Lec3 Chinese Hamster Ovary Mutants Lack UDP-N-acetylglucosamine 2-Epimerase Activity Because of Mutations in the Epimerase Domain of the Gne Gene

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Lec3 Chinese hamster ovary (CHO) cell glycosylation mutants have a defect in sialic acid biosynthesis that is shown here to be reflected most sensitively in reduced polysialic acid (PSA) on neural cell adhesion molecules. To identify the genetic origin of the phenotype, genes encoding different factors required for sialic acid biosynthesis were transfected into Lec3 cells. Only a Gne cDNA encoding UDP-GlcNAc 2-epimerase:ManNAc kinase rescued PSA synthesis. In an in vitro UDP-GlcNAc 2-epimerase assay, Lec3 cells had no detectable UDP-GlcNAc 2-epimerase activity, and Lec3 cells grown in serum-free medium were essentially devoid of sialic acid on glycoproteins. The Lec3 phenotype was rescued by exogenously added N-acetylmannosamine or mannosamine but not by the same concentrations of N-acetylglucosamine, glucosamine, glucose, or mannose. Sequencing of CHO Gne cDNAs identified a nonsense (E35stop) and a missense (G135E) mutation, respectively, in two independent Lec3 mutants. The G135E Lec3 mutant transfected with a rat Gne cDNA had restored activity and cell surface PSA expression. Both Lec3 mutants were similarly rescued with a CHO Gne cDNA and with CHO Gne encoding the known kinase-deficient D413K mutation. However, cDNAs encoding the known epimerase-deficient mutation H132A or the new Lec3 G135E Gne mutation did not rescue the Lec3 phenotype. The G135E Gne missense mutation is a novel mechanism for inactivating UDP-GlcNAc 2-epimerase activity. Lec3 mutants with no UDP-GlcNAc 2-epimerase activity represent sensitive hosts for characterizing disease-causing mutations in the human GNE gene that give rise to sialuria, hereditary inclusion body myopathy, and Nonaka myopathy.

Sialic acids, including N-acetylenuraminic acid (Neu5Ac) and other N- or O-substituted neuraminic acids, are found on the distal end of N- and O-glycans and glycolipids in vertebrate glycoconjugates (1). Sialic acid is an essential component of the sialyl-Lea tetrasaccharide that is recognized by selectins and is required to initiate leukocyte adhesion to inflamed endothelium (2). Sialic acid is also important for different viruses and bacteria to infect host cells (3–5). Polysialic acid (PSA) is a homopolymer of α-2,8-linked sialic acid on N-glycans mainly associated with the neural cell adhesion molecule (N-CAM) in mammals (6). PSA present on N-CAM functions in neural cell recognition and brain development, being important for neural cell migration, axonal pathfinding, and spatial learning (7, 8). Inactivation of sialic acid synthesis by gene targeting causes embryonic lethality in mice (9). Mutations in sialic acid biosynthesis cause sialuria, hereditary inclusion body myopathy (HIBM), and Nonaka myopathy in humans (see OMIM (Online Mendelian Inheritance in Man), accession number 603824). Therefore, it is important to identify the genes involved in sialic acid synthesis to understand the biological functions of sialic acids.

Sialic acid synthesis begins with the generation of ManNAc in the cytosol following the epimerization of UDP-GlcNAc at the C-2 position and hydrolysis. Released ManNAc is subsequently phosphorylated to become ManNAc-6-phosphate. These two steps are catalyzed by a single bifunctional enzyme, UDP-GlcNAc 2-epimerase:ManNAc kinase, encoded by the human GNE gene (10). The N- and C-terminal halves of the protein encode the epimerase and kinase domains, respectively. UDP-GlcNAc 2-epimerization is regulated by strong feedback inhibition by downstream products including CMP-Neu5Ac (11). Point mutations at allosteric sites in the epimerase domain are the cause of sialuria in which patients lose feedback control by CMP-Neu5Ac and secrete large amounts of sialic acid in their urine (12). The loss of epimerase or kinase activities also induces aberrations of muscular motility that are the cause of HIBM or Nonaka myopathy (13, 14). To generate sialic acid, ManNAc-6-phosphate is condensed with phosphoenolpyruvate to form Neu5Ac-9-phosphate by sialic acid synthase (15). An unknown phosphatase induces the release of phosphate and provides the substrate for CMP-Neu5Ac synthetase that forms CMP-Neu5Ac (16). CMP-Neu5Ac is transported into the Golgi via the CMP-sialic acid transporter (17) and used as a substrate of various sialyltransferases.

