Iec32 Is a New Mutation in Chinese Hamster Ovary Cells That Essentially Abrogates CMP-N-acetylneuraminic Acid Synthetase Activity*

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LEC29.Lec32 is a glycosylation mutant that was isolated from a selection of mutagenized Chinese hamster ovary (CHO) cells for lectin resistance. Compared with LEC29 CHO cells, the double mutant exhibited an unusually high sensitivity to the toxic lectin, ricin, indicating increased exposure of galactose residues on cell surface carbohydrates. Structural analysis of LEC29.Lec32 cellular glycoproteins showed a nearly complete lack of sialic acid residues. Genetic analysis demonstrated that the lec32 mutation is recessive and novel. Biochemical analysis showed that the mutant cells contained less than 5% of the cytidine 5'-monophosphate N-acetylneuraminic acid (CMP-NeuAc) present in parental CHO cells (1.6 nmol/mg of cell protein). A sensitive radiochemical assay used to measure CMP-NeuAc synthetase activity showed that the properties of this enzyme in parental CHO cells were essentially identical to those of CMP-NeuAc synthetase in various mammalian tissues. However, no CMP-NeuAc synthetase activity was detected in LEC29.Lec32 extracts. Mixing experiments provided no evidence for an inhibitor in the mutant CHO cells, and two revertants, which expressed only the LEC29 phenotype, had normal CMP-NeuAc synthetase levels. The combined evidence indicates that the lec32 mutation resides in either the structural gene encoding CMP-NeuAc synthetase or in a gene that regulates the production of active enzyme.

The addition of sialic acid to glycoproteins and glycolipids has important physiological consequences and appears to be vital for intercellular adhesion events involved in embryonic development and differentiation (1–3); myelination (4); the cell-mediated immune response via binding to selectins, sialecthesin, or CD22 (4–10); and oncogenic transformation and metastasis (11, 12). The regulation of this terminal event in sialyltransferase and other enzymes may provide a way to control cell-cell interactions and enhance the therapeutic potential of oligosaccharide-modified reagents.

Materials—Asialofetuin, CTP, NeuAc, CMP-NeuAc, N-glycolylneuraminic acid (NeuGc), WGA, ricin, sodium cacodylate, MOPS, N-ethylmaleimide (NEM), polyethylene glycol (M = 1000), Nonidet P-40, and bovine serum albumin (fraction V) were from Sigma. Cytidine 5'-monophosphate N-glycolylneuraminic acid (CMP-NeuGc) was a kind gift of Dr. Akemi Suzuki (Tokyo Metropolitan Institute of Medical Sciences, Tokyo, Japan), and purified CMP-NeuAc synthetase from E. coli was generously provided by Dr. Willie Vann (Laboratory of Bacterial Polysaccharides, Center for Biologics Evaluation and Research, Bethesda, MD). CMP-[14C]NeuAc (313.5 mCi/mmol) was from DuPont NEN, and N-acetyl[14C]neuraminic acid (286 mCi/mmol) was purchased from Amersham Corp. The monoclonal antibody (mAb) anti-NeuAc synthetase; EC 2.7.7.43), 2) the transport of CMP-NeuAc into the appropriate cellular compartments, 3) the action of a sialyltransferase, and 4) the action of neuraminidases that may remove NeuAc residues.

A KB cell mutant lacking a sialyltransferase activity has been isolated by selecting for resistance to the cytotoxic effects of ultraviolet-inactivated Sendai virus (13), and numerous attempts have been made to obtain additional mammalian cell mutants, defective in any of the above steps, by selection for resistance to the sialic acid-binding and cytotoxic plant lectin, wheat germ agglutinin (WGA) (12, 14–17). This latter strategy has primarily yielded mutants with defects in CMP-NeuAc transport, including three different Chinese hamster ovary (CHO) cell lines (12, 18). Two other types of WGA-resistant mutant have either undergone derepression of a gene encoding an α(1,3)fucosyltransferase (19–21) or alteration of a gene that leads to increased levels of CMP-NeuAc hydroxylase (22–24). These latter two phenotypes behave dominantly in somatic cell hybrids, whereas the three other WGA-resistant mutants exhibit a recessive phenotype.

