CMP-SAT with 336 amino acids in the wild-type cells.

Carbohydrate structure analysis on recombinant human IFN-γ expressed in wild-type CHO cells and MAR-11 cells

Recombinant IFN-γ produced by wild-type and MAR-11 CHO cells were purified and the N-linked carbohydrates released by PNGase F digestion were analyzed as previously described (Wong et al. 2005). The glycans were analyzed by the high pH anion exchange chromatography (HPAEC) using the Dionex BioLC system. The elution profiles of the glycans are shown in Figure 2A. The glycans isolated from recombinant IFN-γ produced in wild-type CHO cells contained asialo (0S), monosialylated (1S), disialylated (2S), and trisialylated (3S) oligosaccharides, whereas glycans isolated from recombinant IFN-γ produced in MAR-11 cells contained only asialo glycans as suggested by the HPAEC elution profiles. No
sialylated forms of glycans were found from IFN-γ produced by the wild-type CHO cells were mainly sialylated glycans whereas those produced by MAR-11 cells were all non-sialylated glycans.

**Recombinant human EPO produced by MAR-11 cells reacts strongly with a galactose-binding lectin, RCA-I**

In addition to IFN-γ, recombinant human EPO was also employed as a model protein for glycosylation analyses. EPO is a heavily glycosylated protein which contains three N-glycans and one O-glycan (Hokke et al. 1995). A construct that expresses human EPO was transiently transfected into both wild-type CHO cells and MAR-11 cells. MAR-11 cells were also cotransfected with constructs expressing EPO and CMP-SAT. Glycoproteins in the conditioned media (containing recombinant EPO produced by the cells and the glycoproteins present in FBS) were precipitated with MAA or RCA-I by a precipitation assay. In this assay, conditioned medium from each transfection that contained 20 µg of total protein was incubated with 50 µg of MAA or RCA-I. The precipitated lectin–glycoprotein complex was collected by centrifugation. The total amounts of protein precipitated in each reaction were similar when analyzed by protein quantification (data not shown). The presence or absence of recombinant EPO in the precipitated complex was analyzed by Western blot with a monoclonal anti-EPO antibody. The Western blot results (Figure 3A) show that EPO produced by wild-type CHO cells reacted weakly with RCA-I (lane 1) but strongly with MAA (lane 4). On the other hand, EPO produced in MAR-11 cells reacts strongly with RCA-I (lane 2) but did not react with MAA (lane 5). However, EPO produced in MAR-11 cells that were cotransfected with CMP-SAT no longer reacts with RCA-I (lane 3) but was recognized by MAA (lane 6). These results indicate that the N-linked carbohydrates on the EPO produced in MAR-11 cells lack sialic acids and terminate with galactose residues. Therefore, EPO produced in MAR-11 cells shares a similar glycosylation pattern with that of IFN-γ produced in MAR-11 cells (Figure 2C).