Previously we characterized two loss-of-function CHO mutants in sialic acid synthesis: Lec2 mutants have mutations in the CMP-Neu5Ac transporter gene (18), and the Lec32 mutant (which occurs in the double mutant LEC29.Lec32 (19)) lacks transcripts of the CMP-Neu5Ac synthetase gene (16). We have also described the Lec3 CHO mutant as having reduced sialic acid and belonging to a distinct genetic complementation group...
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(20, 21). Lec3 CHO mutants are slightly resistant to wheat germ agglutinin (WGA) and hypersensitive to ricin. They have reduced sialic acid on their cell surface glycoproteins and glycolipids, although neuraminidase treatment releases significant amounts of sialic acid from Lec3 glycoproteins. They have no apparent defect in sialyltransferase activity. Here we show that two independent Lec3 isolates are mutated in the CHO Gne gene that encodes UDP-Glc-Neu5Ac-epimerase/MannNac kinase. Both mutants lack UDP-GlcNAc 2-epimerase because of different single nucleotide changes in the epimerase domain.

EXPERIMENTAL PROCEDURES

Materials—The 735 mouse monoclonal anti-o2,8-polyosialic acid IgG2a antibody was a gift from Dr. Rita Gerardy-Schahn (Medizinische Hochschule, Hannover, Germany) (22). N-CAM-3 (BD Biosciences) is a monoclonal IgG2a antibody against the extracellular domain of mouse N-CAM. Monoclonal anti-SSEA-1 antibody was prepared by 40% ammonium sulfate precipitation of ascites produced from hybridoma cells obtained from Dr. Barbara Knowles (Jackson Laboratories, Bar Harbor, ME). Monoclonal anti-CSLEX-1 was obtained from Dr. Paul Terasaki (University of California, Los Angeles). Anti-VIM-2 monoclonal antibody was a gift from Dr. Bruce Macher (San Francisco State University, San Francisco, CA). Lectins, including concanavalin A, WGA, and the leukoagglutinin from Phaseolus vulgaris, were purchased from Vector Laboratories (Burlingame, CA). Restriction enzymes and buffers were from Roche Diagnostics GmbH (Mannheim, Germany), Invitrogen (Carlsbad, CA), and New England Biolabs (Beverly, MA). Hybond N membrane was from Amersham Biosciences. DNA fragments were labeled with the Prime-It II random primer labeling kit (Stratagene) with [32P]dCTP (PerkinElmer Life Sciences). Synthetic oligonucleotides, Superscript II, and dNTP were purchased from Invitrogen. DNA sequencing and fluorescence-activated cell scanning (FACS) were performed at Albert Einstein College of Medicine. Other chemicals and reagents were from Sigma and Fisher.

Cell Lines and Cell Cultures—Cloned CHO and mutant lines were isolated previously. The references given describe the molecular basis of mutation for each mutant. For clarity, mutant phenotype names are used throughout the text, and clones are identified where necessary and as follows: parent CHO (clone Pro 5) (23), Lec2 (clone Pro Lec2.4C defective in the CMP-sialic acid transporter) (18), Lec3 (clones Pro Lec3 4B and Gas’ Lec3 8F resistant to WGA) (24), and Lec3 (degenerated from Pro Lec29 Lec3 2, a CHO mutant with two glycosylation mutations) (19). The LEC29 phenotype is due to a gain-of-function mutation resulting in the de novo expression of the CHO Fut9 gene (25), and the Lec32 phenotype is due to a defective CMP-sialic acid synthetase (18). All cells were routinely cultured in α-minimum Eagle’s medium (Gibco) containing fetal calf serum (embryonic stem cell quality, Gemini) in suspension or a monolayer culture at 37 °C. Serum-free growth was accomplished by gradually reducing the fetal calf serum concentration to zero in CD-CHO medium (Invitrogen) as suggested by the manufacturer.

Plasmids and Transfection of CHO Cells—To clone rat Gne and Sas cDNAs into mammalian expression vectors, BamHI fragments and BamHI-NotI fragments were prepared from pFastbacHTA/ratGne (27), hamster washing with 500 μl of Opti-MEM I, Opti-MEM I, 3% fetal calf serum was added, and cells were incubated in a CO2 incubator. Solution A containing 2 μg of cDNA and 50 μl of Opti-MEM I and solution B containing 2 μl of LipofectAMINE 2000 and 50 μl of Opti-MEM I were prepared and left at room temperature for 5 min. Solutions A and B were then mixed and left at room temperature for 20 min. 100 μl of mixed solution were added to each well. The cells were cultured for 1 day and detached with 200 μl of trypsin/EDTA solution (Invitrogen). The detached cells were additionally cultured in suspension for 1–2 days before FACS analysis.

To obtain Lec3 cells stably transfected with a rat Gne cDNA or vector control, the cells were cultured on a 24-well plate and transected using LipofectAMINE 2000 (Invitrogen) after 1 day as directed by the manufacturer. The cells were replated on a 10-cm dish after 2 days and cultured for 2 weeks in α-minimum Eagle’s medium containing 10% fetal calf serum and 1.5 mg/ml G418 (active weight). Colonies were detached and cloned by limiting dilution in a 96-well plate in α-minimum Eagle’s medium containing 1.5 mg/ml active G418.

