Mutants of the CMP-sialic Acid Transporter Causing the Lec2 Phenotype*

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Chinese hamster ovary (CHO) mutants belonging to the Lec2 complementation group are unable to transport CMP-sialic acid to the lumen of the Golgi apparatus. Complementation cloning in these cells has recently been used to isolate cDNAs encoding the CMP-sialic acid transporter from mouse and hamster. The present study was carried out to determine the molecular defects leading to the inactivation of CMP-sialic acid transport. To this end, CMP-sialic acid transporter cDNAs derived from independent clones of the Lec2 complementation group, were analyzed. Deletions in the coding region were observed for three clones, and single mutants were found to contain an insertion and a point mutation. Epitope-tagged variants of the wild-type transporter protein and of the mutants were used to investigate the effect of the structural changes on the expression and subcellular targeting of the transporter proteins. Mutants derived from deletions showed reduced protein expression and in immunofluorescence showed a diffuse staining throughout the cytoplasm in transiently transfected cells, while the translation product derived from the point-mutated cDNA (G189E) was expressed at the level of the wild-type transporter and co-localized with the Golgi marker α-mannosidase II. This mutation therefore seems to directly affect the transport activity. Site-directed mutagenesis was used to change glycine 189 into alanine, glutamine, and isoleucine, respectively. While the G189A mutant was able to complement CMP-sialic acid transport-deficient Chinese hamster ovary mutants, the exchange of glycine 189 into glutamine or isoleucine dramatically affected the transport activity of the CMP-sialic acid transporter.

Carbohydrates added to cell surface proteins and lipids provide major contact and communication elements for animal cells. The biosynthesis of the carbohydrate structures occurs mainly in the luminal parts of the endoplasmic reticulum (ER) and Golgi apparatus and therefore requires specific nucleotide sugar transport systems (1, 2). Nucleotide sugar transporters have been described for CMP-sialic acid, UDP-galactose, UDP-GlcNAc, UDP-GalNAc, GDP-fucose, UDP-xyllose, GDP-mannose, UDP-glucuronic acid, and UDP-glucose (2–4). These proteins function as antiporters in an ATP- and ion-independent manner by exchanging the nucleotide sugar with the corresponding nucleoside monophosphate generated in the organelar lumen through the action of glycosyltransferases and nucleoside diphosphatases (2, 5). The high substrate specificity of the nucleotide sugar transporters, which has been demonstrated in biochemical and genetic analysis (2), makes these molecules ideal targets for the selective inhibition of glycoconjugate maturation. Increased sialylation has been described for tumor cell surfaces and has been shown to correlate positively with malignant potential (6–9). Since numerous sialyltransferases (for a review, see Ref. 10) but probably only a single CMP-sialic acid transporter (2) exist, the transporter may provide an effective target to inhibit cell surface sialylation. Accordingly, the inhibition of the UDP-galactose transporter and CMP-sialic acid transporter by somatic mutations and synthetic inhibitors resulted in strong reduction of the metastatic potential in the murine MDAY-D2 tumor cell line and in human colorectal cancer lines in nude mice (11–13).

Considerable progress in studying the transport of nucleotide sugars into the Golgi lumen has been made by the molecular cloning of nucleotide sugar transporter genes. CMP-Sia-Tr and UDP-Gal-Tr cDNAs were cloned from mammalian species (14–17), and the GDP-mannose transporter from Leishmania donovani (18, 19) and the UDP-GlcNAc- and UDP-Gal-transporters were cloned from yeast (20, 21). Related cDNAs from human, Saccharomyces cerevisiae, and Caenorhabditis elegans were also identified by homology searches in the gene data bases (16, 19, 20). Heterologous expression of the murine CMP-Sia-Tr on the zero background of S. cerevisiae was used to confirm the biological function of the protein (22). The transfected yeast cells acquired the ability to translocate CMP-sialic acid.