**CMP-SAT complements the genetic defect in MAR-11 cells in an EPO/IEF assay**

To further analyze the glycosylation pattern of EPO produced by MAR-11 cells, an isoelectric focusing (IEF) followed by a Western blot assay originally described by Lasne et al. (2002) was employed. In order to focus specifically on the differences in N-linked glycans, the O-glycosylation site on EPO was eliminated by mutating Ser 126 to Val (S126V) according to Delorme et al. (1992). As this mutant form of EPO contains no O-linked glycans, the bands resolved by IEF should correlate only to different glycoforms of the N-glycans. The amounts of EPO produced from different transfections were normalized by ELISA before they were analyzed by the IEF/Western blot assay. As shown in Figure 3B, O-glycan-free EPO expressed by wild-type CHO cells produced a long ladder of bands due to different extents of sialylation (Figure 3B, lane 1) while the MAR-11 expressed EPO that gave only 3 or 4 bands signifying the lack of sialylation (lane 2). However, cotransfection with CMP-SAT into the MAR-11 cells completely converted the recombinant EPO to a sialylation pattern that is similar to the EPO produced...
Fig. 3. Recombinant EPO produced by MAR-11 cells lack sialic acid on $N$-linked carbohydrate and CMP-SAT complements the genetic defect in MAR-11 cells. (A) Wild-type CHO cells and MAR-11 cells were transiently transfected with a construct expressing EPO. MAR-11 cells were also co-transfected with constructs expressing EPO and CMP-SAT. Glycoproteins in the conditioned media were precipitated with RCA-1 and MAA and analyzed by Western blot using an anti-EPO antibody. Lanes 1, 2, and 3: proteins precipitated by RCA-1 and Lanes 4, 5, and 6: proteins precipitated by MAA. Lanes 1 and 4: wild-type CHO cells were transfected with an EPO construct. Lanes 2 and 5: MAR-11 cells were transfected with an EPO construct. Lanes 3 and 6: MAR-11 cells were co-transfected with an EPO construct and a CMP-SAT construct. (B) O-Glycan-free EPO produced by wild-type cells or MAR-11 cells under different conditions were analyzed by IEF. Lanes 1, 4, and 7: EPO produced by wild-type CHO cells; lane 2, 5, and 8: EPO produced by MAR-11 cells; and lane 3, 6, and 9: EPO produced by MAR-11 cells that were co-transfected with a CMP-SAT cDNA. Lanes 1, 2, and 3: untreated recombinant EPO. Lanes 4, 5, and 6: recombinant EPO in the media were treated with neuraminidase. Lanes 7, 8, and 9: recombinant EPO in the media were treated with PNGase F. (C) O-Glycan-free EPO produced in MAR-11 cells that were co-transfected with different amounts of CMP-SAT cDNA. Lane 1, EPO produced in MAR-11 cells. Lane 2, EPO produced in MAR-11 cells co-transfected with 0.2 $\mu$g of plasmid expressing CMP-SAT. Lane 3, MAR-11 cells were co-transfected with 0.1 $\mu$g of CMP-SAT construct. The amounts of CMP-SAT construct used for co-transfection in each of the following lanes were decreased by a 2-fold reduction.

by the wild-type CHO cells (lane 3). Cotransfecting MAR-11 cells with constructs expressing several different sialyltransferases failed to restore the sialylation pattern of recombinant EPO (data not shown). Treatment of these three EPO samples (lanes 1, 2, and 3) with neuraminidase as shown in lanes 4, 5, and 6 indicates that the EPO sample produced by MAR-11 cells is not sensitive to neuraminidase treatment (comparing lane 5 with lane 2). The IEF pattern of wild-type EPO with sialic acid removed is similar to the EPO produced by MAR-11 cells. This result also confirms that the difference in IEF patterns between lane 1 and lane 2 is due to sialic acid. After digestion with PNGase F, these three EPO samples showed one major band by IEF (see lanes 7, 8, and 9).

The degree of sialylation on EPO produced in MAR-11 cells is dependent on the amount of CMP-SAT cDNA used in the transfection. MAR-11 cells grown in 6-well plates were transfected with a total of 2 $\mu$g of plasmid DNA which contained 1 $\mu$g of EPO construct. Various amounts of CMP-SAT construct DNA were used in different transfections. The null vector (pcDNA3.1) was added in order to result in a total 2 $\mu$g of DNA for each transfection. Figure 3C shows the sialylation patterns of EPO produced in MAR-11 cells transfected with different amount of CMP-SAT construct DNA. Lane 1 shows EPO produced in MAR-11 cells as a control. Lane 2 shows EPO produced in MAR-11 cells that were cotransfected with 0.2 $\mu$g of plasmid DNA expressing CMP-SAT and 0.8 $\mu$g of pcDNA3.1 null vector. Lane 3 was cotransfected with 0.1 $\mu$g of CMP-SAT plasmid DNA and 0.9 $\mu$g of pcDNA3.1 null vector. In the subsequent lanes, the amount of CMP-SAT plasmid DNA used for the transfection was similarly reduced by a 2-fold dilution. Our results showed a positive correlation between EPO sialylation and the amount of CMP-SAT expressing DNA used in the transfection. CMP-SAT expressing DNA as low as 1 ng was sufficient to effectively enhance EPO sialylation in MAR-11 cells. These results clearly demonstrate the sensitivity of this EPO/Western blot assay.