Flow Cytometry Analysis—In the CHO cell surface, cells (1 × 10⁶) from suspension culture were washed in PBS, 1% bovine serum albumin and incubated with 1:200-diluted anti-PSA mAb 735 (29) or 1:200-diluted anti-N-CAM (N-CAM13) antibodies for 15 min on ice. After washing once with PBS, 1% bovine serum albumin, the cells were stained with 1:200-diluted fluorescein isocyanate-conjugated anti-mouse IgG1-FITC (30) or anti-IgG FITC (30) and then washed and subjected to FACS analysis.

Lectin Toxicity Assay—Cells (2,000/well of a 96-well dish) were incubated with increasing concentrations of concanavalin A, WGA, or leukoagglutinin from P. vulgaris for 3–4 days at 37 °C. When the control wells lacking lectin were confluent, the medium was removed and the attached cells were stained with 0.2% methylene blue in 50% methanol.

Cloning of a Chinese Hamster Gne cDNA—To clone a cDNA derived from the Chinese hamster Gne gene, subpools of 20,000 clones each were made from a CHO-K1 cell cDNA library (31) (a gift from Drs. Osamu Kuge and Masahiro Nishijima, Institute of Infectious Diseases, Tokyo, Japan) and screened by PCR with primers DGNGE1F (5’-CTCTCACCCTGATMGAGYACTAYGGAAA-3’ forward) and DGNGE2R (5’-TTGRTARTGAGCAGCTTTGATGGC-3’) reverse) designed from the mouse Gne cDNA sequence (GenBank™ accession number NM_015828). From a subpool showing positive signal bands, a 5′ fragment of the hamster Gne gene coding region was amplified with an upstream vector primer and DGNGE2R. A 3′ fragment was amplified with DGNGE1F and a downstream vector primer, and the amplified bands were sequenced. A full-length hamster Gne cDNA was amplified with primers CHO-GNEF/SalI (5’-AAAGCAATGCAAGAGAAATGGGAAATAC-3’, forward) and CHO-GNER (5’-AACCTCGTTCACCTAGCTTCTT-3’, reverse) using the positive cDNA pool as a template. To clone the Gne gene coding region from wild type CHO (Pro5), the cDNA generated using the primers described above was cloned into pcR3.1 (Invitrogen). To obtain FLAG-tagged CHO Gne protein, PCR fragments generated from cDNA were cut with Sall and NotI and cloned into pME-3FLAG (32) to generate pME-3FLAG-CHO-Gne. The cloned full-length cDNA and the 3FLAG-tagged construct were sequenced and shown to be active following transfection and FACS analysis. cDNAs amplified from CHO total RNA were identical in sequence to the CHO-K1 cDNA library Gne sequence except for a change from G at position 568 in CHO to Asp in CHO-K1. The CHO-K1 Gne cDNA was not active in transfectants.

Reverse Transciptase PCR—For reverse transcription, the SuperScript 1st Strand system (Invitrogen) was used. Briefly, 2 μl of total RNA (1 × 10⁶ cells equivalents) from CHO or Lec3 cells were mixed with 1 μl of 500 μg/ml oligo dT 18 primer, 1 μl of 10 mM dNTP, and 5 μl of water and incubated at 65 °C for 5 min. After cooling on ice, 4 μl of 5× first strand buffer, 4 μl of 20 mM dithothreitol, 4 μl of deionized water, 5 μl of 2.5 mM dNTP, 0.5 μl of cDNA, and 1 μl of 10 pm oligonucleotide primer, and 1 μl of 200 units of Superscript II were added. The reaction was incubated at 42 °C for 50 min followed by incubation at 70 °C for 15 min and then stored at −20 °C. Reverse transcriptase products were amplified using the Long Template PCR kit (Roche Applied Science). In a 50-μl reaction, 5 μl of cDNA (1.0 × 10⁶ cells in 10 μl of 10× buffer) (28), 0.1 μl of 10 μM each primer, and 0.75 μl of polymerase were mixed. Reactions were treated at 94 °C for 30 s, 62 °C (for 30 s), and 68 °C (for 1 min). Amplified PCR fragments were eluted from an agarose gel and directly sequenced.

Site-directed Mutagenesis of CHO Gne cDNA—To generate mutations in the CHO Gne cDNA, site-directed mutagenesis was performed using an oligonucleotide-directed mutagenesis method (Stratagene) and pME-3FLAG-CHO-Gne plasmid DNA. The H132A, G135E, and D413K-point mutants were constructed with CHO-GNE Mut1F (5’-AT-CTTCACATTGAGAGAGGACTGACCATGGG-3’) and CHO-GNEM
ut1R (5'-CCCACTGACCTCTCTTGCATGGTAGGAGGT-3'), CHO-GNE2F (5'-AACATCGGATCTCTGACTAGAAGAAGAGAC-3') and CHO-GNE2R (5'-CTCCTCCCTTAAGTTGACCCAGTGGATG- TT-3'), or CHO-GNEMut3R (5'-AGTCCTCGCTGTTAATCTTGGA- GGAACAAAC-3') and CHO-GNENMut3R (5'-GTTTCTGTCCTCCAAGGT- TAACAGCCAGACGACT-3'), respectively. After mutagenesis, all constructs were confirmed by sequencing.