All nucleotide-sugar transporters cloned to date have been identified by complementation cloning in glycosylation mutants. The murine and hamster CMP-Sia-Tr were isolated by expression cloning in a clone of the Lec2 complementation group. Although the Lec2 mutation is known to inhibit translocation of CMP-sialic acid into the Golgi lumen (23), the molecular basis responsible for the asialo phenotype is still unknown. This study was undertaken to determine the molecular basis of the Lec2 phenotype. Thereby, we took advantage of the fact that several independent Lec2 mutants have been isolated in the laboratory (14). The results summarized in this study demonstrate that Lec2 cells carry defects in the CMP-Sia-Tr gene leading to aberrations in the transporter protein. The analysis of these mutants provides a useful system to gain
insight into structure-function relationships of the transporter protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Chinese hamster ovary (CHO) K1 wild-type cells and Lec2 cells (24) were obtained from the American Type Culture Collection (Rockville, MD). The CHO mutants 1E3, 6B2, 8G8, and 9D3 have been described recently (14). All cells, except clone Lec2, were maintained in Dulbecco’s modified Eagle’s medium nutrient mixture F12 (Life Technologies, Inc.) supplemented with 5% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, penicillin (100 units/ml), streptomycin (100 μg/ml) at 37°C in a 5% CO₂ incubator. Lec2 cells were grown in a minimal medium with Glutamax (Life Technologies), supplemented with 10% fetal calf serum. COS cells were grown in Dulbecco’s modified Eagle’s medium with Glutamax (Life Technologies) and 5% fetal calf serum.

**Northern Blot Analysis**—Total RNA was isolated from CHO cells by CsCl gradient centrifugation of guanidinium isothiocyanate lysates (25). RNA (5 μg) was electrophoresed in a 1% agarose, 1× formamide gel in 20 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA and 0.1 mM sodium phosphate, 7% SDS, 1% blocking reagent (Boehringer Mannheim) and visualized overnight at 65°C in high SDS buffer (5× SDS). RNA was transferred to a nylon membrane (Qiagen). Nylon filters were hybridized with a digoxigenin-labeled antisense RNA probe the murine CMP-Sia-Tr cDNA (14). After hybridization, the filters were washed twice in 2× SSC, 0.1% SDS at 65°C for 20 min in a digoxigenin-labeled RNA antisense probe, transferred from the murine CMP-Sia-Tr cDNA. EtBr, ethidium bromide staining of the gel prior to Northern transfer. RT-PCR analysis of mRNA from CHO wild-type cells and the mutants 1E3, 6B2, 8G8, and 9D3. Poly(A)⁺ RNA was reverse transcribed using a primer that is complementary to nucleotides 1292–1185 of the hamster CMP-Sia-Tr cDNA. PCR was carried out in 36 cycles with primers annealing to nucleotides 59–77 and 1187–1168, respectively. The lower of the two bands visible in each lane represents an inactive splice variant lacking exon 2 (nucleotides 17–194). M, DNA molecular mass marker.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)**—Polyadenylated RNA was selected from total RNA by using oligo(dT)25 (Qiagen) according to the manufacturer’s instructions. One μg of mRNA was reverse transcribed using 200 units of Superscript II RNase H (Life Technologies). The resulting plasmids direct the expression of hemagglutinin (HA)-tagged transport proteins under the control of the cytomegalovirus promoter. In these constructs, the stop codon of the transport protein was replaced with the HA epitope tag (GSYPYDVPDYASLRSGTRGAL; the epitope recognized by the anti-FLAG mAb M5 is underlined). C-terminal deletion mutants were generated by subcloning the 1.2-kilobase pair (corresponds to nucleotides 553–575 of the coding sequence of hamster CMP-Sia-Tr) in each of the first PCR were as follows: 30 s at 94°C, 30 s at 46°C, and 1 min at 72°C for 25 cycles. PCR products were gel purified and subjected to fusion PCR using primers ME1 and ME2. Conditions for the fusion PCR were as follows: 30 s at 94°C, 30 s at 46°C, and 1 min at 72°C for 10 cycles. PCR products were gel purified and ligated into the BamHI site of pEVRF0-HA. Plasmid DNA was sequenced by the dyeodeoxy chain termination method (27) using α[32P]dATP (Amersham Pharmacia Biotech) and T7 DNA polymerase (Stratagene). Products of RT-PCR reactions were purified on agarose gels (Qiagick DNA purification kit, Qiagen), denatured by boiling, and sequenced directly.