In this report we describe the isolation and properties of LEC29.Lec32, a cell line obtained from a mutagenized population of CHO cells following selection with WGA. The dominant LEC29 phenotype is due to expression of an α(1,3)fucosyltransferase activity, apparently identical to that previously reported in LEC29 CHO cell extracts (21). The Lec32 phenotype is recessive and novel. We show in this paper that the lec32 mutation reduces CMP-NeuAc synthetase activity to undetectable levels and reduces NeuAc on glycoproteins and glycolipids by 95%.

EXPERIMENTAL PROCEDURES

1 The abbreviations used are: CMP-NeuAc, cytidine 5'-monophosphate N-acetylneuraminic acid; CHO, Chinese hamster ovary; CMP-NeuGc, cytidine 5'-monophosphate N-glycolylneuraminic acid; FCS, fetal calf serum; HPAC-PAD, high performance anion exchange chromatography with pulsed electrochemical detection; MAA, m-amorenuraminic acid agglutinin; mAb, monoclonal antibody; MNN, 1-nitro-3-nitro-1-nitrosoguanidine; MOPS, N-(3-morpholino)propanesulfonic acid; NEM, N-ethylmaleimide; NeuGc, N-glycolylneuraminic acid; PBS++, phosphate-buffered saline containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 0.02% sodium azide; SSEA-1, stage-specific embryonic antigen-1; WGA, wheat germ agglutinin; Le$^a$, Lewis-X.

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SSEA-1 was prepared as described previously (25), and the SNH3 mAb was a gift from Dr. Anil Singhal (Biomembrane Institute, University of Washington, Seattle, WA). Rabbit anti-mouse IgM and IgG were obtained from Zymed (San Francisco, CA), and 125I-protein A (108 cpm/mg protein) was purchased from Amersham Corp. Sheep red blood cells were obtained from PML Microbiologicals (Tualatin, OR). Concanavalin A was purchased from Bio-Rad. 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG) and Ecol-1a (strain V. cholerae) were purchased from Pharmacia Biotech Inc., agarose-bound WGA was from Vector Laboratories (Burlingame, CA), and agarse-bound Maackia amurensis lectin (WGA) was from Pharmacia Biotech Inc., and bovine calf serum was from Hyclone Laboratories (Logan, UT). AG 1-X4 anion exchange resin (100–200-mesh) Cl− form (Bio-Rad, McGaw Park, IL). The protein remaining in these samples (about 3.7–4.3 mg) was removed by passage through a small column of AG50W-X2 (200–400 mesh) H+ form ion exchange resin. Recovery was 71–74% of the original amount. The protein remaining in these samples (about 3.7–4.3 mg) was removed by passage through a small column of AG50W-X2 (200–400 mesh) H+ form ion exchange resin. Recovery was 71–74% of the original amount.

RESULTS

Monoclonal Antibody Binding—The ability of the various cell lines to bind SSEA-1 and SNH3 mAbs was quantified as described (21). Briefly, 106 cells were washed and resuspended in phosphate-buffered saline (PBS−) containing 2% bovine serum albumin (phosphate buffered saline/bovine serum albumin) in a volume of 200 μl. After 1 h of incubation at 4°C in the presence or absence of the first antibody (μg of SSEA-1 or SNH3), the cells were washed with phosphate buffered saline/bovine serum albumin and incubated for 1 h with secondary antibody (2 μg of rabbit anti-mouse IgM or IgG). After washing, a final incubation of 1 h was performed in the presence of 100,000 cpm α125I-protein A. After two additional washes, the percentage of cpm bound was determined from the total cpm added per tube and the cpm bound to the washed cells.