Functional analysis on the biological activities of several previously reported CMP-SAT mutants

Using MAR-11 cells and the EPO/IEF assay system, we then performed a structure–function study on CMP-SAT. Expression constructs that express four previously reported CMP-SAT mutants identified in CHO cells were generated. These constructs express three deletion mutations and one G–E point mutation at position 189 (Eckhardt et al. 1998). One of the deletion constructs in which amino acids from Gly192 to Phe251 were replaced with one Val residue is the mutation identified in the Lec2 cells. In addition, we also generated five C-terminal deletion mutants. Ten constructs used in the structure–function study are summarized in Figure 4A. The wild-type CMP-SAT was used as a control. The $\Delta$101–336 mutation was generated as it resembles the truncated CMP-SAT product in MAR-11 cells. The $\Delta$233–336 mutation deletes the last three putative transmembrane domains and the C-terminal cytosolic tail (Eckhardt et al. 1999). The $\Delta$268–336 mutation deletes the last two transmembrane domains and the C-terminal tail. The $\Delta$297–336 mutation deletes the last C-terminal transmembrane domain and the C-terminal tail. The C-terminal 18 amino acid residues in the cytosolic tail were removed in the $\Delta$319–336 mutation for analyzing the function of the cytosolic tail. One
Analysis on the structural and functional relationships of CMP-SAT.

(A) Ten constructs were generated to express different versions of CMP-SAT. The constructs are numbered in parenthesis on the right. Five deletion mutations of the CMP-SAT were generated by introducing stop codons at the positions indicated. Constructs to express four previously reported CMP-SAT mutants (three deletion mutations and one point mutation) were also generated as indicated. (B) O-Glycan-free EPO produced in MAR-11 cells that were co-transfected with each of the 10 different CMP-SAT constructs are shown. Lane numbers correspond to the construct numbers shown in parentheses on the right side in Figure 4A. EPO produced in MAR-11 cells that were cotransfected with the wild-type CMP-SAT is shown in lane 1 as a control. Lanes 2-6 show EPO produced in MAR-11 cells that were cotransfected with each of the five C-terminal deletion mutations in the respective order in Figure 4A. This includes the construct expressing only the first 100 amino acids that gives the same product produced by the MAR-11 cells (lane 2). Lanes 7-10 showed EPO produced in MAR-11 cells that were cotransfected with four previously reported CMP-SAT mutants (Eckhardt et al. 1998). As shown in lane 6, with the C-terminal cytosolic tail (18 amino acids) removed, the CMP-SAT was able to allow for a very high level of EPO sialylation. However, a careful comparison between lane 1 and lane 6 suggests that the EPO was slightly better sialylated when the wild-type CMP-SAT was present. This could be due to the lack of the ER export signals located at the C-terminal of the transporter (Zhao et al. 2006). A previously reported point mutation (G198E) lost most of its activity in this assay (Figure 4B, lane 10). The results also showed that the CMP-SAT in three previously reported deletion mutations, including Lec2, retained slightly higher activity than that in MAR-11 cells (comparing lanes 7, 8, and 9 with lane 2). This may explain why Lec2 cells contain slightly higher amount of sialic acid on the cell surface than that on MAR-11 cells (Figure 1A). To investigate the cellular localization of Δ101–336 (mutant found in MAR-11) and Δ319–336 (with C-terminal 18 amino acids removed) mutants, HA tag was fused to the N-terminals of Δ101–336 and Δ319–336 constructs. HA tag was also fused to the wild-type CMP-SAT as a control. These three constructs were transiently transfected into the MAR-11 cells. The cells were stained with an anti-HA antibody 2 days after transfection. As shown in Figure 4C, wild-type CMP-SAT is localized to the Golgi as the anti-HA staining shows a complete overlap with an anti-Golgi antibody, anti-giantin. Clearly, both Δ101–336 and Δ319–336 are expressed as shown by positive staining with anti-HA. However, neither of them is restricted to the Golgi (4C). Both show more diffused expression pattern than the wild-type CMP-SAT. In a previous report, FLAG (1–96) which contained a Flag tag and the N-terminal 96 amino acids of CMP-SAT was found to be expressed in the cytosol (Eckhardt et al. 1998). Our Δ101–336 which is similar to FLAG (1–96) is also localized in the cytosol. Another report showed that CMP-SAT with C-terminal 4 amino acids removed remained in the ER (Zhao et al. 2006). The Δ318–336 is likely to be localized in the ER. This, however, remains to be confirmed.