**Immunoblot Analysis**—For Western blot analysis, transiently transfected CHO cells were lysed in 20 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 200 units/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 2% Nonidet P-40. 100 μg of the postnuclear supernatant was used for SDS-polyacrylamide gel electrophoresis and Western blotting as described (28). Blots were probed with 2.5 μg/ml of anti-HA mAb 12CA5 (Boehringer Mannheim) and 5.4 μg/ml anti-FLAG mAb M5 (Eastman Kodak Co., respectively). Primary antibodies were then detected using anti-mouse alkaline phosphatase conjugate (Dianova) and using nitrilotetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

**Transient Transfection of Epitope-tagged Mutants of CMP-Sia-Tr**—2 × 10⁵ cells were seeded on glass coverslips in 35-mm cell culture dishes 24 h before transfection. For transfection, 1 μg of DNA was mixed with 6 μl of Lipofectamine (Life Technologies) in 1 ml of Opti-MEM. After 16 h, the transfection medium was discarded, and cells were washed once with Dulbecco’s modified Eagle’s medium nutrient mixture F12 (Life Technologies) with 10% fetal calf serum and incubated with the fusion protein for 24 h before fixation. For stained with mouse monoclonal anti-FLAG mAb M5 (Sigma) and visualized using a horseradish peroxidase-anti-mouse antibody conjugate in 5% nonfat dry milk in TBS-T. After washing, the blots were stained with a chemiluminescent reagent (Amersham Pharmacia Biotech) and exposed to X-ray film for 1–10 min.
MEM medium (Life Technologies). Cells were washed twice with Opti-MEM medium and incubated with the DNA/Lipofectamine mixture for 6–8 h. Transfection was stopped by adding 2 ml of Dulbecco’s modified Eagle’s medium nutrient mixture F-12 medium containing 5% fetal calf serum, and cells were grown for another 24 h prior to Western blot or immunofluorescence analysis.

Indirect Immunofluorescence—Cells were washed with PBS and fixed in 2% paraformaldehyde in PBS for 15 min. To neutralize residual paraformaldehyde, cells were washed in PBS and incubated twice for 20 min in 50 mM NH₄Cl in PBS. Thereafter, the cells were permeabilized with 0.2% saponin, 0.1% bovine serum albumin in PBS for 15 min followed by an overnight incubation with the primary antibodies at 4 °C. Immunodetection was carried out with the anti-FLAG mAb M5 (5.4 μg/ml), the anti-HA mAb 12CA5 (2.5 μg/ml), and rabbit anti-α-mannosidase II antiserum (29, 30) (1:2000; a kind gift of K. Moremen, University of Georgia, Athens, GA) in 0.2% saponin, 0.1% bovine serum albumin in PBS for 1 h. After washing four times in 0.1% bovine serum albumin/PBS, cells were incubated with anti-mouse Ig-fluorescein isothiocyanate (1:200; Dianova) and anti-rabbit Ig-tetramethyl rhodamine isothiocyanate (1:200; Dianova) for 1 h at room temperature. The incubation was stopped by three washes in 0.1% bovine serum albumin/PBS and a final wash in PBS. Slides were mounted in Moviol and analyzed under a Zeiss Axiophot Epifluorescence microscope.