Enzyme Assays—Cell-free extracts were prepared in extraction buffer containing 1% Nonidet P-40 as described (25), and protein concentration was assayed using the Bio-Rad reagent according to the manufacturer. Sialyltransferase activity was measured essentially as described by Briles et al. (15). Reaction mixtures contained 25 μmol of MOPS buffer (pH 6.5), 5 μmol of NaCl, 0.25 μmol of MnCl2, 50–130 nmol of [3H]CMP-NeuAc (800–2500 cpm/nmol), 1 mg of asialofetuin (as acceptor), and 10 μl of extract containing 100–150 μg of protein. Following incubation at 37°C for 1 h, the reaction was stopped by the addition of 1 ml of ice-cold distilled water. Protein product was precipitated with trichloroacetic acid onto a GF/C glass microfiber filter disc (Whatman, Maidstone, United Kingdom), washed 3 times with 5 ml of ice-cold 10% trichloroacetic acid and once with 5 ml of cold 95% ethanol, and counted with 4.5 ml of Ecolume. CMP-NeuAc synthetase activity was determined initially by a thio-barbituric acid procedure (31), and subsequently, a radiochemical assay was used that was a modification of the method of Edwards and Frosh (32). Unless otherwise indicated, reaction mixtures contained 90 mM Tris-HCl (pH 7.4), 250 μmol of [3H]CMP-NeuAc (560 cpm/nmol), 1 mg of asialofetuin (as acceptor), and 10 μl of extract containing 100–150 μg of protein. Following incubation at 37°C for 1 h, the reaction was stopped by the addition of 1 ml of ice-cold distilled water. Protein product was precipitated with trichloroacetic acid onto a GF/C glass microfiber filter disc (Whatman, Maidstone, United Kingdom), washed 3 times with 5 ml of ice-cold 10% trichloroacetic acid and once with 5 ml of cold 95% ethanol, and counted with 4.5 ml of Ecolume.

Preparation of Nuclei—Nuclei were prepared by a modification of the method of Liu (33). Approximately 1 × 106 washed Pro-5 CHO cells were gently lysed in 0.15 ml of a solution containing 10 mM Tris-HCl, pH 7.4, 0.15 M MgCl2, 0.3 μM sucrose, and 0.25% Nonidet P-40. The lysate was loaded onto a 4-ml cushion of 0.6 M sucrose and centrifuged for 10 min at 2000 rpm at 4°C in a Beckman 50Ti rotor. The supernatant was removed by aspiration, the sample was resuspended in 0.5 ml of water, and insoluble material was removed by centrifugation at 4°C at 10,000 rpm for 10 min in a Biofuge A (Baxter/Scientific Products, McGaw Park, IL). The protein remaining in these samples (about 3.7–4.3 mg) was removed by passage through a small column of AG 50W-X2 (200–400 mesh) H+ form exchange resin. Residues of [3H]CMP-NeuAc were added at the first step of the procedure. The samples were stored frozen at −20°C until thawed, passed through Centrex filter units, and analyzed at room temperature by HPAE-PAD as described above. The standards used were NeuAc, NeuGc, CMP-NeuAc, and CMP-NeuGc.

Mutation and Lectin Resistance—In a selection to obtain new glycosylation mutants expressing an α1(3) fucosyltransferase, a population of 1 × 106 MNNG-treated Pro-5 CHO cells were incubated in 13.3 μg/ml WGA, and surviving colonies were screened for cell surface expression of Lea (29) using the mAb SSEA-1, which binds the Lea determinant (Gal[α1(3)-Fucα1(3)]GlcNAcβ1(4)). Colonies that bound SSEA-1 were

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found to express an α(1,3)fucosyltransferase activity and included the previously described mutants LEC29 and LEC30 (21) as well as novel variants. One new phenotype had an α(1,3)fucosyltransferase activity indistinguishable from that already described for the LEC29 mutant (21). However, this isolate was more resistant to WGA and had a markedly increased sensitivity to ricin, compared with LEC29 cells (Fig. 1). The enormous hypersensitivity to ricin suggested that a second mutation, unrelated to LEC29, had caused an increase in terminal galactose residues on cell-surface glycoproteins. This new mutation was designated lec32, and the mutant phenotype was termed LEC29.Lec32.