Glycine residues in the transmembrane domains of CMP-SAT are crucial for its function

A previous report showed that a G–E mutation at position 189 of CMP-SAT resulted in reduced activity. This loss of activity seemed to be due to the increased size of the side chain in the Glu residue (Eckhardt et al. 1998). The EPO/IEF assay system enabled us to semi-quantitatively analyze the activity of this kind of mutation. According to the model proposed by...
Eckhardt et al. (1999), there are 10 Gly residues in four transmembrane domains of CMP-SAT (domains 5–8). With the exception of the transmembrane domain 7 where two Gly residues are separated by 6 amino acids, the other eight Gly residues in the three transmembrane domains are very close to each other and exist in four pairs. The first pair of Gly residues (G153 and G154) is located in the middle of transmembrane domain 5. The second pair of Gly residues (G177 and G179) is located in domain 6 near the Golgi lumen side. The third pair of Gly residues (G189 and G192) is located in the same transmembrane domain but close to the cytosolic side. The fourth pair of Gly residues (G256 and G257) is located in the middle of domain 8. It is possible that these Gly residues in domains 5–8 are involved in the formation of the transporter channel because they do not have the side chains. To examine this hypothesis, we generated eight mutant constructs. In each such construct, one pair of Gly residues was mutated to a pair of Ala or Ile. If the hypothesis is correct and since the side chain of Ile is larger than the side chain of Ala, mutations that carry Gly to Ala substitution should have higher activities than the ones carrying Gly to Ile substitution. All these mutants should be less active than the wild type. These eight mutant constructs were cotransfected with EPO into MAR-11 cells. The EPO products were analyzed by IEF/Western blot assay and the results are shown in Figure 5A. Lane 1 shows EPO produced by the wild-type CHO cells, and Lane 2, EPO produced by MAR-11 cells. Lane 3 shows EPO produced by MAR-11 cells that were cotransfected with wild-type CMP-SAT. Lanes 4–7 show EPO produced by MAR-11 cells that were cotransfected with mutant CMP-SATs in which a pair of Gly was changed to a pair of Ile. Lanes 8–11 show the EPO produced by MAR-11 cells that were cotransfected with mutant CMP-SATs in which a pair of Gly was changed to a pair of Ala. The results shown in Figure 5A indicate that all these mutants are at least partially active (comparing with lane 2). Mutants with Gly153/Gly154 pair changed to Ala153/Ala154 (lane 8) or Gly177/Gly179 pair changed to Ala177/Ala179 (lane 9) retained the highest activity compared to other mutants. However, when these two pairs of Gly residues were changed to Ile (lanes 4 and 5) the transporter’s activity was reduced dramatically. On the other hand, when Gly189/Gly192 pair was changed to Ala189/Ala192 (lane 10) and Gly256/Gly257 pair was changed to Ala256/Ala257 (lane 11) the transporter’s activity was clearly reduced. The mutant with Gly256/Gly257 pair changed to Ile256/Ile257 (lane 7) had the least activity in all these mutants. These results suggest that all these Gly residues are indeed important for the transport activity. Sequence alignment analyses on CMP-SAT from eight different organisms showed that Gly153 and Gly179 are less conserved. In fact, in human, chimpanzee and Zebrafish, the amino acid at position 153 of the transporter, and UDP-Gal transporter identified from the mouse. The Gly residues are numbered based on their positions in Chinese hamster CMP-SAT. (A) Recombinant O-glycan-free EPO produced by wild-type CHO cells or MAR-11 cells under different conditions. Lane 1, EPO produced by wild-type CHO cells; lane 2, EPO produced by MAR-11 cells; and lane 3, EPO produced by MAR-11 cells co-transfected with wild-type CMP-SAT. Lanes 4–7, Gly pairs in CMP-SAT were changed to Ile pairs. Lanes 8–11, Gly pairs changed to Ala pairs. Lane 4: Ile153/Ile154; lane 5: Ile177/Ile179; lane 6: Ile189/Ile192; lane 7: Ile256/Ile257. Lane 8: Ala153/Ala154; lane 9: Ala177/Ala179; lane 10: Ala189/Ala192; lane 11, Ala256/Ala257. (B) Partial amino acid alignment showing CMP-SAT from eight different organisms. (C) Partial amino acid alignment of CMP-SAT, UDP-GlcNAc transporter, and UDP-Gal transporter identified from the mouse. The Gly residues are numbered based on their positions in Chinese hamster CMP-SAT. (D) MAR-11 cells were transfected with eight mutant forms of CMP-SAT. Each mutant contained HA tag fused to the N-terminal. Two days after transfection, cells were stained with anti-HA for localization of mutant forms of CMP-SAT. Anti-Golgi antibody, anti-giantin, was used to visualize the location of Golgi in the same transfected cell. Gly→Ile mutants are shown on the left and Gly→Ala on the right. The positions of the Gly pairs in the protein are shown on the left.
were stained with anti-HA antibody. As shown in Figure 5D, all eight proteins were expressed and costained with an anti-Golgi antibody, anti-giantin. These results confirmed that the Gly to Ala and Gly to Ile mutants are indeed expressed and localized in the Golgi.