**Northern Blot Analysis**—Total RNA (40 μg) purified by TriZol (Life Technologies) was separated in a 1% agarose gel and blotted onto a Hybond N+ membrane (Amersham Biosciences). The probe to detect CHO Gne transcripts was obtained from a 2.5-kb Sal-NotI fragment of pME-3FLAG-CHO-Gne and radiolabeled using a Random-iT priming kit (Stratagene). All procedures were followed as described in Ref. 30. After blotting, the membrane was incubated in prehybridization solution (0.05 M Pipes, 0.1 M NaCl, 0.05 M NaPO4, pH 7.0, 10 mM EDTA, 5% SDS, 0.1 mg/ml denatured salmon sperm DNA) at 60 °C for 2 h. After adding radiolabeled probe, the membrane was incubated at 60 °C overnight. Washing was done in 2× SSC, 0.1% SDS (2×), and 0.2× SSC, 0.1% SDS (2×), and the membrane was exposed to x-ray film for 3 days at 80 °C. The membrane was reblotted using a radiolabeled glyceraldehyde-3-phosphate dehydrogenase probe after stripping.

**Western Blot Analysis**—To detect FLAG-tagged Gne, cells (5 × 10⁶) were dissolved in 1% Nonidet P-40 in TNE buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM EDTA) containing protease inhibitor mixture (Roche Applied Science) for 1 h. After centrifugation at 15,000 × g for 10 min, the supernatant was immunoprecipitated from the supernatant with biotinylated anti-FLAG M2 antibody-conjugated beads (Sigma) for 1 h at 4 °C, and the beads were washed three times with ice-cold TNE buffer at reverse transcription. One volume of 4% SDS in the sample buffer was added, the beads were boiled for 5 min and then cooled on ice, and the proteins were then separated by 7.5% SDS-PAGE gel (Bio-Rad) and blotted on polyvinylidene fluoride membrane (Amersham Biosciences). Western blots were performed with biotinylated anti-FLAG M2 (Sigma) and horseradish peroxidase-conjugated avidin. To detect N-CAM or PSA/N-CAM, cells (5 × 10⁶) were dissolved in 1% Nonidet P-40 and centrifuged at 14,000 rpm. Anti-N-CAM (N-CAM13) or anti-PSA (735) antibodies were added to the supernatants followed by protein A/G-conjugated beads (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA). Proteins were eluted in 4% SDS sample buffer, boiled, electrophoresed on 7.5% SDS-PAGE gel, western blotted with anti-N- CAM (N-CAM13) or PSA (735) antibodies, and detected with horseradish peroxidase-conjugated mouse IgG antibodies.

**UDP-GlcNAc2:2-Epimerase Assay**—An in vitro assay for UDP-GlcNAc 2-epimerase was performed essentially as described in Ref. 33. To prepare cell lysates, cells (1 × 10⁶) were resuspended in 500 μl of 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride for 10 min on ice. Cells were passed through a 22-gauge syringe using 20 strokes and centrifuged for 20 min in a microcentrifuge at maximum speed. Supernatant protein was then separated by the Bradford method. Sialic acid was released by treating pelleted glycoproteins with 1N HCl (Pierce) at 80 °C for 2 h. After centrifugation for 5 min in a microfuge, the supernatant was monitored for its ability to inhibit sialidase, STX, or PST (see legend) or the CMP-sialic acid transporter (see legend). The Lec3 defect was also rescued by adding ManNAc to the sample buffer. Dried TLC plates were exposed to a phosphorimaging plate for 3 days. After scanning for radioactivity, the TLC plate was stained by orcinol (34). New spots different from UDP-GlcNAc migrated at the same position as nonradiolabeled ManNAc.

**Sialic Acid Assay**—Cells growing in complete or serum-free medium were washed with PBS containing cations and extracted with 1.5% Triton X-100 on ice. Glycoproteins were ethanol-precipitated from ~500 μg of extract protein determined by the Bio-Rad assay. Sialic acid was released by treating pelleted glycoproteins with 1N HCl (Pierce) at 80 °C for 2 h. After centrifugation for 5 min in a microfuge, the supernatant was dried, resuspended in 60 μl of water, and 30 μl were analyzed by high performance anion exchange chromatography with pulsed amperometric detection on a PA 100 column ( Dionex) eluted with a gradient of 7–40 mM NaAc in 100 mM NaOH over 15 min. Sialic acid was detected with the ED40 electrochemical detector. The area under the sialic acid peak was determined compared with a 2-azml control.