Cell Hybridization and Reversion Studies—Since a similar degree of ricin hypersensitivity had been previously observed in the recessive Lec2 CHO mutant (14), LEC29.Lec32 cells were fused to parental CHO cells and to Lec2 mutant cells for complementation analysis. The properties of the resulting hybrids resembled those of the dominant LEC29 phenotype (Table I). Two conclusions may be drawn from this result: 1) LEC29.Lec32 cells contain both a dominant mutation, which activates the expression of a previously quiescent α(1,3)fucosyltransferase gene, and a recessive mutation, whose expression causes the extreme hypersensitivity to ricin; and 2) this latter mutation belongs to a complementation group that is distinct from Lec2. Similar results (Table I) were obtained after fusion to Lec3, another NeuAc-deficient CHO mutant (14), indicating that the lec32 mutation is novel.

The existence of two glycosylation mutations in LEC29.Lec32 cells was further supported by a two-step reversion experiment in which LEC29.Lec32 cells were selected for resistance to ricin. When 0.4 ng/ml ricin was used, surviving colonies appeared at a rate of about 4 × 10−4 and retained the characteristics of LEC29 cells (i.e. the lec32 mutation had been reverted). When one of these revertants was exposed to 7 ng/ml ricin, surviving colonies appeared at a rate of 2 × 10−4 and were indistinguishable from parental CHO cells (i.e. both the LEC29 and lec32 mutations had been reverted). Attempts to isolate double revertants in a single step using the higher concentration of ricin were unsuccessful when 3 × 106 cells were screened, as would be expected for an extremely rare event occurring at a predicted frequency of ≈8 × 10−7.

Monoclonal Antibody Binding Studies—An additional difference between LEC29 and LEC29.Lec32 cells was observed in antibody binding studies with SSEA-1 and SNH3, a mAb that has been reported to bind sialylated lactosamine with α(1,3)fuc (35). Lec3.2.8.1 CHO cells, which do not bind either antibody because they do not produce complex or hybrid N-linked carbohydrates (27) were used as a negative control. The results in Fig. 2 show that LEC29 and LEC29.Lec32 cells bind similar amounts of the Le"-recognizing antibody, SSEA-1. However the binding of the SNH3 antibody was markedly reduced compared with parental CHO or LEC29 cells. As parental CHO cells do not possess an α(1,3)fucosyltransferase activity (20, 25), the SNH3 antibody must recognize sialylated lactosamine units lacking fucose on CHO cells. The reduced binding of SNH3 by LEC29.Lec32 cells provides additional, indirect evidence that the lec32 mutation causes a loss of surface sialic acid residues.

Analysis of Cell Surface Glycoproteins and Glycolipids—Further evidence for a loss of NeuAc residues on LEC29.Lec32 cell surface glycoproteins was obtained by lectin affinity chromatography on WGA and MAA columns (Fig. 3). WGA recognizes α(2,3)- and α(2,6)-linked terminal NeuAc residues (36, 37), while MAA is specific for α(2,3)-linked NeuAc residues (38). In both cases, species that bound to the column were present in parental CHO glycopeptides but were absent in glycopeptides from LEC29.Lec32 cells. To quantitate this difference, HPAEC-PAD analysis was performed using authentic NeuAc and cellular glycopeptides from parental and LEC29.Lec32 cells (Fig. 4). The NeuAc component was reduced in mutant cells to less than 5% of parental cell levels. Analysis of [3H]mannosamine-labeled glycolipids showed the ratio of II3NeuAc-lactosylceramide (Gm3) to lactosylceramide was 0.11 for mutant cells compared with 2.2 for parental CHO cells and a revertant line.2 Therefore, the NeuAc content of glycolipids from LEC29.Lec32 cells was also reduced 95% compared with parental CHO cells.