Discussion

Glycosylation mutant lines isolated from CHO cells by Stanley and colleagues have been invaluable for studying the biological functions of glycoproteins. For example, influenza virus cannot successfully infect Lec1 cells that lack functional GlcNAc transferase I, suggesting that proper N-glycosylation of certain cell surface glycoproteins is required for infection by influenza virus (Chu and Whittaker 2004). Recombinant antibodies produced in Lec13 CHO cells which cannot fucosylate N-linked carbohydrates on the Fc region bind the FcyRIII with much higher affinity than the antibodies with the fucose residues. As a result, these antibodies are more efficient in triggering antibody-dependent cell-mediated cytotoxicity (ADCC) (Shields et al. 2002) and subsequently, the dosage of the therapeutic antibody used for the patients could be reduced. Patients with Gaucher disease lack functional glucocerebrosidase and subsequently accumulate glucocerebrosides in cells of monocyte-macrophage lineage. Complex oligosaccharides present on recombinant glucocerebrosidase produced by CHO cells need to be enzymatically remodeled into a mannose core to facilitate mannose receptor-mediated uptake into macrophages. This step of carbohydrate modification can be eliminated if this protein is produced in Lec1 cells (Van Patten et al. 2007). A recent report showed that IgG with less sialic acid on its carbohydrates that is linked to its Fc region may have enhanced pro-inflammatory activity (Kaneko et al. 2006). As such, therapeutic antibodies that are produced in Lec2 or MAR-11 cells could be more efficient in their ability to kill target tumor cells in vivo.

Although there are already several cell lines in the Lec2 complementation group, MAR-11 is the first one that seems to completely lack CMP-sialic acid transport due to a premature stop codon. With only 100 amino acid residues, the mutated CMP-SAT gene in MAR-11 cells produces the smallest translation product in comparison to all other mutant lines in the Lec2 complementation group. Indeed, compared to Lec2 cells, MAR-11 cells expressed less sialic acid on the cell surface (Figure 1A). Using MAR-11 as host cells we compared the effects of the mutation found in MAR-11 cells with four previously reported mutations. The results suggest that all four previously reported mutations exhibited a slightly higher transporter activity than the MAR-11 mutant (Figure 4B). This may explain why MAR-11 cells contain even less surface sialic acid than the Lec2 cells. Therefore, MAR-11 is a better choice for the production of recombinant proteins devoid of sialic acid.

Using MAR-11 as host cells, the EPO/IEF assay system provides a more sensitive analysis on the structure–function relationship for the CMP-SAT protein. In previous studies, the activity of the CMP-SAT was analyzed by staining the cell surface with relevant lectins (Aoki et al. 2001, 2003; Zhao et al. 2006). Lec2 cells were positively stained by PNA as it recognizes terminal galactose residues on the N-glycans. The staining by PNA is reduced if the Lec2 cells were transfected with a functional CMP-SAT as the galactose on the N-glycan is capped with sialic acid. Clearly, this assay is not sensitive enough for more detailed structure–function relation studies. The experiment where the Gly residues were mutated to either Ala or Ile in different transmembrane domains also demonstrated the advantage of this assay system (Figure 5). The results suggested that the Gly residues may be involved in the formation of a transporter channel. Changing the Gly residues to Ala which has a small side chain did not significantly change the activity of the transporter. However, changing Gly to Ile which has a larger side chain drastically reduced the activity of the transporter (Figure 5). In addition, the G189E point mutation in mutant line 9D3 showed a clearly reduced transporter activity compared to wild type. However, compared to the three deletion mutations in the Lec2 group, this has the highest transporter activity (Figure 4B). Similarly, the EPO/IEF assay system can be applied to structure–function studies for other glycosylation genes. For example, the activity of the UDP-Gal transporter can be studied using Lec8 as host cells. The structure–function relationship for GlcNAc-transferase I can be studied using the EPO/IEF system in Lec1 cells.

In summary, MAR-11 is a CHO mutant line that seems to completely lack CMP-SAT activity due to a premature stop codon. Therefore, MAR-11 cells have a potential application in the biotechnology field to produce recombinant proteins completely devoid of sialic acid. Furthermore, we have developed a sensitive EPO/IEF assay to compare the activities of different CMP-SAT mutants. The EPO/IEF system can also be applied to study structure–function relations of other genes in the N-glycosylation pathway, provided that mutation in the gene results in an altered sialylation pattern of EPO and a mutant cell line deficient in the same gene is available. With this system, we have shown that the glycine residues in the transmembrane domains of CMP-SAT play a crucial role to transport CMP-sialic acid possibly because they do not have side chains.