**RESULTS**

Lec3 Cells Have a Subtle Reduction in Stasylated Glycans on Cell Surface Glycoconjugates—Lec3 cells grown in complete medium with 10% serum exhibited small but significant changes in sensitivity to toxic lectins compared with parent CHO cells, being slightly hypersensitive to concanavalin A and slightly resistant to WGA and leukoagglutinin from P. vulgaris, respectively (Fig. 1A, Ref. 21). However, FACS analyses using these and other fluorescence-tagged lectins at various concentrations did not detect significant binding differences between CHO and Lec3 cells (data not shown). We, therefore, investigated the expression of terminal glycan epitopes that contain sialic acid and fucose using monoclonal antibodies. Lec3 cells were transiently transfected with a CHO cGFPu6B cDNA encoding α-1,3-fucosyltransferase V1B (30) to assess the expression of sialyl-Leα and CD65 (30) recognized by the anti-CSLEX-1 and VIM-2 (CDw65) monoclonal antibodies, respectively, and the nonsialylated Leα determinant recognized by the anti-SSEA-1 monoclonal antibody (Fig. 1B). For comparison, parental CHO transfectants (capable of making all antigens) and the Lec2 transfectants (capable of making only Leα) were transiently transfected with cGFPu6B and analyzed by FACS. In Fig. 1B it can be seen that Lec2 cells that lacked CMP-Neu5Ac transport into the Golgi compartment (17, 35) bound anti-SSEA-1 antibody but did not bind CSLEX-1 or VIM-2 antibodies as expected. By contrast, Lec3 mutants bound CSLEX-1 and VIM-2 antibodies, although both profiles were slightly reduced in intensity compared with parent CHO cells. A much more significant difference was obtained when PSA expression was analyzed in the Lec3 mutant. Previous experiments have shown that N-CAM is essentially the only glycoprotein in CHO cells detected with anti-PSA antibodies (28, 36). Fig. 2A shows that parent CHO cells bound the anti-PSA mAb 735 well. By contrast, the mean fluorescence intensity of mAb 735 was reduced by about two-thirds for Lec3 compared with parent CHO cells, whereas CHO and mutant cells expressed N-CAM at similar levels. As expected, no PSA was detected on the surface of either Lec2 or Lec3 sialic acid mutant cells (Fig. 2A). Similar results were obtained when N-CAM was immunoprecipitated from cell lysates and examined by Western blot analysis (Fig. 2, B and C). N-CAM from CHO and Lec3 cells migrated similarly as a slower and broader band than N-CAM from Lec2 or Lec3 sialic acid mutants (Fig. 2B). However, only CHO cell N-CAM gave a strong signal with anti-PSA mAb 735 (Fig. 2C). The PSA signal was markedly reduced in Lec3 N-CAM. As expected, a PSA signal was not detected in Lec2 or Lec3 N-CAM. Taken together, these results indicated that Lec3 cells synthesize less sialic acid than parent CHO cells and that reduced PSA synthesis on N-CAM is a most sensitive indicator of this defect.

**Complementation of the Lec3 PSA Cell Surface Expression Defect**—To investigate the molecular basis of the lec3 mutation, cDNAs encoding mammalian enzymes involved in sialic acid and PSA synthesis (including Gne, Sas, CMP-sialic acid synthetase, STX, or PST) or the CMP-sialic acid transporter (see legend) were transiently transfected into Lec3 cells, and the cell surface expression of PSA was examined after 48 h. Only the cDNA encoding rat Gne increased PSA on the surface of the Lec3 mutant (Fig. 3B). The very high PSA expression of Lec3 Gne transfectants is presumably caused by Gne overexpression (36).

The Lec3 defect was also rescued by adding ManNAc, the product of UDP-GlcNAc 2-epimerase, to the medium. Both CHO cells and Lec3 mutants, but not Lec2 or Lec3 mutants, expressed high levels of cell surface PSA when grown in 10 mM ManNAc (Fig. 4A) or mannosamine (not shown). Similar results were obtained by Western blot analysis with anti-PSA antibodies, although N-CAM levels were not altered by growth in ManNAc (Fig. 4B). The addition of 1 mM mannosamine to the medium also rescued the Lec3 PSA defect, but up to 5 mM
GlcNAc or 10 mM glucosamine, Glc, or Man did not rescue PSA expression by the Lec3 mutant (data not shown). By contrast, PSA expression was increased in CHO cells under all these conditions. GlcNAc 2-epimerase encoded by a separate gene could, in theory, generate ManNAc from GlcNAc, but this reaction is not the favored biosynthetic route in the cell (37). However, mannosamine is readily converted to ManNAc, which can be phosphorylated by GlcNAc kinase (38, 39). Thereby, both ManN and ManNAc may bypass a block in UDP-GlcNAc 2-epimerase:ManNAc kinase activity. The combined results suggest that Lec3 cells may be defective in UDP-GlcNAc: ManNAc epimerase activity.