Intracellular Pools of NeuAc and CMP-NeuAc—In order to determine whether the lec32 mutation affected the synthesis of NeuAc or CMP-NeuAc, soluble cytoplasmic extracts prepared from both the parental and mutant cells were analyzed by HPAEC-PAD (Fig. 5). The standards used were NeuAc, NeuGc, CMP-NeuAc, and CMP-NeuGc and eluted at approximately 22, 46, 73, and 90 min, respectively. Only the profile for CMP-NeuAc is shown. The LEC29.Lec32 mutant cells were found to contain 10-fold more free NeuAc than the parental CHO cells. By contrast, LEC29.Lec32 cells contained less than 1% of parental cell levels of CMP-NeuAc (or breakdown products derived from it). This difference was extremely significant, and
the combined data suggested that the lec32 mutation affects the synthesis of CMP-NeuAc from NeuAc and CTP.

CMP-NeuAc Synthetase Activity in CHO Cells—The synthesis of CMP-NeuAc may be investigated using a thiobarbituric acid assay (31). With this assay, parental CHO cell extracts had an activity corresponding to slightly less than twice the background of the assay (Fig. 2); 5.1 nmol/min/mg protein). In order to reliably measure low levels of enzyme activity, a radiochemical assay with [14C]NeuAc as substrate was used (see "Experimental Procedures”). Unreacted [14C]NeuAc was separated from CMP-[14C]NeuAc by elution of an anion exchange resin column with a step-wise gradient of NaCl (Fig. 6). Authentic CMP-NeuAc (2 nmol) was used as a standard. The data are from a single experiment that was repeated once.

FIG. 2. Binding of monoclonal antibodies to cell surface antigens. Approximately 10^6 cells were washed and assayed for their abilities to bind the monoclonal antibody SSEA-1 (upper panel) or SNH3 (lower panel) in an indirect binding assay. The results show percentage of 125I-Protein A bound and are from a single experiment that was reproduced once or more for each cell line. The sugar sequences recognized by each monoclonal antibody are indicated: ●, Gal; ■, GlcNAc; ▲, Fuc; △, NeuAc.

FIG. 3. Binding of cellular glycopeptides to NeuAc-recognizing affinity columns. [3H]Glc-labeled cellular glycopeptides were prepared as described under "Experimental Procedures" and chromatographed separately on MAA-agarose or WGA-agarose.

FIG. 4. NeuAc content of cellular glycopeptides. The amount of NeuAc in cellular glycopeptides prepared from ~6 × 10^7 parental or LEC29.Lec32 cells was determined using HPAEC-PAD analysis. Authentic NeuAc (2 nmol) was used as a standard. The data are from a single experiment that was repeated once.

FIG. 5. Intracellular pools of NeuAc and CMP-NeuAc. The levels of NeuAc and CMP-NeuAc in cytoplasmic extracts prepared from ~6 × 10^7 parental (middle panel) or LEC29.Lec32 (lower panel) cells were determined by HPAEC-PAD analysis. Authentic CMP-NeuAc (2 nmol) was used as standard (upper panel). The peak at 73 min corresponds to CMP-NeuAc; authentic NeuAc and NeuAc derived from hydrolyzed CMP-NeuAc eluted at 22 min. The species at 26 and 65 min are unidentified breakdown products of CMP-NeuAc that do not correspond to either NeuGc (elutes at 46 min) or CMP-NeuGc (elutes at 90 min). The data are from one of two experiments that gave similar results.
peak but also caused considerable breakdown of NeuAc, as evidenced by an increase in counts eluting with the initial water washes (data not shown). Attempts to reduce the number of steps in the elution procedure by using different salt concentrations and elution volumes failed to adequately separate substrate and product.