RESULTS

Mutations in CMP-Sia-Tr-negative CHO Cells—CHO mutants used in this study (Lec2, 1E3, 6B2, 8G8, and 9D3) have been determined twice from independent RT-PCR data not shown) the doublets can be explained as N-terminally truncated proteins or by the use of a second downstream ATG. Expression of Epitope-tagged Transporter Proteins—Epitope tags were introduced to visualize translation products predicted from the cloned cDNA sequences. The cDNAs of the wild-type and mutant CMP-Sia-Tr were subcloned into the vector pEVRFO-HA directing the expression of proteins with carboxyl-terminal hemagglutinin (HA) tags. The following constructs were made: Δ[192–251]HA from Lec2 cDNA, Δ[251–296]HA from 6B2 cDNA, Δ[7–66]HA from 8G8 cDNA, and G189E-HA from 9D3 cDNA. In addition, two CMP-Sia-Tr variants with an N-terminal FLAG sequence that lack the C-terminal 171 and 240 amino acids, respectively, were generated. All constructs are summarized in Fig. 2. The constructs were transiently expressed in CHO and COS cells and the expression was analyzed by immunoblotting using the anti-HA mAb 12CA5 and the anti-FLAG mAb M5. The results obtained in COS cells are shown in Fig. 3. The apparent molecular masses of the fusion proteins are in good correlation with the calculated molecular masses (see Fig. 2). However, for the deletion mutants the expression levels are reduced compared with the wild-type and the point-mutated protein. This reduction in the protein level probably reflects a decreased stability of these proteins. Detection of the translation products with the mAb 12CA5 displayed double bands (see Fig. 3A). Since the faster migrating band was not detectable when N-terminally Flag-tagged transporter variants were analyzed (Fig. 3B and data not shown) the doublets can be explained as N-terminally truncated proteins or by the use of a second downstream ATG.

Subcellular Localization of Mutant Transporters—CMP-sialic acid transport activity is strongly associated with Golgi vesicle membranes (3), and epitope-tagged variants of mouse and hamster CMP-Sia-Tr are targeted to the Golgi apparatus (14, 17). To investigate if the mutations observed affect subcellular targeting of the CMP-Sia-Tr, the HA- and FLAG-tagged constructs were transiently expressed in CHO cells. 30 h after transfection, cells were fixed in paraformaldehyde, permeabilized with saponin, and the localization of each construct was determined by indirect immunofluorescence using the anti-HA mAb 12C5 and the anti-FLAG mAb M5. The results obtained in CHO cells are shown in Table I. The amino acid changes resulting from the nucleotide changes and the constructs with C-terminal HA tag are shown. Note that the deletion of an AG dinucleotide (underlined) in the mutant 8G8 leads to a frame shift in the full-length mRNA (a), whereas in the splice variant lacking exon 2 (b) the mutation results in an in frame deletion of amino acids 1–66.
D (192–251)HA (Fig. 4, C and D), D (251–296)HA (Fig. 4, E and F), and D (7–66)HA (Fig. 4, G and H) showed a diffuse staining throughout the cytoplasm. Co-localization of the transporter mutants and α-mannosidase II was not obtained. The same diffuse staining was found for the C-terminally deleted mutants FLAG (1–165) and FLAG (1–96) (Fig. 5, E and G). The FLAG-tagged wild-type transporter again co-localized with α-mannosidase II (Fig. 5, C and D). These data demonstrate that deletion mutants are retained in a pre-Golgi compartment, most probably the ER.

A Missense Mutation Affecting CMP-sialic Acid Transport—While no sialic acid or polysialic acid surface expression was observed after overexpression of transporter mutants derived from sequence deletions and insertions (data not shown), the overexpression of the construct G189E-HA led to a very weak polysialic acid expression. The polysialic acid (PSA) signal was best detectable by immunocytochemistry but was too faint to be displayed in Western blot (see Fig. 6). Starting from this observation, the importance of the glycine residue in position 189 was further investigated by substituting this position by amino acids with different chemical properties. Via site-directed mutagenesis HA-tagged constructs were generated in which Gly189 is exchanged to alanine (G189A), glutamine (G189Q), or isoleucine (G189I). Transient transfection of G189A into 8G8 cells resulted in full complementation, as can be seen by the reexpression of polysialic acid (Fig. 6). In contrast, the exchange of glycine to glutamine and isoleucine resulted in phenotypes that were very close to that of the G189E mutant. Western blot analysis using the anti-HA mAb 12CA5 confirmed equal protein expression for these mutants.