**Lec3 Mutants Have No UDP-GlcNAc 2-Epimerase Activity**—The mammalian Gne gene encodes a bifunctional protein with an N-terminal UDP-GlcNAc 2-epimerase domain and a C-terminal ManNAc kinase domain (10). UDP-GlcNAc 2-epimerase activity was assayed using a cytosol fraction as described in Ref. 33. CHO cell cytosol converted radiolabeled UDP-GlcNAc to ManNAc as shown in Fig. 5A. However, there was no detectable conversion of UDP-GlcNAc to ManNAc by Lec3 cytosol.
The lack of UDP-GlcNAc 2-epimerase activity in Lec3 cell-free extracts does not apparently fit with the significant sialic acid expression at the Lec3 cell surface (Fig. 1). However, this phenotype has been observed previously in cells lacking UDP-GlcNAc 2-epimerase activity (9, 11) and is proposed to be due to the ability of cultured cells to capture sialic acid from serum glycoproteins and to convert it directly into CMP-sialic acid (40, 41). In fact, Lec3 cells grown in serum-free medium had essentially the same amount of sialic acid as Lec2 mutant cells grown in complete medium based on an HPAEC-PAD assay of CHO extracts from Lec3 cells stably expressing a rat Gne cDNA (Fig. 6B). The combined results indicated that the CHO Gne gene may be mutated in Lec3 cells.

Single Point Mutations in the Hamster Gne Gene of Independent Lec3 Mutants—To examine Gne gene transcripts in Lec3 cells, a Chinese hamster Gne cDNA encoding the complete coding region was cloned from a CHO cDNA library by PCR. The Gne cDNA of 2.6 kb was detected in both CHO and Lec3 cells (Fig. 6A). The deduced hamster Gne protein sequence is 94, 95, and 95% identical to the human, mouse, and rat Gne genes, respectively, and the N-terminal half is highly conserved with the N terminus of bacterial UDP-GlcNAc 2-epimerase (Fig. 6B). This suggested that the Lec3 mutant may have arisen from a point mutation. Interestingly, a comparison with glyceraldehyde-3-phosphate dehydrogenase transcripts revealed that the steady state level of Gne transcripts in Lec3 cells was slightly greater than in CHO cells.

To determine the Gne gene coding sequence in CHO and Lec3 cells, cDNAs were prepared by reverse transcriptase PCR from two independent Lec3 mutant lines (Pro Lec3.4B and Gat Lec3.6F, see “Experimental Procedures”) and sequenced. Lec4.4B had a single nucleotide change that gave rise to a missense mutation (G135E), whereas Lec3.6F had a single change that gave rise to a nonsense mutation (E35stop) (see Fig. 6B). Both mutations were also found by sequencing of the PCR products from the genomic DNA of the respective Lec3 mutants (data not shown). The PCR products from either cDNAs or genomic DNA of the mutants were uniform in sequence showing no indication of the presence of a wild type allele in the mutant. Thus, the CHO genome must be hemizygous at the Gne locus and both Lec3 mutants contain only a mutated Gne allele.

The nature of the two CHO lec3 mutations is consistent with the lack of UDP-GlcNAc 2-epimerase activity in Lec3.4B (Fig. 5A). The lec3 nonsense mutation, E35stop, does not encode a functional enzyme. Consistent with this, PSA expression examined by Western blot and FACS analysis was markedly reduced in Lec3.6F cells (data not shown). Moreover, the addition of ManNAc to the culture medium only partially rescued PSA expression in Lec3.6F cells consistent with the probability that this Lec3 mutant does not synthesize a functional ManNAc kinase from the Gne gene. For the lec3 missense mutation, G135E, a Gly at residue 135 is strictly conserved in mammalian and bacterial UDP-GlcNAc 2-epimerases (Fig. 6B). The G135E lec3 mutation therefore appears to identify a residue critical for UDP-GlcNAc 2-epimerase activity, because this mutant had no activity in the in vitro assay (Fig. 5A).

To show that the G135E mutation inactivates Gne function in vivo, FLAG-tagged CHO Gne cDNA was mutagenized to contain the G135E mutation and transfected into Lec3 cells. Two days after transfection, FLAG-tagged proteins were expressed equivalently in the transfected cells (Fig. 7A and data not shown). By FACS analysis, it was shown that CHO Gne cDNA restored PSA expression to Lec3 cells but mutant Gne cDNAs did not (Fig. 7B and data not shown). The very low binding of PSA to control Lec3 transfectants compared with untransfected Lec3 cells growing in suspension (Fig. 2) is because, as shown in Fig. 7B, cells were transfected in monolayer, trypsinized after 48 h, and cultured in suspension for a day before FACS analysis.