CMP-NeuAc synthetase activity has been characterized in various mammalian tissues (39, 40), and the activity in CHO cells was optimized for comparison. The optimum pH for the CHO enzyme was found to be 8.5–9.0 in the presence of Mg\(^{2+}\) (Fig. 7) and 7.0 with Mn\(^{2+}\). However, when Mn\(^{2+}\) was used, the pH values above 8.0 under conditions that minimized the formation of Mn(OH)\(_2\), a similar optimum to that observed with Mg\(^{2+}\) (i.e. pH 9.0) was found, although the levels of activity were only half those observed with Mg\(^{2+}\) (data not shown). Highest levels of activity were obtained when the Mg\(^{2+}\) concentration was at least 20 mM (Fig. 7). Divalent cations other than Mn\(^{2+}\) were unable to effectively substitute for Mg\(^{2+}\), although some activity (10% of that with Mg\(^{2+}\)) was obtained in the presence of Fe\(^{3+}\) (Table II). As reported for the rat liver enzyme (40), Cu\(^{2+}\) and Zn\(^{2+}\) were inhibitory when added with Mg\(^{2+}\).

The optimum temperature over the range tested (22–52 °C) was 37 °C (data not shown). The reaction was essentially linear for at least 60 min and at protein concentrations from about 40–360 μg/mg (Fig. 8). Other reports have indicated that (at least for the partially purified mammalian enzyme) sulfhydryl reagents, such as dithiothreitol, are stabilizing or stimulatory (39, 40). The inclusion of 2.5 mM dithiothreitol in our standard reaction increased CMP-NeuAc synthetase activity by about 30%. It was also observed that concentrations of CTP above 5 mM inhibited activity but that the addition of 0.5 n mole of unlabeled CMP-NeuAc to a reaction mixture had no effect. Kinetic experiments performed under optimal conditions (see "Experimental Procedures") but in the absence of dithiothreitol gave apparent K\(_m\) values of about 0.34 mM for NeuAc and 1.3 mM for CTP. In each case, the value falls within the range of those reported for the CMP-NeuAc synthetase from other mammalian sources (39–41).

Since both the E. coli (42) and rat liver (40) CMP-NeuAc synthetase enzymes are known to be sensitive to NEM, the CHO enzyme was tested. It was found that, whereas very low levels of NEM were slightly stimulatory, the addition of 1 mM NEM to the reaction mixture reduced the CHO activity to about 12%, while the activity of the purified E. coli enzyme was only reduced to 88% under these conditions (Fig. 9).

The mammalian CMP-NeuAc synthetase has been reported by many authors to be localized to the nucleus (39, 40, 43–46). Nuclei prepared from parental CHO cells were found to contain at least 70% of the total extractable activity. Our usual extraction buffer containing 0.15 M NaCl, 25% glycerol, and 1% Nonidet P-40 solubilized 85–95% of total cellular activity. Reextraction of the pellet material with the same extraction buffer released the remaining activity. Increasing the detergent concentration to 2% did not significantly increase the amount of enzyme activity initially solubilized.

LEC29.Lec32 Cells Lack Detectable CMP-NeuAc Synthetase Activity—Initial results with the thiobarbituric acid assay in-

![Fig. 6](image)

**Fig. 6.** A radiochemical assay for CMP-NeuAc synthetase. Parental and LEC29.Lec32 cell-free extracts were incubated with \(^{14}C\)-NeuAc (2 n mole; 17,500 cpm/nmole) to generate \(^{14}C\)CMP-NeuAc as described under "Experimental Procedures." After 1 h, reaction mixtures were fractionated on ion exchange columns under the standard conditions described under "Experimental Procedures." Authentic \(^{14}C\)NeuAc and \(^{14}C\)CMP-NeuAc eluted at the positions shown. Dotted line, parental; solid line, LEC29.Lec32.