**DISCUSSION**

Cells of the Lec2 complementation group are defective in the transport of CMP-NeuAc into the lumen of the Golgi apparatus (23). Clones exhibiting the lec2 defect have been isolated by lectin resistance (24, 31) or immunoselection (14). The lec2 mutation causes the expression of asialo cell surfaces. Among the carbohydrate epitopes missing is PSA. Reexpression of PSA was therefore used to isolate cDNAs encoding the murine and hamster CMP-Sia-Tr cDNAs via complementation cloning (14, 17). The present study was carried out to determine the lec2 mutation at the molecular level. Four independent mutants (1E3, 6B2, 8G8, and 9D3), which arose from chemical mutation...
experiments (32), together with the clone Lec2 isolated via lectin resistance (24), were analyzed by Northern blotting and RT-PCR. Deletions, insertions, and point mutations in the CMP-Sia-Tr coding region were found that demonstrated that the gene defective in these cells encodes the CMP-Sia-Tr. The high frequency by which these mutants occur after chemical mutagenesis (32, 33) makes this approach an attractive way to identify functionally important primary sequence elements and to investigate structure-function relationships of nucleotide-sugar transporters.

Deletions observed in clones 6B2, 8G8, and Lec2, are likely to result from mutations in splice acceptor or donor sites. Support for this assumption comes from the observation that the deleted sequence sections share common boundaries. The extended sequence changes associated with the mutations lec2, 6%2, and 8g8, cause mistargeting of the translation products. While co-localization of the wild-type CMP-Sia-Tr with α-mannosidase II indicated transport to the Golgi apparatus (Ref. 14 and Figs. 4 and 5), all internally deleted or C-terminally truncated transporter mutants were not in the α-mannosidase II compartment but produced a diffuse staining throughout the cytoplasm. The staining pattern observed for the mutants might be explained by retention of the proteins in the ER. The extended primary sequence changes caused by the deletions or truncations most probably lead to misfolded proteins, which do not escape the process of “ER quality control” (34). Consistent with this, reduced expression levels were observed for the mutant proteins.

Analysis of the CMP-Sia-Tr mRNA from clone 9D3 revealed a single missense mutation, resulting in exchange of glycine at position 189 for glutamic acid. Like the other mutants, the steady-state level of mRNA expression in this mutant was comparable with that of wild-type cells (Fig. 1), and Western blot analysis of cells transiently transfected with the epitope-tagged G189E cDNA indicated that this protein was expressed at the same level as the epitope-tagged wild-type protein. In contrast to the mutants described above, this protein co-localizes with α-mannosidase II, indicating correct targeting to the Golgi apparatus. Therefore, the G189E mutation seems to directly affect the transport activity of the CMP-Sia-Tr. Changing glycine 189 to alanine did not influence the activity of CMP-Sia-Tr, measured as polysialic acid expression in clones of

**Fig. 4. Subcellular localization of HA-tagged hamster CMP-Sia-Tr mutants.** CHO cells were seeded onto glass coverslips transfected with the indicated constructs by using Lipofectamine. 30 h after transfection, cells were fixed in paraformaldehyde, permeabilized with saponin, and analyzed by indirect immunofluorescence using anti-HA mAb 12CA5 and anti-α-mannosidase II antisera simultaneously. Bound primary antibodies were visualized with anti-mouse Ig-fluorescein isothiocyanate (A, C, E, G, and I) and anti-rabbit Ig-tetramethyl rhodamine isothiocyanate (B, D, F, H, and K) conjugates. Bar, 25 μm.
the Lec2 complementation group. In contrast, the activities of G189Q and G189I mutants were drastically decreased and resembled that of the G189E mutant. These results suggest that not the charge repulsion between glutamine and CMP-sialic acid, but rather the size of the amino acid at position 189, is a critical factor for the transport activity. Large amino acids at this position may lead to steric hindrance of a hydrophilic “channel” required to translocate CMP-sialic acid through the membrane. This hypothesis is in good agreement with the transporter model proposed recently (17), where Gly189 is part of a transmembrane helix, closely located to the cytosolic face of the membrane. Another explanation would be that the mutation destroys a potential site involved in the binding of cofactors or in protein dimerization. So far, however, there are no experimental data supporting the idea.