Lec3 Mutants for Rapid Identification of Gne Mutations That Alter UDP-GlcNAc 2-Epimerase Activity—To investigate whether complementation of the Lec3 mutant phenotype would...
provide a sensitive test for characterizing human disease-related \textit{GNE} mutations, cDNAs encoding two previously characterized \textit{Gne} mutant alleles (42) were introduced into Lec3 cells. In vitro enzyme assays using recombinant proteins showed previously that the \textit{Gne} mutant H132A has no detectable UDP-GlcNAc 2-epimerase activity, and the D413K \textit{Gne} mutant has no detectable ManNAc kinase activity (42). As expected, introducing the kinase-negative D413K mutation into the wild type CHO \textit{Gne} cDNA corrected PSA expression in Lec3.4B cells (Fig. 7) due to ManNAc kinase activity present in the Lec3 mutant (Fig. 4). By contrast, introducing the H132A UDP-GlcNAc 2-epimerase mutation into the CHO \textit{Gne} cDNA did not rescue the Lec3.4B phenotype (Fig. 7). Notably, however, there was a small but significant increase in PSA expression in H132A \textit{Gne} Lec3 transfectants (Fig. 7). This revealed a slight UDP-GlcNAc 2-epimerase activity that was not detected in the \textit{in vitro} enzyme assay (42) showing that FACS analysis for PSA expression is a more sensitive assay. The same transfection experiments were performed in Lec3.6F cells in which CHO \textit{Gne} and the D413K \textit{Gne} cDNAs corrected PSA expression, whereas the G135E and H132A \textit{Gne} cDNAs did not (data not shown). Both Lec3 CHO mutant lines may, therefore, be used to detect human \textit{GNE} mutations that weaken or inactivate UDP-GlcNAc 2-epimerase activity.

\textbf{DISCUSSION}

Here we have identified the molecular basis of two Lec3 loss-of-function CHO glycosylation mutants as point mutations in the CHO \textit{Gne} gene. One mutation is a nonsense (E35stop), and the other is a missense (G135E), and both fall in the epimerase domain of the \textit{Gne} gene. Each mutant lacks UDP-GlcNAc 2-epimerase activity. The nonsense mutant is pre-
dicted to have also lost ManNAc kinase activity, whereas the missense mutation should not affect the ManNAc kinase activity encoded by \textit{Gne}. However, this point would need to be tested directly in a recombinant enzyme assay, because both mutations are rescued by ManNAc and ManN, probably by GlcNAc kinase that has been shown to phosphorylate ManNAc (38, 39). Lymphoid and hematopoietic cell variants with no detectable UDP-GlcNAc 2-epimerase activity (11) and embryonic stem cells homozygous for a \textit{Gne} gene inactivating mutation (9) are also rescued by ManNAc. In one mutant lacking \textit{Gne} mRNA, biochemical and metabolic experiments identified GlcNAc kinase as the activity generating ManNAc-phosphate (39). CHO cells clearly do not have an alternative epimerase activity that can produce ManNAc, similar to other UDP-GlcNAc 2-epimerase mutant isolates from human and mouse (9, 11). An investigation of a potential role for GlcNAc 2-epimerase in converting GlcNAc to ManNAc concluded that the reverse is true. GlcNAc 2-epimerase plays a catabolic role and diverts ManNAc from the sialic acid synthetic pathway (37). Lec3 mutants are also not affected in the allosteric region of \textit{Gne} responsible for feedback regulation of UDP-GlcNAc 2-epimerase by CMP-sialic acid, because exogenous glucosamine did not increase PSA expression as in the sialuria mutants of Jurkat cells (43).

The \textit{G135E} mutation represents a new inactivating mutation for the UDP GlcNAc 2-epimerase domain of the \textit{Gne} gene. Glycine at this position is conserved in all eukaryotic and prokaryotic UDP-GlcNAc 2-epimerases characterized to date. Other mutations that inactivate UDP-GlcNac 2-epimerase activity were introduced to a rat \textit{Gne} cDNA and include conserved His to Ala mutations induced at H45, H132, H110, H135, and H157 (42). Several human mutations in the \textit{GNE} gene epimerase domain have been identified in patients with HIBM, but none are homozygous and none have been functionally characterized (44). Two compound heterozygotes carry R246Q and D225N or I200F and D378Y mutations, respectively (44). The \textit{G135E} mutation falls at a position distinct from those represented among established HIBM patients (44).

Lec3 mutants were originally selected for their slight resistance to WGA, and it is apparent from Fig. 1 that the sialylation

![Figure 4: Rescue of the Lec3 phenotype by ManNAc. A, cells were cultured in medium containing 10% fetal calf serum and 10 mM ManNAc (bold lines) or the same volume of PBS (thin lines) for 2 days and subjected to FACS analysis with anti-PSA (735) antibodies. B, extracts of the same cells were immunoprecipitated (IP) with anti-N-CAM antibody (N-CAM13) and detected with anti-N-CAM (N-CAM13) (lanes 1–4) or anti-PSA (lanes 5–8) antibodies. Bands marked by an asterisk (*) were heavy chains of antibodies used in immunoprecipitation.]