![Fig. 7](image)

**Fig. 7.** Optimum pH and MgCl\(_2\) concentration for CMP-NeuAc formation. CMP-NeuAc synthetase activity was determined in cell-free extracts of parental CHO cells (about 88 μg of protein) at various pH values in the presence of 10 mM Mg\(^{2+}\) (upper panel) and under the assay conditions described under "Experimental Procedures." Similar results were obtained in two experiments. CMP-NeuAc synthetase activity was also determined in parental CHO extracts (40 μg of protein) under conditions described under "Experimental Procedures" except that the MgCl\(_2\) concentration was varied (lower panel). Similar results were obtained in three experiments.

**TABLE II**

| Cation    | Percentage of maximum activity |
|-----------|-------------------------------|
|           | pH 9  | pH 7  |
| Mg\(^{2+}\) | 100.0 | 36.2  |
| Mn\(^{2+}\) | 3.7   | 100.0 |
| Cu\(^{2+}\) | 3.4   | 27.5  |
| Fe\(^{2+}\) | 1.4   | 12.5  |
| Zn\(^{2+}\) | 2.3   | ≤1.0  |

* Mn(OH)$_2$ precipitate forms at this pH.
LEC29.Lec32 cells lack CMP-NeuAc synthetase activity. CMP-NeuAc synthetase activity of parental CHO (about 90 μg of protein) and LEC29.Lec32 (about 84 μg of protein) extracts was measured over a range of incubation times (upper panel) and at a variety of protein concentrations, for 15 min (lower panel). All other reaction conditions were as described under “Experimental Procedures.”

**DISCUSSION**

Although many WGA-resistant, NeuAc-deficient glycosylation mutants have been characterized (47), the lec32 mutation described in this paper is the first to result in the loss of CMP-NeuAc synthetase activity. Analysis of LEC29.Lec32 glycolipids and cell-surface glycopeptides has demonstrated a reduction of 95% in NeuAc content. Furthermore, the cellular pools of CMP-NeuAc are greatly reduced, while that of free NeuAc is increased about 10-fold in mutant cells compared with parental CHO cells. This latter observation may be the result of two different mechanisms: 1) the lack of CMP-NeuAc synthetase activity should result in a build-up of unused NeuAc substrate; and 2) the deficiency of CMP-NeuAc will lead to release of a previously reported feedback inhibition of UDP-N-acetylglucosamine 2-epimerase (48), the enzyme responsible for the synthesis of N-acetylmannosamine, a precursor in the synthesis of NeuAc.

The properties of CMP-NeuAc synthetase in cell-free extracts prepared from parental CHO cells appear to be nearly identical to those previously described for CMP-NeuAc synthetase studied in other mammalian tissues (39–41). In addition, the CHO CMP-NeuAc synthetase is predominantly localized to the nucleus, as observed for the mammalian enzyme from other sources (39, 40, 43–46).

Molecular biological approaches to the study of CMP-NeuAc synthetase have thus far been confined to the enzyme from bacterial sources such as E. coli (31, 49, 50) and Neisseria meningitidis (32). In both cases, the studies were aimed at understanding the steps involved in the synthesis of bacterial virulence factors, such as capsular polysaccharides, which contain large amounts of α(2,8)-linked NeuAc residues, or providing a source of enzyme for the synthesis of large amounts of CMP-NeuAc. The nature of the lec32 mutation is currently unknown and is the subject of further investigation. Critical reagents, such as a molecular probe for the mammalian CMP-NeuAc synthetase gene and an antibody that specifically recognizes the enzyme must be developed. Attempts to detect the CHO enzyme with an antibody directed against E. coli CMP-NeuAc synthetase were unsuccessful. Transfection experiments aimed at correcting the defect in LEC29.Lec32 cells using two different mammalian expression vectors containing the doted E. coli gene (kindly supplied by Dr. Willie Vann), were also negative (data not shown). The fact that small amounts of NeuAc are present on both glycoproteins and glycolipids of LEC29.Lec32 cells suggests that CMP-NeuAc synthetase activity in LEC29.Lec32 cells was not due to the presence of an inhibitor. In addition, it was shown that LEC29.Lec32 and parental CHO cells have equivalent sialyltransferase activities (14.7 ± 8.5 and 17.2 ± 5.7 nmol/h/mg protein, respectively). Therefore, the biochemical basis of the NeuAc deficiency on glycoconjugates of LEC29.Lec32 cells is a virtual lack of CMP-NeuAc synthetase activity.