The G189E mutation identified a functionally important region of CMP-Sia-Tr. In the mutant 9D3, sialic acid is undetectable by either Western blotting or immunocytochemistry. Overexpression of the mutant G189E in 9D3 cells, however, restored transport activity at a very low level. Due to the high sensitivity of the anti-PSA mAb 735, a very faint PSA signal was visible in immunocytochemistry and Western blot after transient transfection of cells. The signal intensity is however too low to be reproduced in Fig. 6, and sialic acid reexpression was not detectable with *Maackia amurensis* lectin. Thus, the mutant transporter from 9D3 cells is not completely inactive, but the endogenous expression of the mutated protein in 9D3 cells is insufficient to translocate CMP-sialic acid at a rate necessary for detectable amounts of (poly)sialic acid at the cell surface. Transient overexpression of the deletion mutants in CHO cells

**FIG. 6.** Western blot of cell extracts after transfection with mutant constructs. PSA synthesis was determined to test the complementation activity of G189 mutants. Therefore, 8G8 cells were transiently transfected with the HA-tagged wild-type (WT), and mutants where glycine 189 was changed to alanine (G189A), glutamine (G189Q), or isoleucine (G189I), respectively. The empty vector PEVRF0-HA (control) served as a negative control. Two days after transfection, cell lysates were analyzed by Western blot with mAb 735. A parallel immunoblot developed with the anti-HA mAb 12CA5 confirmed that the proteins were expressed at equal levels.

**FIG. 7.** Glycine 189 is part of a sequence segment that is highly conserved in sugar-nucleotide transporters. A comparison of the partial sequences of hamster CMP-Sia-Tr, human and yeast UDP-Gal-Tr, and a putative nucleotide-sugar transporter of *C. elegans* is shown. In all cases, this highly conserved region forms part of a putative transmembrane helix and the following hydrophilic loop. The glycine residue that is changed to glutamic acid in the mutant G189E is indicated by the arrow.

| Species          | Partial Sequence                                      |
|------------------|-------------------------------------------------------|
| Hamster CMP-Sia-Tr | G189E: VTASFGAVYFEVKLESDDT                            |
| Human UDP-Gal-Tr  | G189E: VTASFGAVYFEVKLESDDT                            |
| *S. pombe* UDP-Gal-Tr | G189E: VTASFGAVYFEVKLESDDT                         |
| *C. elegans* ZK370.7 | G189E: VTASFGAVYFEVKLESDDT                         |

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of the Lec2 cells did not lead to a detectable complementation. Together with the above results, this strongly suggests that these mutants are completely inactive.

The change Gly\textsuperscript{180} to Glu occurs in a region that is conserved among CMP-Sia-Tr and UDP-Gal-Tr isolated from mammals and the yeast Schizosaccharomyces pombe. Furthermore, this sequence is found in a putative nucleotide-sugar transporter of \textit{C. elegans}. All transporter sequences containing this motif are listed in Fig. 7. The high conservation strongly suggests that this amino acid stretch is essential for a functionally active transporter. On the other hand, the appearance of this domain in transporters of different specificity argues against a direct involvement in nucleotide-sugar binding or recognition. Additional studies are required to define the functional role of this sequence motif.

An important aspect of this study consists in the fact that a CMP-Sia-Tr mutant exhibiting residual transport activity could be isolated after chemical mutation of CHO cells. Isolation and functional analysis of such mutants provides a powerful way to gain further insight into structure-function relationships for this structurally ambitious group of molecules.

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