Material and methods

Materials

Trypsin was purchased from Promega. N-Glycosidase F (PNGase F) was purchased from Calbiochem (San Diego, CA). Hypercarb SPE cartridges (200 mg sorbent bed weight) were from Thermo Fisher Scientific (Waltham, MA). 2,5-Dihydroxybenzoic acid (DHB) was from Waters Corporation (Milford, MA). N-Linked oligosaccharide standards were from Dextra laboratories, UK. Raffinose was from Sigma (St. Louis, MO). Acetic acid, ammonium bicarbonate, boric acid, hydrochloric acid, sodium acetate, sodium borate, sodium chloride, sodium dihydrogen phosphate, sodium dodecyl sulphate (SDS), sodium hydroxide (NaOH), sulphuric acid, and trifluoroacetic acid (TFA) were all of analytical reagent grade from Merck KGaA, Germany. Acetonitrile and methanol were of HPLC grade from Merck KGaA, Germany. Deionized water was used throughout the analysis.

Cell culture

The parental CHO-K1 cells were originally obtained from Dr. Donald K. MacCallum (University of Michigan Medical School, Ann Arbor, MI). CHO-K1 cells and isolated mutant CHO cells were cultured in Dulbecco’s Modified Eagle’s
Medium (DMEM, Invitrogen/Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen/Gibco), at 37°C with 5% CO₂. Lec2 mutant cells were a kind gift from Dr. P. Stanley (Albert Einstein College of Medicine, NY) and cultured in α-MEM supplemented with proline (40 mg/L) (Invitrogen/Gibco) and 10% FBS.

**Isolation of MAA-resistant CHO cells**

CHO-K1 cells were seeded into 6 cm culture dishes at a density of 10 million cells per dish. After an overnight culture, the medium was removed and the cells were cultured in serum-free DMEM containing 50 µg/mL MAA. The lectin-containing medium was removed after 12 h of incubation and replaced with a fresh DMEM medium containing 10% FBS. The dishes were cultured for 2 weeks until MAA-resistant cells developed into visible colonies. Individual clones were picked and transferred to 24-well plates to grow into cell lines.

**Analysis of cell surface sialic acid content**

Following trypsin digestion of adherent cells, 1 × 10⁷ cells were resuspended in a serum-free DMEM medium and incubated at 37°C for 1 h to let the cells recover from the trypsin treatment. Cells were then spun down and resuspended in 200 µL of 10 mM phosphate buffer, 10 mM sodium acetate, and 0.14 M NaCl (0.14 mol/L). 50 mU of sialidase (Calbiochem) was added into the cell suspension and incubated at 37°C for 30 min. After incubation, the cell pellet was spun down and the supernatant collected. The sialic acid content in the supernatant was analyzed using the thiobarbituric acid (TBA) method. Briefly, 25 µL of 25 mM periodic acid in 0.125 M HCl was added into the supernatant and incubated at 37°C for 30 min. After incubation, 40 µL of 2% sodium arsenite (Sigma) in 0.5 M HCl was added. The mixture was then incubated at room temperature until the yellow coloration was no longer visible. 0.4 mL of 0.1 M thiobarbituric acid (Sigma), pH 9, was added and the tube was heated to 100°C for 8 min. 1.5 mL of 95% (v/v) butanol with 5% (v/v) HCl and 0.7 mL of H₂O was added and the solution was spun down at 13 000 rpm for 5 min. The upper aqueous phase was removed and the absorbance at 549 nm measured. A standard curve was generated using 1–5 nmol of sialic acid. The parental CHO cells, MAR-11 and Lec2 cells were also adapted to suspension culture and grown in HyQ medium (HyClone) in the absence of serum. 1 × 10⁷ suspension-grown cells were collected by centrifugation and the sialic acid content on the cell surface was analyzed as described above.