**TABLE III**

| Cell line          | CMP-NeuAc synthetase activity | nmol/min/mg protein |
|--------------------|-------------------------------|---------------------|
| CHO Pro 5          | 3.7 ± 1.4                     |                     |
| LEC29.Lec32        | 0.2                           |                     |
| CHO Pro 5 + LEC29.Lec32 | 1.9 ± 0.3                   |                     |

**Fig. 8.** LEC29.Lec32 cells lack CMP-NeuAc synthetase activity. CMP-NeuAc synthetase activity of parental CHO (about 90 μg of protein) and LEC29.Lec32 (about 84 μg of protein) extracts was measured over a range of incubation times (upper panel) and at a variety of protein concentrations, for 15 min (lower panel). All other reaction conditions were as described under “Experimental Procedures.”

**Fig. 9.** Inhibition of CHO CMP-NeuAc synthetase by NEM. The CMP-NeuAc synthetase activity of parental CHO cell extracts (about 100 μg of protein) and purified E. coli CMP-NeuAc synthetase (about 0.03 μg of protein) were assayed in the presence of NEM (0.005–1 mM) with standard reaction conditions described under “Experimental Procedures.” In the absence of NEM, 100% activity for the CHO extract was 3.01 nmol/min/mg protein, and for the E. coli enzyme, it was 46,167 nmol/min/mg protein.

Distributed that LEC29.Lec32 cells had no CMP-NeuAc synthetase activity, and this was confirmed with the more sensitive radiochemical method (Fig. 6). LEC29.Lec32 cell-free extracts contained no detectable activity (≤0.2 nmol/min/mg protein), while extracts from the parental Pro5 cells, as well as those from two independently isolated revertants, contained 3.7–4.0 nmol/min/mg protein. Similar results were obtained when assays were run at room temperature for longer time periods (data not shown), making it unlikely that a temperature-sensitive mutation has occurred. Mixing of equal amounts of parental and mutant cell extracts yielded about 103% of the expected levels of activity (Table III), showing that the lack of CMP-NeuAc synthetase activity in LEC29.Lec32 cells was not due to the
thease is not completely inactivated by the lec32 mutation. This would be consistent with a mutation in the coding region of the CMP-NeuAc synthetase gene that affects the activity, stability, or localization of the enzyme. It is also possible that the lec32 mutation involves a mutation in an upstream control element, a gene encoding a positive or negative transcriptional regulatory factor, or a gene rearrangement, all of which could allow small amounts of enzyme to be synthesized. Although a regulatory factor, or a gene rearrangement, all of which could encode a gene positively or negatively, thease is not completely inactivated by the lec32 mutation. In this case, the nuclear localization of the enzyme responsible for producing CMP-NeuAc would clearly be functionally important. For any enzyme involved in the regulation and synthesis of carbohydrate ligands for cell adhesion molecules, further studies of CMP-NeuAc synthetase may be of considerable importance. The nuclear localization of this enzyme (which has long puzzled investigators) provides yet another incentive for probing both the range of its normal cellular functions and the regulation of the gene that encodes this enzyme. It has been proposed that the compartmentalization of this enzyme may be required to protect its product from the action of a cytoplasmic membrane-bound hydrolase. Recent work involving the glycosylation of nuclear proteins has suggested that a sialyltransferase, whose activity is critical for the normal functions of the various nuclear proteins that never transit the Golgi apparatus, may exist in the nucleus. This theory is supported by a report in which five glycosyltransferases, including a sialyltransferase, were found to be associated with rat liver nuclei. In this case, the nuclear localization of the enzyme responsible for producing CMP-NeuAc would clearly be of functional importance.

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