![Figure 5: Lec3 CHO Glycosylation Mutants. A, cell cytosol extracts were incubated with UDP-[\textsuperscript{14}C]GlcNac for 3 h at 37 °C as described under "Experimental Procedures," and reaction products were separated by TLC. The TLC plate was exposed on an image plate for 3 days and read by the phosphorimaging device (left panel). Lane 1, buffer only; lane 2, wild type cells; lane 3, Lec3 cells; lane 5, vector transfected Lec3 cells. B, extracts were made from Lec3 cells stably transfected with a rat \textit{Gne} cDNA or vector alone and immunoprecipitated with anti-N-CAM (N-CAM13). After blotting, proteins were detected with anti-N-CAM or anti-PSA (735) antibodies. Bands marked by an asterisk (*) were heavy chains of antibodies used in immunoprecipitation.]}
defect in Lec3 mutants is extremely subtle. This is because serum glycoproteins in normal culture medium rescue the Lec3 defect presumably by exogenously providing sialic acid to the cytosol (9, 40, 41). This was demonstrated by the fact that Lec3 cells grown in serum-free medium had only 15% of the glycan-bound sialic acid of glycans from Lec3 cells grown in complete medium. Similar results were obtained with mouse cells lacking Gne (9) and in human lymphoid cells with GNE mutations (11). Interestingly, the Lec3 Gne mutations were severe enough to result in a mild sialic acid-deficient phenotype (Fig. 1) despite the large extent of rescue by serum glycoproteins. Clearly, mutations with a weak effect on UDP-GlcNAc 2-epimerase activity might not survive WGA selection. However, the data reported here show that even weak CHO Gne gene mutants should be selectable using anti-PSA antibodies to sort for low PSA expressors or to selectively kill wild type cells.

Selections for sialic acid-deficient mutants target the most terminal sugar on cellular glycoconjugates and consequently select for mutations affecting the addition of any sugar that would preclude sialic acid addition. Even selection strategies that appear focused, such as selecting for mutants with altered incorporation of synthetic sialic acid analogues like ManLev (43), do not avoid this problem. In CHO cells, WGA selections for cells expressing reduced or enhanced amounts of sialic acid have given rise to a wide spectrum of mutations in many different genes that reduce the synthesis of sialyltransferase substrates (45). Thus, it is interesting that Jurkat cells sorted for expression of reduced or increased amounts of ManLev comprised mainly sialic acid pathway mutants including many GNE mutants (43). This is presumably because of the diploid nature of the genome in Jurkat cells that precluded the isolation of recessive mutations. All Jurkat GNE mutants expressed both wild type and mutant GNE alleles. Thus, only dominant GNE mutations that reduced feedback regulation of UDP-GlcNAc 2-epimerase by CMP-sialic acid were obtained (43). By contrast, both of the Lec3 CHO mutants described here express

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**Fig. 6.** Lec3 cells have point mutations in the Chinese hamster Gne gene. A, Northern blot analysis. Total RNA (40 μg) from wild type or Lec3 (G135E) cells was electrophoresed, transferred to paper, hybridized with an ~2.6-kb CHO cDNA Gne probe, and exposed on x-ray film for 6 days (upper panel). After stripping, the blot was hybridized to a glyceraldehyde-3-phosphate dehydrogenase probe (lower panel). B, sequence alignment of the UDP-GlcNAc 2-epimerase region of mammalian GNE/Gne genes and bacterial homologous genes. Sequences shown are CHO Gne (GenBankTM accession number AB107226), human GNE (AF051852), Legionella pneumophila neuC (AJ007311), Streptococcus agalactiae neuC (AB028896), Escherichia coli neuC (M84026), Pseudomonas aeruginosa (AF498403) and Campylobacter jejuni neuC2 (AL139078) genes. The lec3 mutations are noted (*).
GNE mutation. A, Lec3 cells were transfected with CHO or mutant FLAG-tagged GNE. After 3 days, the cells were extracted with 1% Nonidet P-40, immunoprecipitated onto anti-FLAG (M2) antibody beads, and subjected to Western blot analysis using the same antibodies. Lane 1, vector alone; lane 2, CHO Gne; lane 3, Lec3 G135E Gne; lane 4, human H132A Gne; lane 5, human D413K Gne. B, the same cells were subjected to FACS analysis with anti-PSA (735) antibodies (Fig. 7). Under these conditions, the very small amount of PSA expression induced by the Gne mutant allele. This makes them ideal hosts for functionally characterizing Gne mutations.

The most significant effect of the lec3 mutation in CHO cells is the reduced addition of PSA to N-CAM. A similar result was obtained with the human GNE cDNA. Therefore, at least a double mutant would be required to characterize human GNE kinase domain mutations in CHO cells. This would perhaps be easiest to generate by an RNA interference strategy.

In conclusion, Lec3 CHO mutants are well suited for fundamental studies of mutations that alter UDP-GlcNAc 2-epimerase activity including human GNE mutations that cause sialuria and the myopathic disorders, HIBM and Nonaka myopathy (13, 14, 44).

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