**Production and purification of recombinant human IFN-γ in CHO cells and MAR-11 cells**

The pcDNA3.1 vector (Invitrogen) expressing the human IFN-γ was transiently transfected into the parental CHO cells and the MAR-11 cells by electroporation. The cells were then cultured in T75 cell culture flask. The culture media from both cell lines were collected and the recombinant IFN-γ was purified as described previously (Wong et al. 2005). After purification, the IFN-γ was quantified by reversed-phase HPLC using the Shimadzu VP system (Shimadzu Corporation, Japan). A Vydac C18 column, 1 × 250 mm, 5 µm (218TP51) from GraceVydac was used for separation. A binary mobile phase system was employed: Buffer A contained 0.1% (v/v) TFA in water and Buffer B contained 0.1% (v/v) TFA in acetonitrile. The elution pro-gram began with 35% B for 10 min, 35–65% B for 30 min, 95% B for 10 min, and finally, 35% B for 35 min. A constant flow rate of 0.06 mL/min was used. The typical injection volume for a sample ranged from 15 to 50 µL. The IFN-γ standard, RDI-302 from Fitzgerald Industries International, USA was used for calibration.

**Analysis of the oligosaccharides from recombinant IFN-γ using high pH anion exchange chromatography (HPAEC)**

An amount of 50–100 µg of IFN-γ was used for oligosaccharide analysis. The IFN-γ sample was buffered with 50 mM ammonium bicarbonate to pH 8.2 before adding trypsin. The amount of trypsin used was ~4 µg for every 100 µg of IFN-γ. Upon addition of trypsin, the mixture was incubated for 6 h at 37°C. The trypsin was inactivated at 80°C for 10 min. The mixture was cooled before adding 5 µL of PNGase F. N-Linked oligosaccharides from Dextra Laboratories were used as standards. These were prepared by dissolving 20 µg in 1 mL of water each. The IFN-γ samples and oligosaccharide standards were cleaned with Thermo Hypercarb SPE cartridges according to manufacturer’s instructions. The oligosaccharides obtained after cleanup were dried using a Labconco CentriVap before reconstituting in 150 µL of water. The Dionex BioLC system (Dionex Corporation) with integrated amperometry was used for HPAEC analysis. A PA100 analytical column (2 × 250 mm) was used for separation. The three eluents used were 500 mM CH₃COONa, 500 mM NaOH, and water. The gradient program for the elution of both neutral and charged oligosaccharides began with an equilibration period of 25 min with 20% NaOH and 80% water, a start injection on isocratic mode for 10 min, followed by a ramp gradient for CH₃COONa to reach 46% whilst maintaining NaOH at 80% until 75 min. The waveform used was E₁ = +0.05 V, t₁ = 400 ms; E₂ = +0.75 V, t₂ = 200 ms; E₃ = −0.15 V, t₃ = 400 ms. The flow rate was kept constant at 0.25 mL/min and the volume of sample/standard injected was 30 µL. The remaining solution was retained for the following analysis.

**Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)**

MALDI-TOF analysis was employed as an orthogonal method for the structural confirmation of oligosaccharide structures. The sample preparation for MALDI-TOF analysis involved the additional steps of permethylation and cleanup after the treatment for HPAEC. The protocol for NaOH permethylation was described elsewhere (Dell et al. 1993). After permethylation, the oligosaccharides were cleaned with Sep-Pak C18 cartridges (Waters Corporation) which were previously conditioned by 5 mL each of water, acetonitrile, ethanol, and finally, 10 mL of water. The permethylated oligosaccharides were eluted using 2 mL each of 15, 35, 50, and 75% v/v acetonitrile as individual fractions. Each fraction was dried using the CentriVap before reconstituting in 30 µL of 80% (v/v) methanol. One microliter from each fraction was spotted onto the 96 sample plate and water. The gradient program for the elution of both neutral and charged oligosaccharides began with an equilibration period of 25 min with 20% NaOH and 80% water, a start injection on isocratic mode for 10 min, followed by a ramp gradient for CH₃COONa to reach 46% whilst maintaining NaOH at 80% until 75 min. The waveform used was E₁ = +0.05 V, t₁ = 400 ms; E₂ = +0.75 V, t₂ = 200 ms; E₃ = −0.15 V, t₃ = 400 ms. The flow rate was kept constant at 0.25 mL/min and the volume of sample/standard injected was 30 µL. The remaining solution was retained for the following analysis.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)
program, GlycoMod (http://www.expasy.ch/tools/glycomod/) was used to interpret the mass spectra.

**Molecular cloning and sequencing analysis of the CMP-SAT cDNA in wild-type CHO and the MAR-11 cells**

Total mRNA was isolated from the parental CHO and the MAR-11 mutant cells using the RNaseaueous-4PCR Kit (Ambion). The first strand cDNA was synthesized using a First Strand cDNA Synthesis Kit (Promega) following the manufacturer’s instructions. CMP-SAT cDNA was amplified from the total mRNA by PCR using Chinese hamster CMP-SAT specific primers. The PCR products from both samples were cloned into a TOPO vector (Invitrogen). The DNA sequences of the CMP-SAT inserts were determined by an ABI PRISM 3100 Genetic Analyzer (HITACHI).

**Transient expression of O-glycan-free human erythropoietin (EPO) in wild-type and the mutant MAR-11 CHO cells**

cDNA encoding the open reading frame of human EPO was cloned into pcDNA3.1. The O-glycan glycosylation site was removed from human EPO (O-glycan-free) by mutating the Ser residue at position 126 to Val according to Delorme et al. (1992). The site-directed mutagenesis to generate Ser126Val mutant EPO was carried out using the QuikChange XL site-directed mutagenesis kit (Stratagene) following manufacturer’s instructions. Constructs to express four previously reported Chinese hamster CMP-SAT mutants (Eckhardt et al. 1998) were also cloned into the pcDNA3.1 vector by overlapping PCR or site-directed mutagenesis. Three of them carry a different deletion each: Lec2 (192G→251F was replaced with a Val), 6B2 (Δ251–296), and 8G8 (Δ7–66). One of them carries a point mutation at position 189 (G189E). In order to ensure the same translation efficiency, a Kozak sequence (GCCACC) was added upstream of the translation start codon (ATG) in each construct.

**Isoelectric focusing (IEF) analysis of O-glycan-free EPO produced by wild-type CHO cells and MAR-11 CHO cells**

Unless specified, 1 µg of DNA construct that expresses O-glycan-free EPO and 1 µg of DNA construct that expresses CMP-SAT or a sialyltransferase were cotransfected into the wild-type or MAR-11 CHO cells growing in a 6-well plate. Seventy-two hours after transfection, conditioned culture media from the transfected cells were collected. The sialylation patterns of EPO in different samples were analyzed by IEF followed by Western blot according to Schriebl et al. (2006). The pH range for IEF was either 3–10 or 2.5–7. To ensure equal loading for IEF, the concentration of EPO was predetermined by ELISA using an EPO ELISA kit (Roche).

**Immuno-fluorescence staining**

HA tag was fused to the N-terminals of CMP-SAT and various mutant cDNAs by PCR. MAR-11 cells were plated on glass coverslips and grown overnight before transfection with different HA-tagged constructs. Immunofluorescent staining was performed according to previously published methods (Eckhardt et al. 1998; Zhao et al. 2006). Briefly, 2 days after transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma), and blocked in 10% normal goat serum. After blocking, cells were incubated with a 1:100 dilution of anti-HA monoclonal antibody (Sigma) and a 1:200 dilution of rabbit anti-giantin serum (Abcam) in PBS for 1 h. Cells were washed three times with PBS and then incubated with goat anti-mouse antibody conjugated with Alexa Fluor 594 and goat anti-rabbit antibody conjugated with Alexa Fluor 488 (both from Invitrogen) in PBS with 1% BSA for 1 h. Cells were washed three times with PBS before mounting on glass slides with a Prolong Gold antifade mounting medium (Invitrogen). Fluorescence images were obtained using a Carl Zeiss META confocal microscope.

**Construction of expression vectors to express different mutant versions of CMP-SAT**

The wild-type Chinese hamster CMP-SAT was cloned into the pcDNA3.1 vector between the EcoR I and Xba I sites. Nine constructs to express different mutant forms of CMP-SAT were also cloned into the pcDNA3.1 vector (Figure 5A). Constructs to express five different truncated forms of Chinese hamster CMP-SAT were generated by introducing stop codons at amino acid positions 101, 233, 268, 297, and 319, respectively, using the QuikChange XL site-directed mutagenesis kit (Stratagene). Introduction of a stop codon at position 101 gives rise to the same truncated CMP-SAT product as in MAR-11 cells.
Conflict of interest statement

None declared.

Abbreviations

ADCC, antibody-dependent cell-mediated cytotoxicity; CMP-SAT, CMP-sialic acid transporter; DHB, 2,5-Dihydroxybenzoic acid; HPAEC, high pH anion exchange chromatography; IEF, isoelectric focusing; MAA, *Maackia amurensis* agglutinin; RNAi, RNA interference.

References


